# Session 8D

# Biological Control and Integrated Crop Management

Session Organiser      Dr K F A Walters

Poster Papers      8D-1 to 8D-27

DEVELOPMENTS IN THE MARKET FOR BIOPESTICIDES

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ABSTRACT

The markets for biopesticides are growing, although more slowly and from a smaller base than is commonly believed. The main reason for growth is the larger research effort in recent years, which has resulted in improved products based on knowledge of how and why biopesticides work. This has attracted larger agrochemical companies into the business which should, in turn, help promote future growth.
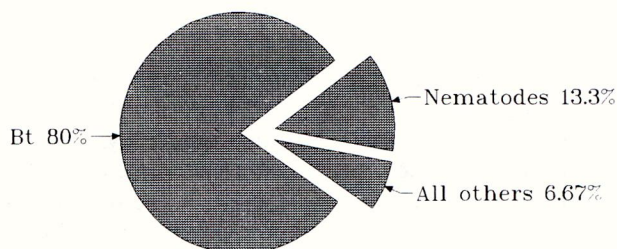
INTRODUCTION

Surveys show that around two-thirds of all Americans rank chemical pesticides as causing their most serious concerns about food safety, despite the fact that most people are much more likely to suffer from microbial contaminants in food. This anxiety should provide the perfect incentive for a growing market in non-chemical products for crop protection and crop production. Yet, despite optimistic predictions, the volume and value of biopesticides has remained small and, at $75m, is still less than 1% of the total world market for agrochemical crop protection of $20-$25b. In the past, biological products did not work very well and most were more expensive than chemical equivalents. Recently, established products have been improved and new lower-cost products introduced that, together, have led to market growth.

THE MARKET

At present the market for biopesticides is around $75m per annum and can be divided between three groups of products (Figure 1).

Figure 1.    Division of the $75m biopesticides market



The market for biopesticides was steady for many years at around $20-25m. Within the past five years, the market size has increased to around $75m and its future potential has also

increased substantially due to the introduction of new and better products, including many based on *Bacillus thuringiensis* (*Bt*) and to the entry of new companies into the biopesticides business (Lisansky, 1992). However, intense competition for the main (*Bt*) markets has resulted in lower prices; as a consequence the volume of sales has increased, but total market value has remained fairly static. In addition, seasonal variations in pest infestations in the small number of principal *Bt* markets (forestry, cotton, brassica crops) cause unpredictable changes in annual volumes. These factors can mislead those making future market projections, which include many predictions for sales well in excess of $300m by the year 2000, with some estimates ranging as high as $750m.

At present, we estimate that the growth rate will be around 10% per year for at least the next three years and will result in a market of $120-$130m by 2000.


HISTORY OF THE MARKET

Over the past four decades, the potential for biopesticides in the crop protection market has been viewed differently from different perspectives. Proponents have oversold the value, virtues and prospects of biopesticides while sceptics have generally concentrated on their many deficiencies. Biopesticides were once neglected by the agrochemical industry. They were subsequently promoted by venture capitalists as commercially and technically important components of crop protection in the future. Agricultural biotechnology would transform the market laggers into market leaders. Now biopesticides are perceived more realistically, not as a pest control panacea but as useful and valuable components of integrated pest management systems.

At present the main participants in the biopesticides business are the principal *Bt* producers, Abbott, Novo and Sandoz, who divide most of the market among themselves (Figure 2). A number of agrochemical companies are entering the biopesticides business. These include Ciba with several strains of *Bt* and interests in the nematode and insect businesses; Cyanamid with a *Bt* product for use against Diptera; Hoechst with a viral insecticide and Kemira with several fungal products useful against fungal plant diseases. Venture capital companies starting to sell biopesticide products include Biosys which sells nematodes and pheromones; Ecogen which sells a range of conjugated *Bt* products, a fungal herbicide, nematodes and pheromones; EcoScience which sells a newly developed range of fungal insect control products; MicroBio, a UK based company which sells nematodes; and Mycogen, which sells a range of products based on killed genetically engineered pseudomonads with *Bt* toxins.

The Producers of *Bacillus thuringiensis*

During the 1960s and 1970s, the market for biopesticides was exclusively for a single product based on a strain of *Bt* used to control lepidopterous insects. Two companies, Abbott and Sandoz, dominated the tiny (<$20m) market for *Bt*. However, because the product seemed easy and cheap to make and was thought to generate a good profit margin, a large number of companies (estimated at over 12 per year for around 20 years) considered making and selling *Bt*. "New" strains of *Bt* that were claimed by their discoverers to be more potent than existing commercial strains were continually offered by researchers and small companies.

Several of these strains were shown to be more potent than those in commercial use when potency was measured in laboratory bioassays. However, a potency improvement of around 10-fold was generally thought to be required in order to see an appreciable difference in field efficacy. One major international company which was unfamiliar with this, announced its new product with public claims of 5-fold improvements in laboratory potency only to be embarrassed by subsequent failures in use. The rights to these new strains were usually available for a single large payment. Many companies (estimated at around 3 per year for 20 years) tried to make and sell *Bt*. All except one failed and most regret the attempt.

By the early 1980s, several strains of *Bt* had been discovered with activity against other insect groups, particularly Diptera (mosquitoes and blackfly) and Coleoptera (including Colorado potato beetle). The principle was established that *Bt* was not just a control agent for caterpillars, but could be used against many pests. Novo Nordisk, a company known for expertise in fermentation, joined Abbott and Sandoz as a principal *Bt* producer. Although Novo had not previously marketed crop protection products, it has persisted using a combination of acquisition, opening new markets (mainly in Central Europe), product innovation and competitive pricing.

At present, we estimate that the *Bt* market of $60m may be divided approximately as shown in Figure 2.

Figure 2. Division of the $60m *Bt* market
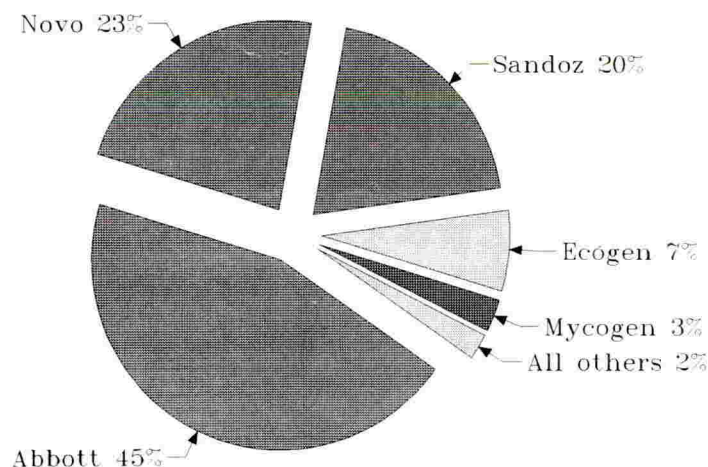


## The Agrochemical Companies

Twenty years ago, the development of biopesticides was considered unpromising by virtually all the major agrochemical companies. Despite the many observations that microorganisms could control pests, commercial exploitation of that ability was modest. Microorganisms were thought too narrow in spectrum, too expensive in manufacture, too fastidious in formulation and too erratic in performance while being simultaneously too

common to patent. Agrochemical companies that understood the necessity of synthesizing and screening thousands of different chemicals every year, targeting the most promising candidates, and developing formulations tailored to each chemical, pest, crop and environment, did not extend that procedure to microorganisms. The fact that most microorganisms failed most of the time was taken as evidence that all microorganisms would fail all the time. The industry remained uninterested in the meagre efficacy and market niches offered by biopesticides. Proponents of biopesticides were challenged to show that microorganisms were as good as chemicals, without spending even a fraction of the resources normally spent on developing chemicals. To be of interest to the agrochemical industry, a biopesticide had to be like a chemical in every respect yet substantially less expensive.

## The deficiencies of biopesticides

A key deficiency of biopesticides was their generally poor and erratic performance in practice. Laboratory tests would often be an inaccurate guide to how well a biopesticide would work in the field. Companies involved in biopesticides were frustrated by the fact that occasionally biopesticides would work very well, controlling pest infestations completely over substantial periods of time, but that this quality of performance was neither reliable nor predictable.

The mode of action of biopesticides is generally different from that of chemicals. For example, *Bt* causes insects to stop feeding long before they die so damage to the crop ceases quickly. However, what farmers see, and what it was thought they would not accept, was live insects in the crop. Agrochemical companies perceived this as an obstacle to effective marketing of biopesticides.

These differences between chemicals and biopesticides were further reflected in their patentability. Strong "composition of matter" patents could be obtained for new chemicals; for biopesticides relatively weak process or use patents were the best that could be obtained. Another problem with biopesticides was equally fundamental. The roots of the agrochemical industry were in chemicals, not biologicals. Many employees were chemists who had conceptual difficulties working with biopesticides. They called biopesticides "compounds" and expected them to behave as such, which they did not.

When industry analyzed the biopesticides market it found a business in which the main market (*Bt*) was taken by producers with spare, written off, capacity. Other prospective markets open to biopesticides were all thought to be small niche markets. Generally markets were not growing and new products had either failed to appear or were performing poorly. Registration, although acknowledged to be somewhat simpler and less expensive than for chemicals, might become more difficult in the future. Most companies had either had poor experiences with biopesticides or were familiar with those of other companies. These experiences were often accepted as valid predictors of future results. Finally, companies could point to the lack of interest by their competitors to support their own inaction.

## Recent developments

More recently, the major agrochemical companies have taken a greater interest in biopesticides. This may be due to the pressures on the industry over the past few years. Most

markets for conventional pesticides have been growing much more slowly than in the past; some are shrinking. Competition in the main agrochemical markets of the US and Western Europe is becoming more difficult as re-registration and use-reduction programmes depress sales. Although Central Europe and Asia offer new opportunities, it is questioned whether the industry as a whole will ever return to the profitability of the past.

The agrochemical industry's interest in biopesticides is therefore strategic and tactical, but it is not especially commercial. Companies want to ensure that they do not miss a key opportunity (should one ever arise) and staying involved in biopesticides, even in small way, gives them ongoing information on technological developments. For bigger companies, joint ventures, R&D programmes and even a few products for sale are a relatively inexpensive form of technical and market intelligence. In addition, companies want to be seen to be doing something positive towards what are perceived as safer methods of crop protection and involvement in biopesticides can have significant public-relations value. In particular, participation in the low to medium technology end of the biopesticides business, such as insects, nematodes and other products not requiring registration, is relatively low-risk. Although profits may be small, any losses are likely to be similarly constrained.

For these reasons, virtually all the major companies have biopesticide products in development, manufacture or marketing. However, the industry remains unconvinced of the commercial future of biopesticides. No major company anticipates that the sales volume of any biopesticide will approach that for chemicals. Despite lower development and registration costs than chemicals, individual biopesticides are unlikely ever to obtain the broad and varied markets achieved by chemicals. Overall, biopesticides will remain niche products, best suited to higher-value uses and requiring a correspondingly higher marketing input.

The Venture Capital Companies

The advent in the early 1980s of venture capitalists and entrepreneurs enthused about agricultural biotechnology led to the formation of a number of new companies planning to succeed with products overlooked by the agrochemical majors. It was thought that innovative technology would overcome the perceived limitations of biopesticides. The spectrum of pests controlled by each product would be broadened, control would be more rapid and more persistent and the costs of production and marketing would decline as more products obtained larger markets. A high percentage of the companies formed during this era still exist, but almost none in their original form or with their original purpose. Developing, making, registering and selling biopesticides turned out to be, at best, a difficult business with lower margins and smaller prospects than hoped for at first. All the new, venture capital financed companies, have adapted to the fact that biopesticides based on microorganisms requiring registration could not form the basis of a sound business. They now sell a variety of other products that do not require registration such as those based on insects and nematodes.

THE FUTURE

It is likely that a continual decline will occur in the use of chemical pesticides in the main developed markets of the US and EU due to developments that include re-registration procedures in the US and EU which will reduce the number of chemicals available or will restrict their permitted uses; pesticide use-reduction programmes in the US and other

Figure 3.    Distribution of active ingredients in 281 biopesticides available in 1993
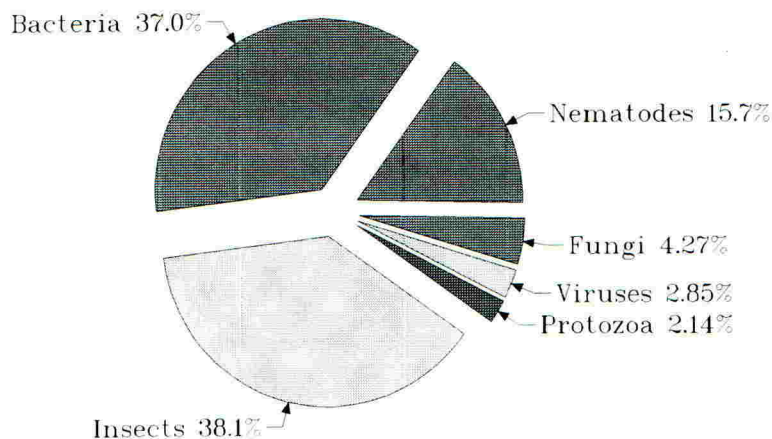


developed countries which will result in fewer products on the market and smaller volumes of them being sold and chemical discovery and development programmes which are becoming less productive while new registrations are becoming more difficult and expensive.

This decline in agrochemical usage will provide a increasing number of niche markets for which biopesticides may be appropriate. Market factors in favour of biopesticides include consumer preferences for pesticide-free produce and a growth in the market for organic or reduced-pesticide products; the development of more sustainable agricultural systems using integrated pest management programmes; the stabilization and harmonization of regulations governing registration of biopesticides containing either naturally occurring or engineered organisms; and the presence of many more major companies in the biopesticide business.

We anticipate that biotechnology will continue to improve the quality of the scientific work being done on biopesticides. As a consequence, more strains of *Bt* and other microorganisms will be developed with a greater range and quality of activities as crop protection and crop production products. Most of these new products will be used in higher-value niche markets since before biopesticides can be used in field crops, they will have to cost less than at present. However, competition may make some biopesticides into commodities, as has already happened for *Bt* in forestry and cotton. This will be good news for consumers but less beneficial for producers or venture capital investors. In the longer-term, we anticipate that spray-on products for crop protection are likely to be superseded by plants genetically engineered to be resistant to insects, diseases and weeds.

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EFFECTS OF DIFFERENT BACTERIAL ISOLATES ON GROWTH AND PATHOGENICITY OF THE SLUG-PARASITIC NEMATODE *PHASMARHABDITIS HERMAPHRODITA*

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ABSTRACT

A wide range of bacteria associated with the slug parasitic nematode *Phasmarhabditis hermaphrodita* were isolated. A small selection were identified and used in growth experiments and bioassays against slugs. *P. hermaphrodita* was found to be capable of growth and reproduction on the majority of the tested bacteria, but its reproductive capacity was influenced by bacterial species. Monoxenic growth with different bacteria was found to influence the pathogenicity of the nematode to the slug *D. reticulatum*, but this did not reflect differences in pathogenicity of the bacteria alone. No evidence was found that *P. hermaphrodita* is mutualistically associated with one species of bacterium. *Moraxella osloensis* was selected as the bacterium for rearing *P. hermaphrodita* as a biocontrol agent for use against slugs.

INTRODUCTION

The rhabditid nematode *Phasmarhabditis hermaphrodita* is a parasite capable of killing several species of pest slugs; it is capable of being produced *in vitro* and has been used successfully in field experiments to control slugs (Wilson *et al.* 1993a;1993b;1994a;1994b; Glen *et al.* 1994). *P. hermaprhodita* is now sold in the U.K. to domestic gardeners as a biocontrol agent for slugs and sales to commercial growers are planned within the next few years. The commercialisation of this nematode has been rapid, because it can be produced using technologies similar to those used in commercial production of entomopathogenic nematodes (families Heterorhabditidae and Steinernematidae). Heterorhabditid and steinernematid nematodes are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus* respectively, which are carried monoxenically in the nematodes' intestine. When the nematodes penetrate the host insect they release the bacteria which help to kill the insect and provide an ideal medium for nematode growth and reproduction. The nature of the symbiosis is complex and varies between different species

of nematode, but generally both bacteria and nematodes are thought to cause the insect's death. Whenever entomopathogenic nematodes are produced commercially they are grown in monoxenic cultures with the appropriate species of *Xenorhabdus* or *Photorhabdus* (Friedman, 1990). The aims of this study were to investigate the effects of different bacteria on growth of *P. hermaphrodita* and its pathogenicity to the slug *Deroceras reticulatum*, and to determine if *P. hermaphrodita* is symbiotically associated with a specific bacterium. Initially, bacteria associated with *P. hermaphrodita* were isolated and a selection were tested for their ability to support monoxenic growth of *P. hermaphrodita*. Five of the bacterial isolates which supported good growth were then used to produce *P. hermaphrodita* in monoxenic cultures, and these nematodes were bioassayed to test their ability to kill *D. reticulatum*. The pathogenicity of the bacteria to *D. reticulatum*, in the absence of *P. hermaphrodita*, was tested by injecting high doses of bacteria into the slug haemocoel.


MATERIALS AND METHODS

Collection and maintenance of experimental organisms

Slugs (*D. reticulatum*) collected from baited traps in rough grassland at Long Ashton Research Station (LARS) were kept at 10°C with a 12 h day length in non-airtight plastic boxes lined with moistened absorbent cotton wool. *P. hermaphrodita* were produced in cultures with a mix of unknown bacteria (xenic cultures) on foam chips as described by Wilson *et al.* (1993b), or they were isolated from individuals of *D. reticulatum* infected with *P. hermaphrodita* when collected from the field, or from *D. reticulatum* infected in the laboratory with xenically cultured *P. hermaphrodita*. Bacteria were isolated from three sources: 1) from the intestine of infective larvae of *P. hermaphrodita* following surface sterilisation; 2) from thriving foam-chip cultures of *P. hermaphrodita* which produced nematodes pathogenic to slugs; and 3) from field-collected *D. reticulatum* infected with *P. hermaphrodita*. In all cases isolation methods used were those described by Wilson *et al.* (1994b). Individual bacterial isolates were selected on the basis of colonial morphology, subcultured and maintained on slopes of nutrient agar at 4°C. Bacteria selected for further studies were identified using the Analytical Profile Index (API) system (la Balme les Grottes, 3830 Mantalieu Vercieu, France) and other standard bacteriological tests.

Monoxenic growth of *P. hermaphrodita* on different bacteria.

Sterile juveniles of *P. hermaphrodita* were obtained by immersing gravid adults overnight in 0.02% sodium ethyl-mercurithiosalicylate, then transferring the J1/J2 larvae to quarter strength Ringer's solution containing 500 units ml$^{-1}$ each of benzyl-penicillin and streptomycin sulphate for 24 hours. The juveniles were then washed twice in fresh sterile quarter strength Ringer's solution. One bacteriological loopfull of overnight nutrient broth culture of the test bacteria was spread over one half of the surface of a 3 cm diameter Petri dish containing a kidney-based nutrient medium. Ten sterile nematode larvae were placed at the edge of the dish opposite the bacteria, so that nematodes had to move at least 15 mm over bacteria-free medium to reach the test bacterium. Any contaminating bacteria associated with juveniles formed visible colonies in the "clean" half of the plates, and these plates were

discarded. Plates were incubated for three weeks at 15°C after which time the nematodes were flooded off into a known volume of water and counted.

## Injection of bacteria into *D. reticulatum.*

Nutrient broth cultures of test bacteria were grown at 25°C for 24 h. *D. reticulatum* (at least 20 per treatment) were then injected into the haemocoel in their tail region with 10μl of the appropriate bacterial culture using a micro-applicator fitted with a hypodermic needle. The slugs were kept for a period of one week at 10°C before mortality was recorded. Slugs injected with 10μl of sterile nutrient broth and untreated slugs were used as controls.

## Bioassay of nematode/bacterial combinations against *D. reticulatum.*

Nematodes were grown monoxenically in foam-chip cultures with selected bacterial strains in flasks using the methods described by Wilson *et al.* (1993b). Their ability to kill *D. reticulatum* was tested in a soil based bioassay in plastic boxes (Wilson *et al.,* 1993a). Soil aggregates (diameter 12-25 mm) were moistened with tap water, or tap water with one of five nematode doses as they were added to fill the box. Ten slugs were added to each box (at least two boxes were used for each nematode dose). The boxes were left at 10°C for five days after which slugs were transferred individually to Petri dishes lined with moistened filter paper. They were kept in these dishes for a further nine days after which time mortality was recorded.

## RESULTS

### Isolation of bacteria associated with *P. hermaphrodita.*

Over 150 isolates of bacteria as distinguished by colonial morphology were obtained. No bacterial isolate seemed to predominate, and none was isolated consistently. Bacteria selected for further study were identified as *Providencia rettgeri* (P.r.), *Serratia proteamaculans* (S.p.), *Moraxella osloensis* (M.o.), *Flavobacterium odoratum* (F.o.), *Flavobacterium breve* (F.b.), *Aeromonas hydrophila* (A.h), *Aeromonas* sp. (A.sp.), *Bacillus cereus,* (B.c.) *Sphingobacterium spiritovorum* (S.s.), *Pseudomonas paucimobilis* (P.p.) and three isolates of *Pseudomonas fluorescens* (P.f.), (isolate nos 1, 140 and 141). The isolate of *M. osloensis* has previously been referred to as *M. phenylpyruvica* (Wilson *et al.* 1994b) but has recently been identified as *M. osloensis* (A. von Graevenitz, pers. comm.).

### Monoxenic growth of *P. hermaphrodita* on different bacteria.

Figure 1 shows total numbers of nematodes per 3 cm Petri-dish following three weeks growth with thirteen different isolates of bacteria (mean relative values taken from three separate experiments). Most nematodes were found in cultures with *P. rettgeri*, but numbers were not significantly higher than in cultures with *S. proteamaculans*, *P. fluorescens* (isolates 140 and 141), *Aeromonas* sp. *M. osloensis*, *S. spiritovorum* or *F. odoratum*.
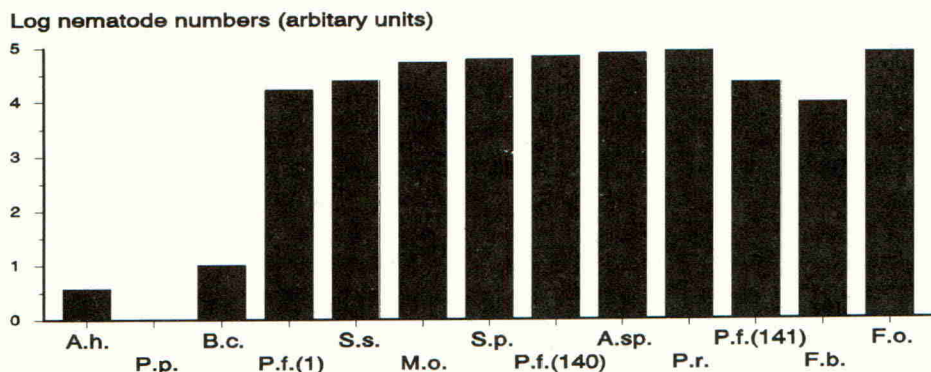
**Log nematode numbers (arbitary units)**



Figure 1. Numbers of nematodes per 3 cm Petri dish when grown for three weeks in monoxenic culture with 13 bacterial isolates (mean relative values from three experiments).

## Injection of bacteria into *D. reticulatum.*

Of the nine isolates of bacteria injected into slugs, only *A. hydrophila* and *P.fluorescens* (isolate 140) were pathogenic. Mortality of slugs injected with other bacteria or with sterile nutrient broth was not significantly different from untreated slugs (Figure 2).
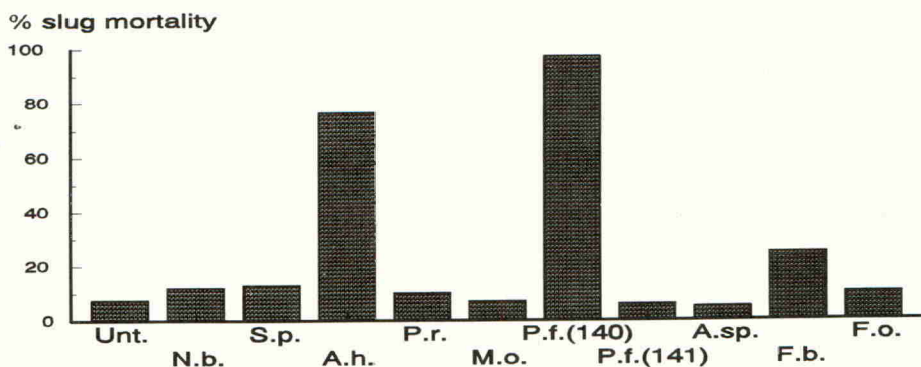
**% slug mortality**



Figure 2. Percentage slug mortality (7 DAT) of untreated slugs (Unt.) or slugs injected with 10µl of sterile nutrient broth (N.b.) or nutrient broth cultures of different bacteria.

## Bioassay of nematode/bacterial combinations against *D. reticulatum.*

There was a significant positive relationship between nematode dose and slug mortality for nematodes grown in monoxenic culture with *M. osloensis* and *P. fluorescens* (141). The $ED_{50}$ estimate (the number of nematodes per bioassay box needed to kill 50% of slugs after fourteen days) for nematodes grown with *M. osloensis* was 28,900 ($\log_{10}$ SE = 0.0605), significantly less than that for nematodes grown with *P. fluorescens* (141) ($ED_{50}$, 49,700,

$\log_{10}$ SE = 0.0947). There was no evidence of nematode induced mortality for nematodes grown in monoxenic culture with *P. fluorescens* (140), *S. proteamaculans* or *P. rettgeri*.
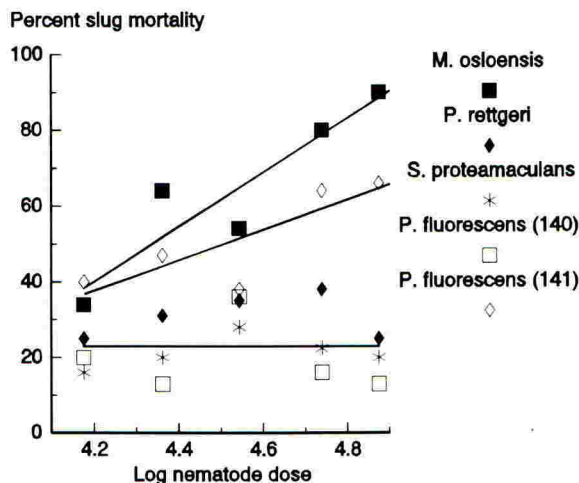
**Percent slug mortality**



Figure 3. Relationship between mortality of *D. reticulatum* and dose of nematodes grown in monoxenic culture with five different bacteria (14 DAT).

## DISCUSSION

In order for *P. hermaphrodita* to be commercialised as a biological control agent, it was necessary to select a species of bacterium capable of supporting prolific growth of *P. hermaphrodita* in monoxenic *in vitro* cultures. It was also necessary that this bacterium produced infective larvae capable of killing slugs. In this study, *P. hermaphrodita* was found to be capable of growing in monoxenic culture with a wide range of bacteria, although its growth and reproduction was strongly influenced by different bacterial isolates. However, *P. rettgeri*, the bacterium which supported best *in vitro* growth of *P. hermaphrodita*, produced nematodes which were not reliably pathogenic to slugs. It is not known why nematodes grown in monoxenic culture with certain bacteria differ in their ability to infect and kill slugs. However, the experiments in which different bacteria were injected into slugs in the absence of nematodes, showed that differences in pathogenicity between nematode/bacterial combinations do not result from differences in the pathogenicity of the bacteria alone. Both *P. fluorescens* (141) and *M. osloensis* produced nematodes which were pathogenic to slugs, but neither bacterium caused mortality in slugs, even when injected directly into the haemocoel in high numbers. Conversely, nematodes grown on *P. fluorescens* (140) were not pathogenic to slugs, even though this bacterium was pathogenic when injected into slugs. (*A. hydrophila*, which was pathogenic when injected into slugs did not support growth of *P. hermaphrodita*).

These and other experiments, *M. osloensis* produced nematodes that were consistently pathogenic to *D. reticulatum* and other slug species. It gave good yields of infective larvae of *P. hermaphrodita*, which were not significantly less than those achieved with *P. rettgeri*.

Therefore it was selected as the preferred bacterial isolate to produce *P. hermaphrodita* for field experiments and for selling to U.K. gardeners.

Selection of the best bacterium to grow heterorhabditid and steinernematid nematodes is generally a matter of isolating the nematode's natural symbiont. However, from this and other studies we believe that *P. hermaphrodita* may not be symbiotically associated with a specific bacterium. No single species was isolated consistently from within dauer larvae of *P. hermaphrodita* or from slugs infected with *P. hermaphrodita*. However, our studies of bacteria associated with *P. hermaphrodita* were done using a limited selection of nematode strains collected from one location, and in some cases, kept in laboratory culture prior to initiating studies on bacterial associations. Investigations of bacteria associated with a wide range of *P. hermaphrodita* isolates, preferably from many distinct geographical locations, should be done to confirm that no symbiont exists. It may be possible to find a bacterium which is superior to *M. osloensis* in supporting nematode growth and producing pathogenic nematodes, but without knowledge of the underlying mechanisms of nematode pathogenicity, selection would have to rely on screening large numbers of bacteria for their ability to support nematode growth, and bioassaying the resulting nematode/bacterium combinations.

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A COMPARISON OF BAITS CONTAINING DIFFERENT ISOLATES OF ENTOMOPATHOGENIC NEMATODES FOR THE CONTROL OF THE HOUSEFLY (*MUSCA DOMESTICA*) IN LARGE-SCALE LABORATORY ENCLOSURES

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ABSTRACT

The efficacy of a bait system for housefly control was tested with five different commercial isolates of entomopathogenic nematodes, against two strains of houseflies. The experiments were carried out in 10 m$^3$ polythene cubicles. For a comparison insecticidal baits containing methomyl or azamethiphos were also tested. After six days exposure, methomyl was most toxic to both housefly strains, *Heterorhabditis megidis* killed 91.6% to 95.7% of the houseflies, and was as effective as azamethiphos.

INTRODUCTION

The use of conventional insecticides for the control of houseflies (*Musca domestica*) in England and Wales is causing increasing levels of resistance (Chapman *et al.*, 1993). There is an urgent need for new methods of housefly control which will minimise the development of insecticide resistance. Renn (1990) described the development of a bait for the control of adult houseflies containing the entomopathogenic nematode *Steinernema feltiae* (= *bibionis*) which in the laboratory was able to attract and kill up to 100% of houseflies over a six day period. These experiments were completed at a high humidity (85% r. h.) in order to prevent desiccation of the nematodes. In the U. K., air humidities of between 45% and 75% have been recorded in animal units (Webb, pers. comm.) and these levels may cause fatal drying of the nematodes and thus render the bait system inoperative. Renn (1990) concluded that a system for reducing moisture loss from a nematode-containing bait for houseflies would be essential if the system were to be effective in practice.

To meet this objective a bait formulation for housefly control has been developed which minimises drying of the nematodes. The laboratory experiments reported here used this bait formulation to examine the pathogenicity of five isolates of entomopathogenic nematodes towards two strains of adult houseflies. They have also compared the efficacy of commercial housefly bait formulations of methomyl and azamethiphos with that of the nematodes. All of the experiments reported here were performed on a large scale using 10 m$^3$ polythene cubicles.

## MATERIALS AND METHODS

### *M. domestica* Strains

Two strains of *M. domestica* collected from pig-rearing units in the U.K. were used. Strain S, collected in 1985, was found to have low levels of resistance to a number of insecticides, including azamethiphos and methomyl (Renn, 1990). Strain 122 was collected in 1991 during a housefly resistance survey and was shown to have moderate resistance levels to methomyl and azamethiphos (Chapman *et al.*, 1993). Adult females of 1-3 days old were used in all tests.

### Nematodes

The details of the bait design used in this study are currently the subject of a patent application. Five isolates of nematodes, available in the UK as commercial products, were formulated into the bait system. The nematode species were: *S. feltiae* (= *bibionis*) (1) (Nemasys$^{Tm}$), *S. feltiae* (= *bibionis*) (2) (BioSafe N$^{Tm}$), *Steinernema carpocapsae* (BioSafe$^{Tm}$), *Heterorhabditis megidis* (Nemasys H$^{Tm}$) and *Heterorhabditis bacteriophora* (= *heliothidis*) (Fightagrub$^{Tm}$). One million nematodes of each isolate were used in each bait. For testing, each bait was placed on an 18.5 cm black filter paper on the floor of the cubicle 10 cm away from the mid-point of one of the vertical sides (described below).

### Insecticides

The manufacturers of the commercial formulation of azamethiphos (Alphacron$^{Tm}$) recommend that 10 g m$^{-2}$ should be applied to 2.5% of the wall and ceiling area of the building being treated. To achieve this, 1.45g of the 10% azamethiphos formulation was mixed with 7.25 ml water and this was spread evenly across a square piece of mounting board with a 38 cm side. Once the formulation had dried, one of these treated boards was taped to each of four vertical sides of a polythene cubicle (described below). Methomyl bait (Golden Malrin$^{Tm}$) was applied at 100g m$^{-2}$ of floor area by evenly spreading 2.9g of the dry formulation across the base of a 9 cm petri dish. Four of these baits were placed on the floor of each cubicle, so that one bait was 10 cm away from the mid-point of each vertical wall.

### Assessment of Baits

Experiments were carried out in 10 m$^3$ polythene cubicles (Morgan and Barson, 1994). Access into the cubicles, for placement of baits and assessment of the treatments, was via a 1.5 m zip fastener. Lactose solution (2.5% w/v), absorbed on to cotton wool, was supplied *ad libitum* as an alternative food source to the bait treatments.

The first set of tests were carried out to assess the effects of varying the numbers of nematode-containing baits per cubicle on housefly mortality. They were completed using the bait system formulated with *S. feltiae* (1) and strain S houseflies. Either one, two or four baits per cubicle were used. There were three replicate cubicles for each of the nematode treatments and three controls without nematodes. Approximately 100 female *M. domestica* of strain S were introduced into each cubicle. Experiments were carried out at 25°C with a 12 h

light regime of 700 lux intensity. The humidity within each cubicle was maintained at 55-70% r. h. by spraying tap water from a hand-held pot-plant sprayer. Mortality was assessed at 24h intervals after introduction of the houseflies by removing and counting the dead flies from each cubicle. The final count was on day 6, when the tests were terminated.

After the results of the first set of tests were evaluated, a second set of experiments were designed to compare housefly mortalities caused by the five different nematode isolates after formulation or the two insecticides. The two strains of houseflies were exposed to either one bait formulated with a nematode isolate, or an insecticide. There were three replicate cubicles for each treatment and three control replicates without a bait. All other experimental procedures were as described above.

Statistical analyses

Mortality counts for individual treatments of each *M. domestica* strain were compared using chi-square 2 x 2 contingency tables.

RESULTS

Effects of varying the numbers of nematode-containing baits

In replicates with one housefly bait containing *S. feltiae* (1) 58.8% of adult females had died by day 3 and 92.8% by day 6 (Table 1). These mortalities were significantly higher (P<0.001) than those observed in replicates containing either 2 or 4 baits. Therefore, for all subsequent tests only one nematode-containing bait was used in each replicate cubicle.

TABLE 1. Percentage mortalities of housefly strain S after exposure to different numbers of baits containing *S. feltiae* (1).

| Numbers of baits | Percentage Mortality | | N tested |
|---|---|---|---|
| | 3 days | 6 days | |
| 1 bait | 58.8 | 92.8 | 279 |
| 2 baits | 41.6 | 88.4 | 284 |
| 4 baits | 22.5 | 71.8 | 294 |
| Control | 1.3 | 1.3 | 300 |

Effects of different nematode isolates or insecticides

Mortality of female *M. domestica* strain S

Control mortality was 1.3% after 6 days (Table 2). After 3 days *H. megidis* and *S. feltiae* (1) were significantly more pathogenic than the other nematode isolates having both killed 58.8% of the strains flies. All houseflies of strain S died after 3 days exposure to

methomyl bait; this mortality was significantly greater than the 86.4% killed by azamethiphos bait (P<0.01; Table 2). Methomyl and azamethiphos baits were significantly more toxic to the houseflies than any of the nematode isolates after 3 days exposure (P<0.05; Table 2).

After six days exposure there was no significant difference in mortality from azamethiphos (97.7%), *H. megidis* (95.7%) or *H. bacteriophora* (96.7%) baits (P>0.05). Methomyl bait was still significantly more toxic to female houseflies of strain S than either azamethiphos or the five nematode isolates (P<0.01).

The mortality of houseflies of strain S exposed to *S. feltiae* (1) and (2), *S. carpocapsae, H. megidis, H. bacteriophora* or azamethiphos increased significantly from day 3 to day 6 (P<0.001).

TABLE 2.   Percentage mortalities of houseflies of strains S and 122, after exposure to baits containing different nematode isolates or insecticides.

| | Strain S | | | Strain 122 | | |
|---|---|---|---|---|---|---|
| | 3 days (%) | 6 days (%) | N tested | 3 days (%) | 6 days (%) | N tested |
| *S. feltiae* (1) | 58.8 | 92.8 | 279 | 76.2 | 90.0 | 290 |
| *S. feltiae* (2) | 49.6 | 85.3 | 299 | 48.3 | 91.6 | 286 |
| *S. carpocapsae* | 39.7 | 77.3 | 282 | 34.1 | 75.2 | 334 |
| *H. megidis* | 58.8 | 95.7 | 277 | 54.1 | 91.6 | 296 |
| *H. bacteriophora* | 50.3 | 96.7 | 304 | 34.3 | 74.3 | 329 |
| Azamethiphos | 86.4 | 97.7 | 309 | 72.9 | 88.1 | 303 |
| Methomyl | 100.0 | 100.0 | 300 | 97.7 | 100.0 | 304 |
| Control | 1.3 | 1.3 | 300 | 0.7 | 4.0 | 300 |

Mortality of female *M. domestica* strain 122
Control mortality was 4.0% after 6 days (Table 2). With the exception of methomyl bait, a slightly different pattern of mortalities emerged from that observed with strain S. On day 3, there was no significant difference in the numbers of strain 122 female houseflies killed by either azamethiphos (72.9%) or *S. feltiae* (1) (76.2%) (P>0.05). However, these mortalities were significantly greater than those caused by formulations of either *S. feltiae* (2) (48.3%), *S. carpocapsae* (34.1%), *H. megidis* (54.1%) or *H. bacteriophora* (34.3%) (P<0.001; Table 2). After 3 and 6 days, methomyl bait had killed 97.7% and 100% respectively and, was significantly more toxic to females than either azamethiphos or each of the nematode isolates (P<0.01).

By day 6 the numbers of strain 122 killed by all the bait treatments had risen significantly from day 3 (P<0.01) (Table 2). By this time, there was no significant difference in mortality between formulated *S. feltiae* (1), *S. feltiae* (2), *H. megidis* and azamethiphos bait

treatments (P>0.05). There was no difference in mortality between *S. carpocapsae* (75.2%) and *H. bacteriophora* (74.3%) (P>0.05) after 6 days exposure. However, the numbers of strain 122 houseflies killed by these two treatments were significantly lower than the number killed in all other treatments at this time (P<0.001).

Comparison of female *M. domestica* mortality between strains S and 122
On day 3 there was no significant difference in mortality between strains exposed to baits formulated with *S. feltiae* (2), *S. carpocapsae*, *H. megidis* (P>0.05; Table 3). *S. feltiae* (1) killed significantly more houseflies of strain 122 than strain S (P<0.001). Whereas significantly more houseflies of strain S were killed by *H. bacteriophora*, azamethiphos and methomyl than strain 122 (P<0.001; Table 2). On day 6, significantly more houseflies of strain S had succumbed to *H. megidis*, *H. bacteriophora* or azamethiphos compared with strain 122 (P<0.05). However, more of strain 122 died after exposure to *S. feltiae* (2) (P<0.05). There was no significant difference in mortality between the strains after 6 days exposure to *S. feltiae* (1), *S. carpocapsae* or methomyl.

DISCUSSION

When the mortality of strain S caused by different numbers of baits formulated with nematodes was examined, the mortality of houseflies was inversely proportional to the numbers of baits used in each cubicle. In cubicles containing four baits the houseflies would have had a greater number of choices to investigate than in cubicles with one or two baits. The interference from the greater number of baits may have caused the flies to spend less time on each of them. Therefore this shortened period of investigation would in turn, have given the nematodes less of an opportunity to parasitise houseflies. Such interference is well known in choice tests with plant-feeding insects, where Lewis and van Emden (1986) showed that the palatability of a food source is relative to the available alternatives.

At the end of the experiments some variation in the numbers of houseflies killed by each of the formulated nematode isolates was observed. There may be several factors which could be operating simultaneously to give different levels of housefly kill, including variations in the nematodes and the houseflies. Dunphy and Thurston (1990) state that this type of variation of nematode pathogenicity has been attributed to environmental adaptations of the nematodes, their behavioural differences and the number of symbiotic bacteria within the infective juvenile nematodes.

The response of both housefly strains to the formulated nematode isolates was also different by the end of the experiments. It was observed that *H. megidis* and *H. bacteriphora* killed larger numbers of housefly strain S than strain 122, while *S. feltiae* (2) killed larger numbers of strain 122 than strain S. The reasons for these differences are not clear. All entomopathogenic nematodes are able to penetrate their hosts via their natural bodily openings (mouth, anus and spiracles). But, the heterorhabditid nematodes also possess a dorsal tooth, which can be used to break thinner parts of the insects cuticle and allow entry into the heamocoel. Sawicki and Lord (1970) suggested that a thicker housefly cuticle may explain the differences in the responses of susceptible and resistant insects since it may delay the entry of insecticides. If the cuticle was thicker in the more resistant strain 122, the

Heterorhabditids may have been less able to break through the strain's cuticle and so this extra route of infection would have been impaired or lost altogether. Alternatively the different mortalities of each housefly strain may have been a result of differing behaviour patterns. Strain S had been in laboratory culture longer than strain 122, so its behaviour may be quite different since strain 122 had more recently been exposed to insecticidal baits.

The results show that when *H. megidis* was formulated into this bait system it was the most consistently pathogenic of the nematode isolates and killed over 90% of both housefly strains. This study demonstrates that this formulation for entomopathogenic nematodes is successful in killing considerable numbers of insecticide-resistant houseflies in large arenas and is as effective as a commercial insecticide bait containing azamethiphos. Renn (1994) has shown that housefly larvae can be controlled with encapsulated nematodes and the simultaneous use of the two techniques will ensure control of *M. domestica* in animal rearing facilities.

ACKNOWLEDGEMENTS

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BIOLOGICAL CONTROL OF SCIARID FLIES AND SHORE FLIES IN GLASSHOUSES

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ABSTRACT

Laboratory and glasshouse experiments were conducted in Ohio, USA and Bridgemere, UK using *Steinernema feltiae* and *Steinernema carpocapsae* isolates for control of sciarid (*Bradysia*) and shore fly (*Scatella*) larvae. The predatory mite *Hypoaspis miles* was also evaluated for the control of both insect groups. The most effective nematodes against *Bradysia* larvae were *S. feltiae* and *S. carpocapsae* Umeä isolate. *H. miles* was also very effective and provided control equal to the standard insecticide treatment (Diazinon). Shore fly control was more difficult to assess, but in one experiment, both *S. feltiae* and *H. miles* reduced fly numbers for 1-4 weeks, but total numbers trapped during the experiment were not significantly different from the untreated. Similar results were obtained with drench applications of insect growth regulators.

INTRODUCTION

Sciarids and shore flies are sometimes considered as simply nuisance pests, but it is now clear that sciarid larvae can stunt or kill young seedlings and transplants by feeding on the roots, sometimes causing heavy losses in propagation. Shore flies are polyphagous feeders on several classes of algae. Shore fly larvae do not damage plants directly, but the adults cause indirect injury to ornamentals by leaving fecal spots on leaves (Foote, 1977; Zack and Foote, 1978). Adults of both insect groups can aid in the spread of several plant pathogens, including *Pythium, Verticillium, Cylindrocladium, Sclerotinia* and *Theilaviopsis* (Stanghellini, 1991).

Because of restrictions on the use of conventional insecticides in glasshouses, biological controls, including entomopathogenic nematodes, have been evaluated for the control of these flies. Recent studies using isolates of the nematodes *Steinernema feltiae* and *Steinernema carpocapsae*, have shown their efficacy against sciarid fly larvae (Buxton, 1993, Gouge and Hague, 1993; Lindquist and Piatkowski, 1993). *Hypoaspis miles* predators have also been used for sciarid control in research and commercial glasshouses (Matteoni, 1993). There is little information published on the efficacy of these biological control agents against shore flies

(*Scatella sp*). In this paper, the results of trials with biological control of sciarid and shore flies are presented.

## METHODS

Experiments in Ohio, USA used individual plants in cheesecloth cages in the glasshouse to separate different treatments. Test plants in the first trial were potted chrysanthemums (variety Nob Hill), while the trial against shore flies used maize plants. Nematodes were made up to recommended rates (Table 1) in distilled water and applied in 30-60 mls of water per 10.5 cm diameter pot. Microscopic examination just prior to application was used to determine that the nematodes were active. Nematode species and isolates within species used in one or more experiments included *S. feltiae* (Otio, Mexican and No 27 isolates) and *S. carpocapsae* (All and Umeä isolates). All nematodes were obtained from commercial sources and handled as suggested by each supplier. Application times, rates and intervals varied, depending upon the nematode and experimental design. *H. miles* was obtained from a commercial insectary, and subsequently reared in the laboratory on *Tyrophagus sp* mites. A natural infestation of sciarid or shore flies was present in the pots at the time of the first treatment. Efficacy of treatments was determined by weekly counts of the number of adult flies on 5 x 5 cm yellow sticky traps (two per cage) placed face up on the pot edges. There were three replicates of each treatment. Later trials, using the same methods investigated the effect of insect growth regulator insecticides on a naturally occurring population of shore flies.

In the UK, trials were conducted in a commercial nursery growing a range of nursery stock cuttings in plastic trays, including azaleas, rhododendrons and skimmias, using a compost of 50/50 peat and bark placed over a heating element and misted over frequently to maintain high humidity. The plot size was 10 trays (approx 1m x 2m). Plastic screens were used to separate groups of trays and the whole area was covered with plastic sheeting to prevent emigration or immigration of flies. Treatments evaluated included *S. feltiae* (Strain 76, marketed as Nemasys in the UK) and *Bacillus thuringiensis var Israeliensis* (marketed as Vectobac in the USA). Efficacy of treatments was determined by weekly counts of the number of adult flies on a 17.5 x 6.5 cm yellow sticky trap. There were four replications of each treatment.

RESULTS

TABLE 1. Numbers of sciarid fly adults on potted chrysanthemums at Wooster, Ohio.

| Treatment | Rate ($10^6$/m²) | Timing (wks after planting) | No of + applications | Mean no of flies trapped # | S.E. |
|---|---|---|---|---|---|
| *S. carpocapsae* All | 2.5 | 3 | 1 | 105.7 b | 24.3 |
| *S. carpocapsae* All | 2.5 | 3 | 3 | 83.5 b | 19.7 |
| *S. feltiae* Otio | 2.5 | 3 | 1 | 79.5 b | 19.7 |
| *S. feltiae* Otio | 2.5 | 3 | 3 | 55.8 bc | 8.6 |
| *S. carpocapsae* Umeä | 4.0 | 3 | 1 | 56.7 bc | 8.1 |
| *Hypoaspis* | 25/pot | 1 | 1 | 1.7 d | 0.3 |
| Diazinon ME * | | 3 | 1 | 12.5 d | 4.6 |
| Untreated | - | - | - | 140.5 a | 52.3 |
| LSD (5%) | | | | 28.2 | |

+ multiple applications were made one week apart.
* 120 mls of solution applied per pot
# means followed by the same letter are not significantly different.  (Duncan's Multiple Range Test)


In the Ohio trial, the Umeä strain of *S. carpocapsae* gave better control than the All strain, while *S. feltiae* Otio strain was best overall   (Table 1).   *H. miles* predatory mites were significantly better than any other biological treatment, even though only one application of the mites was made.


TABLE 2. Numbers of sciarid fly adults in a trial at Bridgemere Nurseries, UK.

| Treatment | Rate ($10^6$/m²) | Mean no flies per trap* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 28 Nov | 5 Dec | 12 Dec | 19 Dec | 2 Jan+ | 9 Jan | 16 Jan |
| Untreated | - | 48.4a | 30.3a | 19.5a | 31.6a | 51.4a | 28.6a | 19.6a |
| *S. feltiae* | 1.0 | 98.4a | 22.5a | 12.9ab | 19.8b | 26.9a | 12.2a | 9.4b |
| *S. feltiae* | 1.5 | 42.3a | 15.8a | 10.5b | 19.5b | 29.6a | 14.3a | 12.0ab |
| SED | | 30.0 | 7.2 | 3.5 | 4.6 | 13.2 | 7.4 | 3.6 |

* Nematodes applied twice;  on 21/11 and 11/12.
+ Two week count; traps not assessed during Christmas week.  Means followed by the same letter are not significantly different (Duncan's Multiple Range Test).

In the nursery trial there was no effect of treatments until two weeks after the first application of nematodes; this was expected as only sciarid larvae are affected, not adult flies. There was no significant difference between the two application rates of nematodes (Table 2); both reduced the numbers of sciarid flies compared with the untreated controls, although the differences were not significant on every sampling date. The *S. feltiae* strain used was strain 76.

TABLE 3. Numbers of shore fly adults on potted maize plants at Wooster, Ohio.

| Treatment+ | Application rate | No applications | Mean total no trapped | SE |
|---|---|---|---|---|
| *S. feltiae* 27 | $2.5 \times 10^6/m^2$ | 3 | 254.2a | 17.6 |
| *H. miles* | 25/pot | 1 | 252.2a | 36.0 |
| Untreated | - | | 320.5a | 18.9 |

+ Treatments applied two weeks after trapping began. Trapping continued for eight weeks in total. Means followed by the same letter are not significantly different (Duncan's Multiple Range Test).

Overall, there were no significant differences in catches of shore fly adults after application of control agents in the experiments in Ohio (Table 3), but some reduction occurred in weeks 4-8 following the *S. feltiae* applications and weeks 8-10 after *H. miles* application. (Table 4).

TABLE 4. Numbers of shore fly adults at Wooster, Ohio, over a ten week period.

| Treatment | Mean number of flies per week | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *S. feltiae* 27 | 15.5 | 14 | 23 | 27.5 | 28 | 24 | 18 | 18.3 | 44.2 | 41.7 |
| *H. miles* | 10.7 | 11 | 21.7 | 35.8 | 36 | 38.7 | 32 | 14.8 | 23.7 | 27.8 |
| Untreated | 8.2 | 19 | 27.2 | 46.8 | 45 | 38.7 | 24 | 25.8 | 52.5 | 33.3 |

Numbers of shore flies caught on the sticky traps continued to rise on the untreated pots during the first four weeks of the experiment, but after this time numbers were variable. As occurred in the sciarid fly trials, there was a delay in the effect seen from nematode treatment. This is because the nematodes have little or no effect on shore fly pupae or adults, so pupae continued to emerge as flies for some time after treatment. The reason for the even longer interval between applications of *H. miles* predators and reduction in shore fly numbers is not known.

TABLE 5. Numbers of shore fly adults on potted impatiens plants at Wooster, Ohio.

| Treatment | Rate (gm/l)+ | No. Applications++ | Mean total no. flies per trap* | S.E. |
|---|---|---|---|---|
| fenoxycarb 25WP | 0.16 | 2 | 27.8 a | 7.3 |
| fenoxycarb 25WP | 0.32 | 2 | 13.3 a | 1.8 |
| cyromazine 75WP | 0.01 | 2 | 18.5 a | 3.7 |
| cyromazine 75WP | 0.01 | 3 | 13.0 a | 0.7 |
| untreated | | | 19.0 a | 4.0 |

+   150 mls drench solution per pot.
++ Two applications:  16 June, 23 July;  Three applications:  16 June, 23 June, 23 July.
*   Treatments applied one week after trapping began.  Trapping continued for 10 weeks in total.  Means followed by the same letter are not significantly different (Duncan's Multiple Range Test).

At the time of the first drench of insect growth regulator, a mixed population of adult shore flies and larvae and pupae was present.  Because insect growth regulator materials only affect actively growing larvae, the adults were able to continue oviposition, and this may be the reason why overall counts of adult flies were not significantly different from the untreated pots (Table 5).

TABLE 6. Numbers of sciarid fly adults in a second trial at Bridgemere Nurseries, UK.

| Treatment | Rate mls/100 litres | 3/11 | 10/11 | 17/11 | 24/11 | 31/11 | 7/12 |
|---|---|---|---|---|---|---|---|
| Untreated | - | 33.3 | 40.5 | 30.5 | 41.3 | 34.0 | 24.3 |
| *Vectobac | 600 | 38.0 | 28.3 | 20.0 | 19.5 | 28.0 | 15.5 |
| *Vectobac | 1200 | 33.3 | 21.3 | 17.0 | 23.0 | 19.5 | 14.0 |
| SED (5%) | - | 26.3 | 25.3 | 24.5 | 33.0 | 30.2 | 20.5 |
| | | ns | ns | ns | ns | ns | ns |

*A commercial formulation of *Bacillus thuringiensis* var *israeliensis* containing 1200 ITUs per mg was applied on 27/10, 3/11 and 10/11 as a drenching wet spray to compost in a UK nursery.

No reduction in catches of adult sciarids was noted in the UK experiment until 10/11; but this was expected as *Bacillus thuringiensis var Israeliensis* is only active against sciarid larvae (Table 6).  Treatments consistently reduced the numbers of adult flies during the trial, but the differences were not statistically significant.  It was noticeable that sciarid larvae which had been affected by the treatments came to the compost surface before dying.

DISCUSSION

The trials in both the USA and UK showed that nematodes could give excellent control of sciarid fly larvae, *Bradysia spp*, but there were differences in effectiveness of different species and strains. Overall, *S. feltiae* was more effective than *S. carpocapsae* but in one trial, *Hypoaspis* predatory mites were better than several applications of nematodes. UK work showed that strain 76 of *S. feltiae* could give good control of sciarids for at least 5-6 weeks, while the *Israeliensis* strain of B.t. was also effective under commercial conditions. A major benefit of all these biological materials is their safety to the operator, to the environment, and to young plant material. However, because of their mode of action they tend to kill the larvae more slowly than chemical insecticides, therefore applications of biological materials should be made as early as possible.

With shore flies, the trials work so far in the USA has shown only a limited control from *S. feltiae* and *H. miles*, and insect growth regulator insecticide but more detailed work on this pest is needed. In petri-dish tests, Gouge (1994) showed that larvae of *Scatella stagnalis* could be infected by infective juveniles of both *S. feltiae* and *S. carpocapsae*. However, in a commercial nursery, shore flies can survive in very wet areas, such as under the benches, which may be unsuitable for the biological control agents and can act as sites for reinfestation of the crop. Because these wet areas encourage growth of algae, shore fly numbers can build up very rapidly, so nursery hygiene as well as chemical or biological controls are important for the control of this pest. In addition, these flies can readily breed outside and then enter glasshouses via the vents and re-infest the crop, so screening of the vents may be beneficial.

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## CONTROL OF SCIARIDS IN GLASS- AND PROPAGATION HOUSES, WITH *STEINERNEMA FELTIAE*

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ABSTRACT

*Bradysia* spp in propagation houses and glasshouses can be controlled with applications of the nematode *Steinernema feltiae*. The nematodes are not effective at temperatures in excess of 25-26°C. *S. feltiae* can be applied through conventional sprayers by flood irrigation, but not through high pressure systems fitted with fine nozzles as damage to nematodes may occur.

INTRODUCTION

Sciarid flies are serious pests of glasshouse ornamentals in the UK (Binns, 1973; Freeman, 1983), the most important species belonging to the genus *Bradysia*. Biological control of sciarids has become important partly because of an increase in insecticide resistance (White and Gribben, 1989) but also because many growers are now using biological methods for controlling other insect pests where the use of insecticides is undesirable.

*Steinernema feltiae* is the nematode species most used to control sciarids (Richardson and Grewal, 1991), the infective juvenile of the nematode enters the sciarid larva through the mouth or anus (Gouge, 1994) and rapid death follows due to septicaemia caused by the introduction of bacteria of the genus *Xenorhabdus* into the insect haemocoel. In mushrooms sciarid control with *S. feltiae* is well documented (Grewal and Richardson, 1993; Nickle and Cantello, 1991) and preliminary experiments in the UK have also reported satisfactory control in propagation houses (Gouge and Hague, 1993), and in glasshouses (Gouge and Hague, 1994).

This paper describes three experiments, one in a propagation house and two in glasshouses, conducted under commercial growing conditions.

MATERIALS AND METHODS

Experiment 1  Control of *Bradysia amoena* in a propagation house

Two UK strains of *S. feltiae* were used in this experiment. One was recovered from soil at the University of Reading, cultured in *Galleria mellonella* at 23°C and then sent to Biosys, Palo Alto, California, who produced a commercial formulation for application. The other strain is marketed as Nemasys.

Typically, propagation houses are maintained at a relatively high temperature and humidity, providing excellent conditions for sciarid reproduction. Small seedlings/cuttings are particularly vulnerable to sciarid attack under these conditions. The propagation house used for this work was 200 $m^2$ with bays containing 6 rows of trays, each tray containing 198 cells (plugs) with individual seedlings. Mixed propagules of rose, hydrangea, clematis and actinidia were grown in a 4:3 bark:peat media. The house was maintained at 25°C by underfloor heating and misted automatically to give a constant relative humidity of 92%. The pH of the medium was 4.1 and the plants were exposed to a 16 h photoperiod. The two isolates were extracted from the formulated material according to the manufacturer's instructions. The nematodes were applied at a rate of 960,000 nematodes $m^{-2}$ close to the recommended dose. Nematode viability in each sample was tested before application. Fifty $m^2$ of propagules were treated with each nematode, with a further 50 $m^2$ untreated.

## Estimation of the sciarid populations and nematode persistence in plugs

Yellow sticky traps were hung 40 cm above the crop with one trap per 12.5 $m^2$ of propagules, to estimate numbers of adult sciarids. Traps were changed every 7 days. Six plugs were taken at random at the beginning of the experiment and then every 7 days until the completion of the experiment. Nematodes in the plugs were assessed by placing one *G. mellonella* larva in each plug and the number of nematodes in the *Galleria* larvae counted.

## Control of sciarids in glasshouses

### Experiment 2 Control of *Bradysia paupera* in a cyclamen crop
Mixed cultivars of cyclamen were grown individually in 8 cm plastic pots in a peat based compost. All pots were top watered daily, the temperature in the glasshouse was maintained at 20-26°C and there were 14,000 plants per treated bay. The formulation of *S. feltiae* was applied at the following rates per pot: 0; 2,500; 5,000; 10,000; 20,000. Applications were done with a Brinkman hydraulic sprayer and samples of the nematodes were examined visually for any damage after passage through the sprayer. The effect on the sciarid population was assessed by suspending sticky traps 0.4 m above each bay, each sticky trap covering 32 $m^2$ of bench area: the first traps were hung one week before nematode application to assess the initial sciarid population and traps were replaced weekly until the completion of the experiment.

### Experiment 3 Control of *Bradysia tritici* in Asiatic lilies
The glasshouse in which this experiment was to be done was fitted with an overhead irrigation system which utilised a Dosatron injector set with 4 Bar back pressure and fine nozzles. *S. feltiae* passed through this system lost nearly 75% of their viability and therefore the overhead irrigation method was replaced by a Carroila motorised sprayer (Truchem Ltd) applying the nematodes by flood irrigation. Hybrid Asiatic lilies have a very short growth period (7-10 weeks) and sciarid control is normally achieved with gamma HCH followed by Lindane smokes weekly. Prior to sale a final treatment with gamma HCH is given to ensure that flies do not appear after the plants are sheathed in plastic. These methods of control are expensive so alternative methods with *S. feltiae* were investigated. Three Asiatic lily bulbs were planted in Levingtons MCI compost (pH 4.6) in 1.2 l pots. The glasshouse was fumigated and therefore the sciarid population was assumed to be minimal prior to the start of the experiment. Two blocks of 1,500 pots were treated with the following applications:

1.  600,000 nematodes m$^{-2}$ on days, 0, 20 and 38 giving a nominal dosage of 24,000 nematodes per pot.  2.  Gamma-Col (15 ml per 100 l of water $\equiv$ 150 ml/pot) on days 0, 27 and 40.  3.  Two applications of 600,000 nematodes m$^{-2}$ on days 0 and 20 followed by Gamma-Col on day 40.

The population of *B. tritici* was monitored by caging 4 pots from each treatment on days 0, 7, 14, 20 (prior to second nematode application), 31 and 45, and counting the number of adult sciarids emerging.  Nematode persistence in the pots was monitored throughout the experiment using *G. mellonella* larvae as live bait (Fan and Hominick, 1991).

RESULTS

Experiment 1

In the propagation house the viability of the nematode formulations was about 95%. One week after application of the nematodes, *B. amoena* was almost completely eliminated and this degree of control was maintained until the end of the experiment (Fig 1).  Both nematode strains persisted throughout the experiment although there was a 75% fall in the nematode population in the plugs after 60 days (Fig 2).

Fig 1.  The mean number of *B. amoena* caught on sticky traps per 12.5 m$^2$ of propagation tray
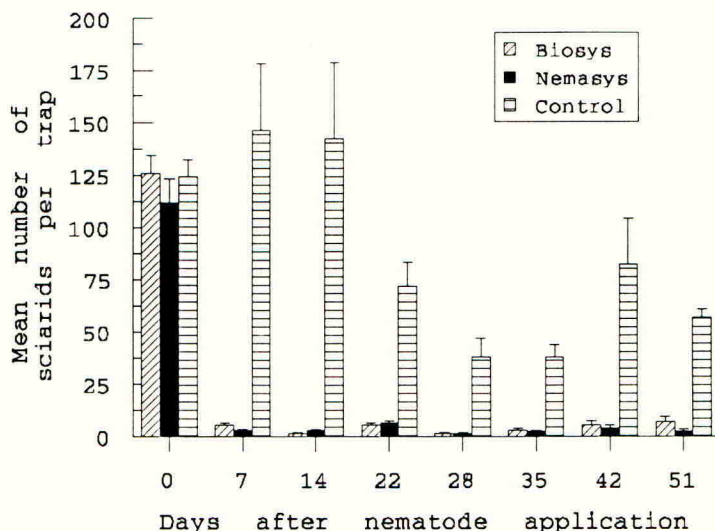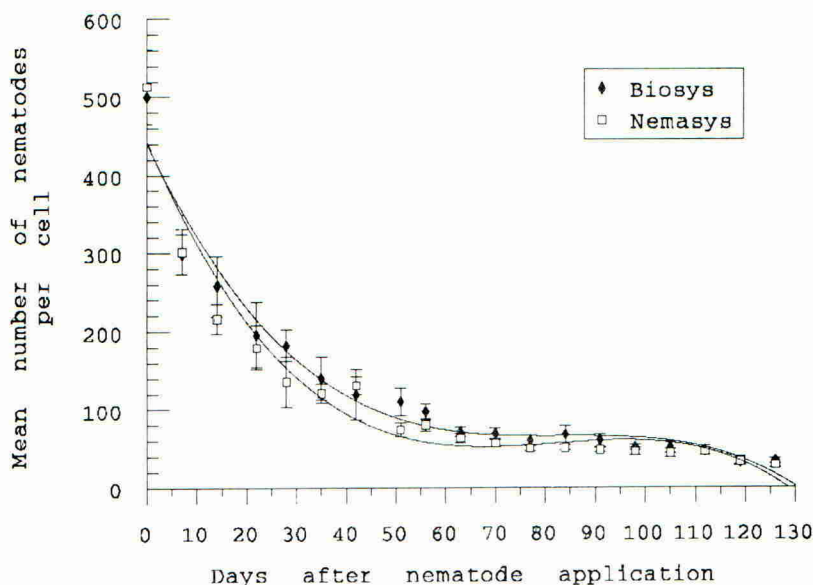
Fig 2.  The mean number of nematodes recovered from single cells in a propagation tray



## Experiment 2

*B. paupera* populations declined markedly 7 and 14 days after application of the nematodes, the most effective control being obtained from the highest dose of 20,000 nematodes per pot (Fig 3).  Although the number of sciarids caught from the untreated plots was always greater than the number from the nematode treated plots, it was noted that the population of *B. paupera* in the untreated plots also fell markedly (Fig 3).  Traps placed in the glasshouse at the beginning of the experiment also caught the sciarid parasitoid *Synacra holconata* (Hymenoptera: Diapriidae) which may have caused reductions in the sciarid population in the glasshouse.

## Experiment 3

The number of *B. tritici* emerging from Gamma-Col treated pots was significantly higher than from the other treatments (Table 1).  The low number of flies emerging from the nematode treated pots still rendered the crop unsaleable and they had to be treated with Gamma-Col before sale.  The grower was most satisfied with the combination of nematodes with Gamma-Col.  The relatively poor performance of the three applications of nematode was unexpected, but examination of the temperature records within the glasshouse revealed that at midday from days 21-31 the temperature was between 28 and 32°C, nematodes being almost completely eliminated by day 31.  The cost of treating 200 m$^2$ of Asiatic lilies (4,700 pots) by normal methods, eg Gamma-Col and Lindane smokes, is about £375, compared to about £185 for two nematode treatments followed by a final Gamma-Col application and thus there is a considerable economic saving as well as reduction in the use of insecticide on the crop.

Fig 3. The mean number of *B. paupera* caught on sticky traps for 36 m$^2$ of glasshouse bench area
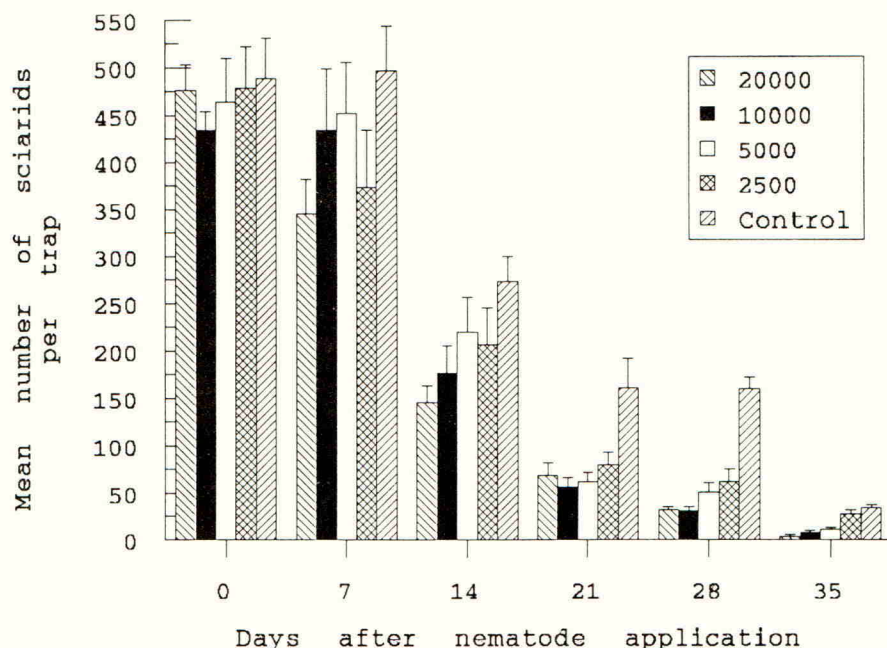


Table 1. The mean number of *B. tritici* emerging from treated pots (S.E. values in brackets).

|  | Days after treatment | | | | | |
|---|---|---|---|---|---|---|
|  | 0 | 7 | 14 | 20 | 31 | 45 |
| *S. feltiae* | 0 | 0 | 0 | 3 (1.1) | 3 (0.9) | 1.25 (0.5) |
| Gamma-Col | 0 | 0 | 20 (1.6) | 7 (1.3) | 45 (5.7) | 0.5 (0.4) |
| *S. feltiae* + Gamma-Col | 0 | 0.25 (0.21) | 0 | 1.75 (0.9) | 3 (0.35) | 0.25 (0.21) |

## DISCUSSION

In the propagation house *S. feltiae* suppressed *B. amoena* populations to a satisfactory level, confirming the result with another Bradysia species, *B. paupera* (Gouge and Hague, 1993). In glasshouses and propagation houses it is difficult to undertake satisfactory experiments because treatments cannot be isolated from migrating adult sciarids either from

the untreated plots or from outside the house. These adult sciarids can easily become infected (Gouge, 1994) and will transfer nematodes to both treated and untreated plots.

Temperature is a major factor influencing control. At temperatures above 26°C for prolonged periods *S. feltiae* will lose effectiveness and even at normal temperatures (15-26°C) the population will decline (Fig 2). For the most satisfactory results *S. feltiae* should be used between 15 and 26°C.

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NEMATODE CONTROL OF LEAFMINERS: EFFICACY, TEMPERATURE AND TIMING

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ABSTRACT

The maintenance of high relative humidities has been shown to enhance the survival/efficacy of entomopathogenic nematodes in the foliar environment. The minimum length of time high humidity is required is critical in determining the potential of nematodes as biological control agents for leafminers. This study has shown that, in the laboratory, application of *Steinernema feltiae* results in a high mortality at all larval instars of *Liriomyza huidobrensis,* provided high humidity (>90% r.h.) exists throughout the pest larval stages. However, high humidities need not be sustained for this long. Late second/early third instars suffered high mortality when nematodes were left on the leaf surface for only 10 h at 15-25°C. It took longer for nematodes at 15°C and 25°C to achieve the same mortalities when applied against late first/early second instar larvae. At 20°C there was no difference between instars in the time required to achieve equivalent mortalities.

INTRODUCTION

The first outbreaks of the dipteran leafminer *Liriomyza huidobrensis* in the United Kingdom were recorded in 1989. Although early infestations were associated with chrysanthemum and other ornamentals, the pest quickly spread to vegetable crops (Cheek *et al.*, 1993). The larvae of *L. huidobrensis* feed on the spongy mesophyll and palisade layers of the leaf and the adults puncture the leaf surface when ovipositing and feeding. Both cause cosmetic and physiological damage, and even lightly mined leaves show significant reductions in photosynthesis (Parrella *et al.*, 1985).

In recent years entomopathogenic nematodes have been considered as potential control agents for leafminers. Adult nematodes enter affected plants via leafminer oviposition holes/feeding sites or tears in the leaf surface (Harris, *et al.*, 1990), attacking the larvae which they subsequently encounter in the underlying tissues. Preliminary investigations into the use of *Steinernema carpocapsae* (Harris *et al.* 1990) and *Heterorhabditis bacteriophora* (Olthof & Broadbent, 1991) for the control of *Liriomyza trifolii* have both given promising results. However, a major factor limiting their successful use is the need to maintain high humidity after application. While *S. carpocapsae* application resulted in a mean leafminer mortality of 67% at relative humidities greater than 92%, mortality was considerably reduced at lower humidities (Hara, *et al.*, 1993). High humidity is likely to be particularly important while nematodes are still on the surface of the leaf, but once nematodes enter mines, protection from desiccation is afforded by the leaf tissues.

This paper compares the mortality of *L. huidobrensis* after *S. feltiae* are left for varying lengths of time, at high humidity, on the surface of the leaf. The effect of application of *S. feltiae* to different life stages of *L. huidobrensis* at different temperatures is reported.


## MATERIALS AND METHODS

### Nematode products and insect cultures

Commercial formulations of *S. feltiae*, (Nemasys) were used in all experiments. Nematodes were stored at 5°C in water for up to two months, and after each experiment stocks were subjected to an infectivity bioassay with *Galleria mellonella* as described by Fan & Hominick (1991). Only results from experiments using nematode populations with infectivity levels greater than 15% were analysed. *L. huidobrensis* was reared in perspex cages on a variety of vegetable plants (Williams & Macdonald, in press). Cultures were maintained at $25 \pm 3°C$ with a 12:12 h light:dark regime and an artificial dawn and dusk. All experiments were conducted under the same light:dark regime.

### Infestation of plants and treatments

Between 50 and 100 mixed sex *L. huidobrensis* adults, all older than 24 hours, were released into each perspex cage containing six to eight week old tomato plants cv. Moneymaker. After a 24 h egg laying period, flies were removed and the resulting larvae allowed to develop to the instar required for each investigation. Treatments included a suspension of 10000 *S. feltiae*/ml with 0.02% of a non-ionic wetting agent Agral (a.i. alkyl phenol ethylene oxide), and a control without the nematodes. Treatments were sprayed until run off at a rate of 1400-1600 l/ha, using a hand held sprayer.

### Efficacy against different instars

Three sets of 30 tomato plants were infested simultaneously. When the appropriate instar had developed, half of each set was sprayed with *S. feltiae*, the other half as a nematode free control. Each set was immediately placed in a cabinet at $20 \pm 1°C$ and >90% r.h. until prepupae were about to emerge. The plants were then cut at the base, sealed in plastic bags and returned to $20 \pm 1°C$. The total number of pupae that developed from each plant was recorded.

### Time of entry by *S. feltiae* into a leaf

Infested tomato leaves were detached at the base of the petiole and placed on a plastic gauze suspended over a container of water. Each container of leaves was sprayed with either a nematode suspension or water. A sample of ten leaves was removed from each treatment, and the containers were then sealed to ensure high humidity was maintained. The containers were incubated at temperatures between 15 and 25°C, with the first 12 hours in darkness. At intervals from 1 to 24 h after the container was sealed, further samples of ten leaves were removed. In order to fix the nematodes on the leaf surface, the leaf samples were immediately dried by blowing cool air across them until no surface moisture was apparent. Each dried leaf was placed on a petri dish filled with 1.2% Japanese agar, and the number of larvae present

counted using a microscope. Leaves were then returned in their uncovered petri dishes to the temperature from which they were taken. When late third stage larvae had developed, the dishes were sealed to prevent prepupae escaping. All leaves were assessed for pupal production.

RESULTS

At 20°C and constant humidities of >90% r.h. very few larvae survived to pupation after application of *S. feltiae* (Fig.1). There was a significant difference in pupal production between the *S. feltiae* treated plants and the water control (F= 25.97, d.f. = 1,84, p<0.001). There was no significant difference between the pupal production after application of the nematodes to different instars (F= 0.36, d.f. = 2,84, p>0.5) suggesting that the instar to which *S. feltiae* was applied was not important.

Figure 1. Efficacy of *S. feltiae* against three *L. huidobrensis* larval instars.
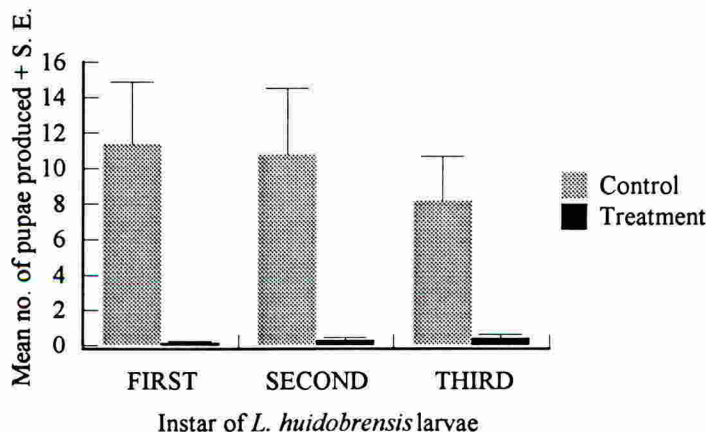


There was a significant linear relationship for 2nd/3rd instar larvae between percentage larval survival and log time elapsed after application of *S. feltiae*, when data from all temperatures were combined (y = 57.41 - 35.38 log time, F = 36.11, d.f. = 1,23, p<0.001; Fig. 2a). This relationship explained 61% of the variance. *L. huidobrensis* mortality increased from an average of 67.3 ± 7% when *S. feltiae* were fixed after 2 h to 88.7 ± 3% after 10 h on the leaf surface. After a 24 hour period, mortality was not significantly improved (83 ± 4%). Fitting individual lines for each of the temperatures did not improve the strength of the relationship, and no significant effects of temperature on the survival of 2nd/3rd instar larvae were recorded (F = 0.91, d.f. = 2,18, p>0.05).

When *S. feltiae* was applied to the younger (1st/2nd instar) larvae the relationship between percentage larval survival and log time explained only 47.3% of the variance (Fig 2b). The strength of the relationship was increased by fitting individual lines for each of the temperatures investigated (F= 7.19, d.f.= 2,36, p<0.01). When nematodes were left on the leaf surface for 12 h at 15°C, mortality was 48.5 ± 14% compared to a mean of 87 ± 8% at 20°C after the same time period. At 25°C, 57 ± 10% larval mortality was recorded after 11 h.

Figure 2. Percentage survival of *L. huidobrensis* larvae after *S. feltiae* was exposed to >90% r.h. for varying lengths of time on the leaf surface. Nematodes applied to (a) late second/early third instar larvae or (b) to late first/early second instar larvae.
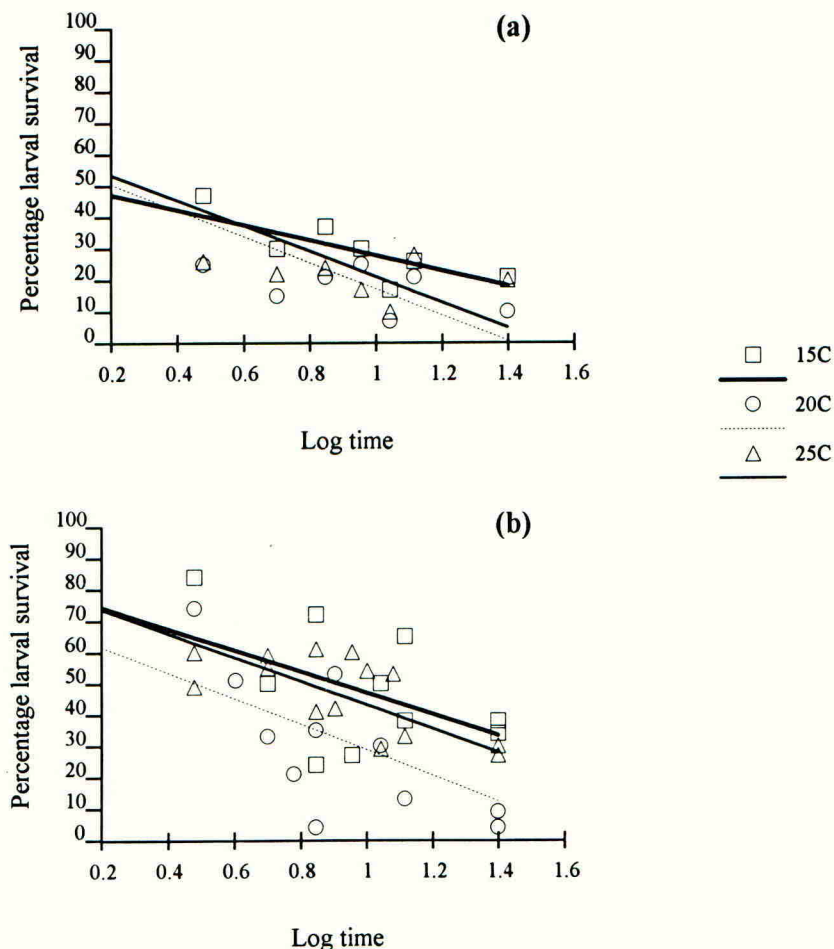


Comparison of the slopes of the regression lines, between percentage larval survival and log time, showed no differences for different larval instars at the same temperature. At 20°C there was no significant difference between the length of time taken by the nematodes to successfully parasitise a high proportion of each larval instar of *L. huidobrensis*. ($F = 3.18$ d.f.=1,22 $p>0.05$). At 15°C the intercepts of the lines were significantly different ($F = 9.92$, d.f. 1,16 $p<0.01$) and showed that *S. feltiae* parasitised more 2nd/3rd instar *L. huidobrensis* in the same period of time than when applied to 1st/2nd instar larvae. At 25°C, there was also a significant difference in the intercept between instars ($F = 21.22$, d.f. 1,20 $p<0.001$) which showed that 2nd/3rd instars suffered a higher mortality at this temperature.

DISCUSSION

At high humidities *S. feltiae* is extremely effective against the leafminer *L. huidobrensis.* The larval instar to which the nematodes are applied is not critical if this high humidity can be maintained for the duration of the larval stage of the leafminer lifecycle.   However, in commercial cropping systems, most host plants would not be able to tolerate such high humidities for these long periods of time without suffering damage (Clover, 1994).  At lower humidities of 80 $\pm$ 10% r.h.  the second instar of *L. huidobrensis* is more susceptible to applications of *S. feltiae* than other instars (Williams & Macdonald, in press).  This result is consistent with work done on *L. trifolii* (Lebeck *et al.*, 1993).

High humidity need not be maintained for extensive periods, only long enough to enable the entry of the nematode into the oviposition puncture or tear in the leaf surface (Williams & Macdonald, in press).  The results of this study indicate that the period of time required for a nematode to successfully enter the leaf tissues, and subsequently cause the death of the larvae, may vary with pest instar and temperature.  Temperatures in the range 15-25°C do not effect the time required by the nematodes on the leaf surface when *S. feltiae* are applied to late second, early third instar larvae. High larval mortality is achieved if nematodes are left for up to 10 h on the surface of the leaf, with little significant improvement when left for longer. However, with younger (1st/2nd) *L. huidobrensis* larvae, there does appear to be an effect of temperature at 15°C and 25°C. At these two temperatures the larvae required longer to achieve the same mortality during the first 24 hours than when the nematodes were applied at either 20°C or at a later instar.

The observation that different temperatures did not significantly effect larval survival when *S. feltiae* are applied to late second/early third instar larvae could be explained by the leaves being more extensively mined at this stage.  *L. huidobrensis* larvae tend to graze through both palisade and spongy mesophyll tissues of the leaf, probably making more opportunities for the nematodes to enter either through feeding punctures underneath which larvae have mined or through an increased number of tears in the leaf surface.

With younger larvae, the longer time required by nematodes on the surface of the leaf could result from less extensively developed mines.  It is known that the feeding rate of *L. huidobrensis* decreases considerably with temperature.  Thus at lower temperatures less carbon dioxide may be produced by the larvae with the result that the nematodes could be taking longer to respond to this cue, which has been shown to be important in host finding (Gaugler *et al.*, 1990).  However, this would not explain why it takes longer at 25°C to achieve the same level of pest mortality as at 20°C and further work is required to investigate these observations.

The initial results of this study show that temperature might be important when applying nematodes to younger larval instars of *L. huidobrensis* if high humidity cannot be sustained for long periods.    In commercial production systems, conditions of high humidity and temperatures of 20°C are rarely maintained consistently.   Therefore, targeting nematode applications to different instars at the appropriate temperature might be an important element in a leafminer control strategy.

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THE EFFECTS OF TEMPERATURE AND ULTRA-VIOLET IRRADIATION ON CONIDIA OF *METARHIZIUM FLAVOVIRIDE*

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ABSTRACT

Conidia of *Metarhizium flavoviride* can be formulated in vegetable and/or mineral oils and applied as a biological pesticide against locusts and grasshoppers. Use against these pests involves tolerance to environmental constraints such as low humidity, high temperatures and high levels of UV irradiation. Formulating in oils overcomes the constraint of low humidity and dried conidia can tolerate at least three hours exposure to 55°C without loss of viability. UV irradiation remains a serious constraint and there was a marked interaction between temperature and irradiation; the adverse effect of a given dose of irradiation was 2-3 times greater when the dose was given while the conidia were at 55°C compared with conidia held at 25°C. The addition of chemical sunscreens to the formulation did not increase tolerance to UV.

INTRODUCTION

The International Institute of Biological Control (IIBC), The International Institute of Tropical Agriculture (IITA), Cotonou, Benin, and the Département de Formation en Protection des Végétaux (DFPV) Niamey, Niger are collaborating in a programme for biological control of the desert locust *Schistocerca gregaria* and Sahelian grasshoppers (Prior *et al.*, 1992). Research is aimed at using the conidia of entomopathogenic fungi formulated in an oil mix suitable for Ultra Low Volume (ULV) application. Oil formulation of conidia increases their efficacy, especially under conditions of low humidity (Prior *et al.*, 1988; Bateman *et al.*, 1993). Isolates of *Metarhizium* species, usually *M. flavoviride* obtained from West Africa , have proved most suitable for this work.

The formulated mycopesticide will mainly be used in environmental conditions of high temperatures, low humidity, and high light intensities. Conidia of *M. flavoviride*, when dry and oil formulated, can tolerate temperatures of 55°C for a few days (McClatchie *et al.*, 1994) and low humidities (Bateman *et al.*, 1993), but radiation in the ultraviolet (UV) region is very harmful to conidia (Moore *et al.*, 1993). The most damaging ultraviolet wavelength, UVC, is largely filtered out by the atmosphere, so conidia are therefore mainly exposed to UVB (wavelengths ranging from 280-320 nm) and UVA (from 320-400 nm) with the former being the most damaging for conidia of *Metarhizium flavoviride* (Moore *et al.*,1993).

Half-lives of 100 and 360 minutes have been reported for *Metarhizium anisopliae* exposed to UV irradiation although under field conditions plant architecture, cloud cover, the position of conidial deposition and other factors will influence the rate of inactivation

(Fargues *et al.*, 1988; Inglis *et al.*, 1993). Secondary uptake, from sprayed vegetation, has a significant beneficial effect in causing mortality (Lomer *et al.*, *1993* ) and increasing the persistence of conidia may be crucial in optimising control by secondary infections (M. B. Thomas, pers. comm).

Many sunscreens have been developed by the cosmetic industry to filter out these wavelengths by acting as either physical or chemical absorbers. Included in the chemical absorbers are para-amino benzoates, cinnamates, salicylates and benzophenones (Shaath, 1990). Relatively little work has been conducted on the use of these chemicals as protectants of fungi, most studies having been carried out using bacteria and viruses, but Moore *et al.*, (1993) demonstrated protection by some of these chemicals in oil formulations of conidia of *M. flavoviride*. That work used conidia directly harvested from agar plates and other work has demonstrated that the moisture content of the conidia can greatly influence their properties. For example, ensuring that the conidia are dried when in the oil diluent increases their tolerance to high temperature (McClatchie *et al.*, 1994).

In the present work the effects of interactions between temperature and simulated sunlight on the viability of conidia of *M. flavoviride* were examined at IIBC UK . Chemical sunscreens were also examined to determine their efficacy.

## MATERIALS AND METHODS

### Conidial suspensions

The isolate investigated was *M. flavoviride* IMI 330189 (International Mycological Institute) obtained in 1989 from *Ornithacris cavroisi* (Orthoptera: Acrididae) found at Niamey, Niger.

Production involved a two phase system; the first being liquid production of blastospores and mycelium, based on the system of Jenkins and Prior (1993). Brewers yeast and sucrose (20:30 g of each) was added to 1 litre of water, heated to boiling point and homogenized at high speed for 30 s. 75 ml of the homogenate was added to each 250 ml flask and autoclaved. After cooling, 1 ml of conidial suspension (approximately $8 \times 10^6$ conidia/ml) was added to each flask which was placed on a shaker for three days. The solid phase used 75 ml of the culture added to 500g of autoclaved rice in a plastic bag. The inoculated rice was thoroughly mixed to ensure complete coverage of the rice with the inoculum. The bags were then loosely inflated with air from a laminar flow, sealed and incubated for 6-10 days at around 28-32°C. After conidiation the bags were carefully opened, excess moisture removed from the inner surface of the bags and the rice left to dry for 24 h ; subsequently the rice was put on trays to dry for a further 48 h. Extraction was by sieving conidia off the rice; the conidia were put into Shellsol T, dried non-indicating silica gel was added and the formulation stored at 8°C until exposed to sunlight simulation.

### Solar Simulation

For exposure to simulated sunlight, 0.07 ml of the conidial formulation was pipetted onto 33mm diameter petri-dishes and spread over the whole dish giving an oil depth of about 100 μm corresponding, very approximately, to the diameter of a large droplet in ULV

spraying.

Sunscreen formulations were irradiated using a 1000w (J s$^{-1}$) solar simulator (Oriel Corp, Stratford, Ct, USA) which produces a collimated uniform beam (152 x 152 mm) and, by using a specific arrangement of filters can simulate a range of UV radiations. The one used was with an aluminized mirror which reflects the visible and infra-red towards the target along with the UV. This is obtained with minimal sample heating, the temperature not exceeding 30°C, well below damaging levels (McClatchie *et al.*, 1994). UVC was removed using a blocking filter The samples were therefore exposed to irradiation closely simulating the natural situation with UVB, the wavelengths most harmful to *M. flavoviride* (Moore *et al.*, 1993), UVA and longer wavelengths.

The conidia were maintained at different temperatures by floating the petri-dishes containing the conidial samples in a water-bath directly under the beam from the sunlight simulator.

Conidial Viability

Viability of conidia was assessed by germination tests on petri dishes of Sabouraud Dextrose agar. A drop of oil suspension was placed onto the agar with a tiny spatula and then spread thinly over the surface. The plates were incubated at 26°C for 22 or 24h before germination counts were made, depending on the particular experiment and, where these numbers were low, after a further 24 h incubation. Conidia were examined under the microscope (X300 magnification) and germination recorded when the germ tube was visible; 300 conidia were examined for each assessment.

Experiment 1 The effect of different temperatures on conidia of *Metarhizium flavoviride* exposed to simulated solar radiation.

Samples of formulation were exposed to 1, 2 or 3h of sunlight simulation at one of three temperature, 25°, 40° and 55°C. Comparable controls were exposed to three hours at these temperatures in a water bath without exposure to UV. There were four replicates of each treatment. Germination was assessed after 24 h and 48 h of incubation.

Experiment 2 The effect of different chemical sunscreens on viability of conidia exposed to UV irradiation at two temperatures.

Three chemical sunscreens were selected on the basis of work (Moore *et al.*, 1993 ; T.R. Hunt unpublished data) which had produced variable results: Eusolex 8021 (Merck) a mixture of 61% Eusolex 8020 (4-Isopropyl-dibenzoylmethane) and 39 % Eusolex 6300 (3-(4-methylbenzylidene)-camphor), Oxybenzone (2-hydroxy-4-methoxybenzophenone) and ethyl cinnamate. These were added to the formulations at concentrations of 2% or 4%. Control formulations contained no sunscreens. The formulations (five replicates of each) were exposed to UV irradiation for 4 h at either 25° or 40°C . Additionally all formulations were exposed to 40°C for 4 h without exposure to UV irradiation as controls.

Where necessary germination results were transformed by the arcsine transformation of the square root of the proportion of the germination data.

## RESULTS

### Experiment 1

The three temperatures influenced germination of the conidia as reflected by the levels of germination after 24 h incubation, with those exposed to 25°C showing highest levels and those exposed to 55°C the lowest germination levels (table 1). The final levels, after 48 h incubation were all around 97%. Exposure to simulated solar radiation resulted in decline of viability, especially at the higher temperatures (figure 1). At 25°C 3h exposure to simulated solar radiation resulted in a loss of viability from 97% to 84%; at 55°C the same sunlight simulator exposure resulted in viability declining to 57%.

TABLE 1. The germination (percent ± S.D.) of conidia of *Metarhizium flavoviride* exposed to 25°, 40° or 55°C for 3 h.

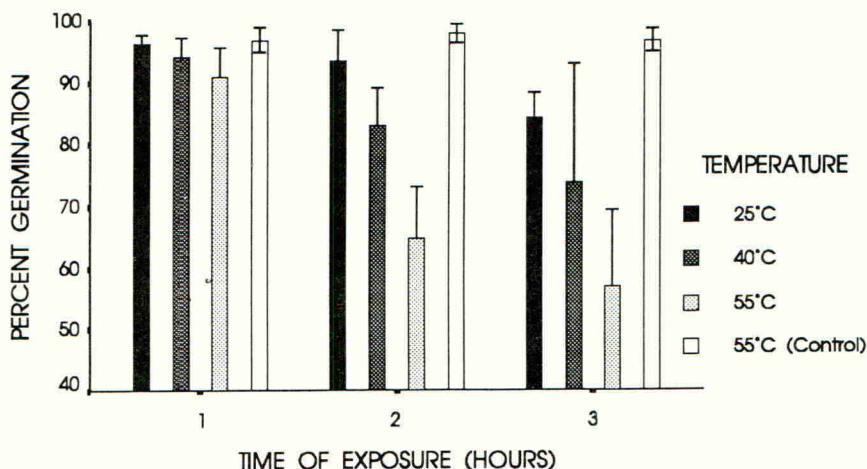| Incubation h. | 25° | 40° | 55° |
|---|---|---|---|
| 24 | 84.6 ± 2.61 | 76.4 ± 2.18 | 63.6 ± 5.52 |
| 48 | 97.7 ± 0.66 | 97.4 ± 0.85 | 96.8 ± 1.16 |



FIGURE 1. Germination of conidia of *Metarhizium flavoviride* after exposure to simulated solar radiation at different temperatures. Error bars are 95% confidence limits.

### Experiment 2

Control germination levels (4 h exposure to 40°C) ranged between 72-88% with no significant difference between the treatments. The three sunscreens tested did not provide any

protection to the conidia; for both temperatures the control treatment (with no added sunscreens) showed the highest levels of germination (table 2). There were no effects of adding 4% sunscreen as opposed to 2%.

TABLE 2. The germination (percent ± S.D.) of conidia of *Metarhizium flavoviride* exposed to simulated solar radiation at two temperatures.

| Treatment | | 25°C 4 h sunlight | 40°C 4 h sunlight | 40°C no sunlight |
|---|---|---|---|---|
| Ethyl | 2% | 53.3 ± 13.09 | 42.3 ± 12.09 | 86.3 ±  4.24 |
| cinnamate | 4% | 53.6 ±  9.80 | 37.7 ± 13.57 | 87.5 ±  4.46 |
| | | | | |
| Oxybenzone | 2% | 55.5 ±  9.12 | 36.9 ± 13.40 | 86.1 ±  4.75 |
| | 4% | 56.6 ± 15.56 | 47.1 ± 12.54 | 88.4 ±  6.60 |
| | | | | |
| Eusolex 8021 | 2% | 63.5 ± 18.51 | 29.0 ± 17.49 | 75.2 ± 19.43 |
| | 4% | 58.7 ± 18.11 | 26.3 ±  4.61 | 76.9 ±  3.84 |
| | | | | |
| Control | | 65.2 ±  8.21 | 54.2 ±  7.24 | 79.9 ± 12.48 |

## DISCUSSION

The work demonstrated that oil formulations of *Metarhizium flavoviride* could tolerate temperatures of up to 55° C for at least 3 h, in line with previous work (McClatchie *et al.*, 1994). However high temperature did appear to delay germination as did exposure to UV and higher temperatures resulted in a greater deactivation of fungal conidia with exposure to a constant dose of simulated UV irradiation. Addition of the sunscreens did not result in increased survival of conidia.

The rapid inactivation of conidia exposed to UV has serious implications for practical field control. If flying swarms are the target the exposure to UV would be of very short duration. However with locusts and grasshoppers, secondary uptake of the mycopesticide from vegetation is likely to be a vital component of good control (Nguyen, 1980; Lomer *et al.*, 1993). Under those circumstances inactivation would be slower because of protection of the conidia from direct sunlight afforded by the plant architecture (Fargues *et al.*, 1988; Inglis *et al.*, 1993) but even so additional protection may be needed. Chemical sunscreens were shown to provide some protection with an enhanced UV dose of simulated solar radiation and older conidia are more resistant (Moore *et al.*, 1993) but the present and some previous work have found no benefit of chemical sunscreens with a more realistic UV dose (T.R. Hunt unpublished data). Isolates vary in their susceptibility to UV and selection or genetic engineering may result in increased UV resistance for a mycopesticide . This UV resistance and formulations to protect against UV are likely to be of great significance in field control.

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IMPROVED BIOLOGICAL CONTROL BY CHANGING POLYOLS/TREHALOSE IN CONIDIA OF ENTOMOPATHOGENS

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ABSTRACT

The use of entomopathogenic fungi to control agricultural pests has been limited by the narrow range of relative humidity and temperature in which spore germination and host infection can occur in both protected and field environments. We have examined the effect of water activity ($a_w$) and carbohydrate concentration on polyol and trehalose accumulation in conidia of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* to improve inoculum quality. Modified conidia of all three species, containing increased quantities of glycerol and erythritol, germinated more quickly and over a wider $a_w$ range than unmodified control conidia. Conidia with elevated trehalose concentrations remained viable during a 17-week storage period for longer than those from control treatments. Conidia with enhanced levels of low molecular weight polyols were more pathogenic against larvae of *Galleria mellonella* than unmodified conidia over a range of water availabilities. Enhanced viability, accelerated germination and an extended water availability range for conidial germination can thus result in improved virulence under sub-optimal environmental conditions in the field.

INTRODUCTION

Fungal biological control agents have attracted much interest as an alternative method for controlling insect pests and plant pathogens (Gillespie, 1988). However, they can be ineffective if conidia loose viability or are unable to germinate and infect at sub-optimal water availability (Doberski, 1981). Although methods for optimizing production of spore numbers in liquid or solid substrate fermentation have been developed, little attention has been given to the manipulation of spore physiology as a means of improving inoculum quality.

Polyhydroxy alcohols (polyols) are known to accumulate in fungal cells at low water availability (van Eck *et al.*, 1993) and act as compatible solutes to enable essential cell functions to be carried out. It has been suggested that trehalose enhances desiccation tolerance of fungi grown under reduced water availability (Harman *et al.*, 1991). Previous studies have shown that significant accumulations of low molecular weight polyols occurs in conidia of entomopathogens as osmotic concentration was modified with KC1 (Hallsworth & Magan, 1994a) and with modifications of carbohydrate source and concentration (Hallsworth & Magan, 1994b) although there were differences between *B. bassiana*, *M. anisopliae* and *P. farinosus*. Trehalose was found to accumulate at intermediate water activities ($a_w$) and with slightly increased concentrations of carbohydrates in *B. bassiana* and *P. farinosus* but not *M. anisopliae*.

This paper presents work on the effect of physiological manipulation on accumulation

of polyols and trehalose in conidia of these three entomopathogens, germination at reduced water availability, and virulence of modified conidia against larvae of *G. mellonella.*

## MATERIALS AND METHODS

### Organisms and media

Fungal isolates used in this study were *B. bassiana* (Isolate 206, HRI, Wellesbourne) and *M. anisopliae* (V90), and *P. farinosus* (V51) both from Rothamsted Experimental Station, Harpenden. Stock cultures were stored at 5°C and had been sub-cultured on Sabouraud Dextrose Agar (SDA, Oxoid Ltd).

Media used in this study were SDA ($0.988$ $a_w$), SDA + KCl ($115$ g $l^{-1}$; $0.930$ $a_w$), glycerol ($141.4$ g $l^{-1}$; $0.0952$ $a_w$), trehalose ($378$ g $l^{-1}$; $0.972$ $a_w$) and trehalose+KCl ($378$ and $54$ g $l^{-1}$ respectively; $0.955$ $a_w$; *M. anisopliae* only). The media were inoculated with 2 mm agar plugs from the growing margins of 14 d old cultures of each fungus. After 30 days conidia were wet harvested by flooding cultures with 10 ml AnalaR water containing 1% Tween 80 and agitation with a glass rod. Suspensions of conidia were filtered through glass wool into 30 ml Universal bottles and centrifuged immediately for 12 min at 4000 rev min$^{-1}$ in a MSE Cenetaur 2. The centrifuged conidia were resuspended in AnalaR water and frozen at -80°C before lyophilization using a Edwards EF4 Modulyo freeze dryer. All experiments were conducted with three replicates per treatment.

### Extraction and detection of polyols and trehalose

Five mg dry conidia plus 1 ml AnalaR water were sonicated for 120 s using a Soniprep 150 (Fisons), at an amplitude of 28 µm. A Dionex series 4500 HPLC (Dionex (UK) Ltd, Camberly, Sussex) fitted with a pulsed electrochemical detector was used for the determination of polyols and trehalose. Fifty µl standard solutions of glycerol, erythritol, arabitol, mannitol and trehalose were used to calibrate the HPLC and for comparisons with conidial extracts. The method has been described in detail elsewhere (Hallsworth & Magan, 1994a). The limits of detection using this system was between 1.6 and 4.2 ng µl$^{-1}$ for polyols and 2.8 to 5.9 ng µl$^{-1}$ for trehalose. Polyol and trehalose content was calculated as mg g$^{-1}$ dry spores.

### Germination of modified conidia

Conidia from the SDA, SDA+KCl, glycerol, trehalose and trehalose+KCl treatments were harvested and suspended in polyethylene glycol (PEG) 600 or PEG 600+200 of the same $a_w$ as the test germination medium. Conidia were spread immediately on the surface of germination medium in range 0.887 to 0.989 $a_w$. This germination medium was based on SDA with 30% of the normal nutrient concentrations present. A 9 mm cork borer was used to randomly remove discs of medium from the various treatments after 11 and 240 h. They were stained with lactophenol/cotton blue, covered with a coverslip and examined at x200 and x400 magnification to assess number of germinated spores. Spores were considered to have germinated if the germ tube was longer than the diameter of the spore. A total of 100 conidia from each disc and from each of the three replicate plates were counted.

Germination after long-term storage of conidia

Conidia were harvested from cultures grown on SDA, and trehalose or trehalose+KC1 media. The harvested spores stored in sterile Petri dishes in a desiccator for 17 weeks at 70% equilibrium relative humidity (ERH) at 25°C. Conidia were suspended in PEG 600 (0.989 $a_w$) and spread onto SDA plates which contained PEG 600 to maintain an equivalent $a_w$. This allowed comparison between the germination of conidia obtained from SDA, trehalose and trehalose+KC1 treatments.

Bioassays of modified conidia with *Galleria mellonella*

Fifteen 19 d old larvae were inoculated with 1 µl conidia of each treatment suspended in PEG 200 (approx. $2 \times 10^7$ conidia $ml^{-1}$) on the top and in the centre of the cardavers. Each group of 15 larvae were incubated in triplicate in separate jars at two different relative humidities, i.e. 100 and 86.5% ERH, for up to 15 d at 25°C in the dark. The cardavers were subsequently placed at 100% ERH to allow production of conidia which was taken to be indicative of mycosis.

RESULTS

Polyols and trehalose content of modified conidia

Figure 1 shows an example of the changes obtained in the polyols and trehalose content of conidia of *B. bassiana*. On SDA, mannitol was predominantly present in conidia of this species. However, in the presence of SDA+KC1, the quantities of mannitol were significantly reduced, while that of the lower molecular weight polyol erythritol was increased. In the presence of glycerol, conidial content of glycerol and erythritol were significantly increased when compared to those from SDA alone. By contrast, media containing trehalose+KC1 resulted in a significant increase, almost 50-fold, in trehalose content when compared to SDA. The trend for *M. anisopliae* and *P. farinosus* was similar although the accumulation of specific polyols was species dependent.

Germination of modified conidia

Figure 2 shows that at a $a_w$ of 0.951 germination of conidia from SDA+KCl treatments were relatively low when compared to that of conidia containing modified levels of polyols. For example, there was a significant increase in the germination of conidia from the glycerol and SDA+KC1 treatments when compared to SDA. For *M. anisopliae* although no conidia from the SDA treatment had germinated after 25 h, 60-70% had germinated of conidia of the three species with a high trehalose content.

Germination after long term storage

Figure 3 shows that conidia which contained a very high content of trehalose germinated significantly better than those from SDA. This was found for all three species. For *B. bassiana* and *P. farinosus* there was also an improvement in the germ-tube extension of modified conidia (Hallsworth and Magan, unpublished data).

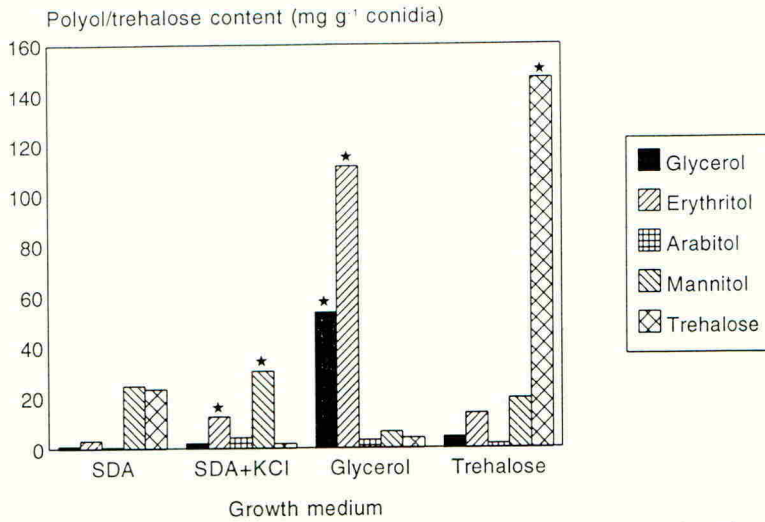Polyol/trehalose content (mg g⁻¹ conidia)



Figure 1. Summary of the mean concentrations of different polyols and trehalose in conidia obtained from media based on Sabouraud Dextrose Agar (SDA), SDA+KC1, glycerol, trehalose or trehalose+KC1. Asterisk show significant treatment differences (P < 0.05).
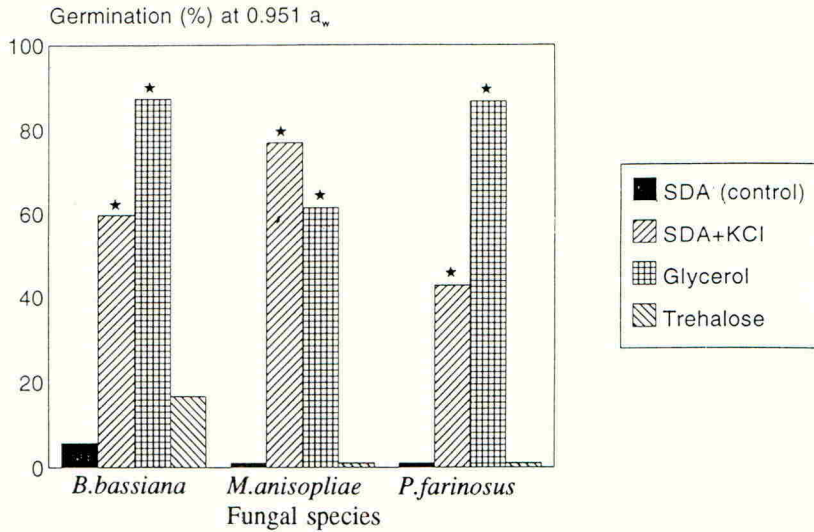


Figure 2. Mean percentage germination of conidia from different treatments at 0.951 water activity ($a_w$) for *B. bassiana* (after 44 h), *M. anisopliae* (25 h) and *P. farinosus* (61 h). Asterisks indicated significant treatment differences (P < 0.05).

Virulence of modified conidia against larvae of *G. mellonella*

The death of *G. mellonella* larvae after 9 and 15 d at two different ERH levels after inoculation with modified conidia are shown in Table 1. This shows that conidia grown on media modified with glycerol were more virulent than those grown on SDA causing significantly more mycosis at both 96.5 and 100% ERH.
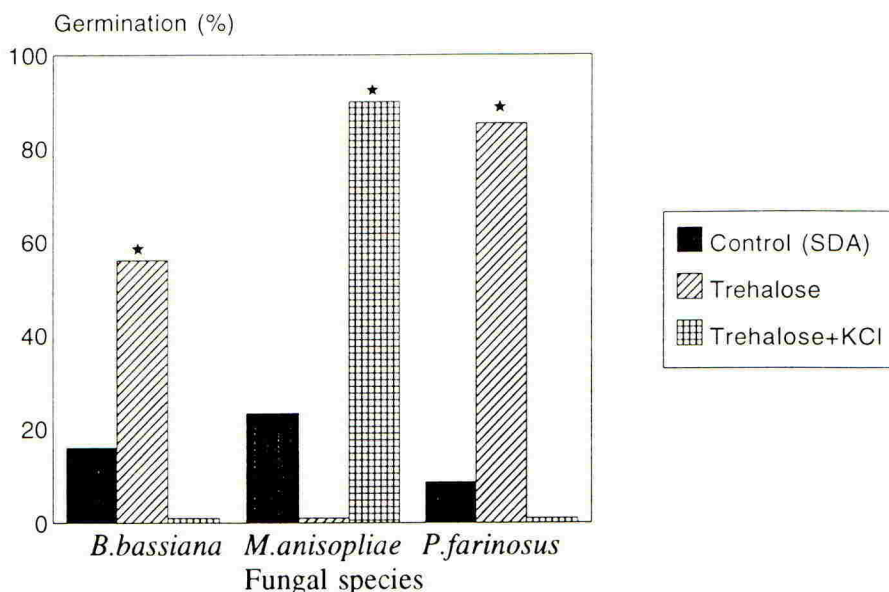
Figure 3. Comparison of germination of conidia containing low and elevated amounts of trehalose after 17 weeks storage. Asterisks show significant treatment differences (P < 0.05).

Table 1. Mean percentage death of larvae of *Galleria mellonella* at two relative humidities (%, ERH) and 25°C. Results are given for percentage killed after 9 and 15 days..

| | *B. bassiana* | | | | *M. anisopliae* | | | | *P. farinosus* | | | |
| | Control(SDA) | | Glycerol | | Control(SDA) | | Glycerol | | Control(SDA) | | Glycerol | |
| Time (d) | 9 | 15 | 9 | 15 | 9 | 15 | 9 | 15 | 9 | 15 | 9 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ERH(%) | | | | | | | | | | | | |
| 100 | 57 | 100 | 80 | 97 | 67 | 100 | 97 | 97 | 77 | 100 | 90 | 100 |
| 86.5 | 12 | 12 | 34 | 59 | 23 | 35 | 90 | 97 | 49 | 100 | 90 | 100 |

DISCUSSION

This study has shown that manipulation of carbohydrate content or water availability of growth media can significantly influence the accumulation of polyols and trehalose in conidia of entomopathogens. Of particular interest was the ability of conidia with increased amounts of the low molecular weight polyols such as glycerol and erythritol to germinate more rapidly over a wide $a_w$ range. There is potential for producing fungal inocula with accumulations of specific polyols for improving biological control agents in the field. Previous reports with inocula of entomopathogens in liquid culture have increased glycogen content of blastospores and mycelial biomass resulting in improved germination, but consideration of polyols/trehalose, or germination at reduced $a_w$ were excluded (Lane *et al.*, 1991).

The improved viability and germination of conidia containing elevated amounts of trehalose in the conidia after long term storage suggests that modification of its content may be useful for improvements in desiccation tolerance and to improve formulations. Previously, spores of *Trichoderma harzianum* and *Aspergillus japonicus* with increased trehalose content were shown to have better viability during storage (Harman *et al.*, 1991; Gornova *et al.*, 1992). However, in these studies water availability was not considered.

Bioassays with *G. mellonella* larvae at different relative humidities show the potential which exists for using inocula with better competence by modification of polyol concentrations resulting in more rapid infection and killing of the larvae. This study has demonstrated that even at reduced ERH the pathogenicity of these entomopathogens can be significantly improved. Potential thus exists for manipulation and improvement of mass production of inocula with improved field performance.

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THE POTENTIAL OF OIL-BASED SUSPENSIONS OF *METARHIZIUM ANISOPLIAE* CONIDIA FOR THE CONTROL OF THE HOUSEFLY (*MUSCA DOMESTICA*), A PEST OF INTENSIVE ANIMAL UNITS

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ABSTRACT

Conidia of the entomopathogenic fungus *Metarhizium anisopliae* suspended in linseed oil were pathogenic to male and female houseflies from 0-2, 1-3, 4-6 and >6 day-old age groups. Fecundity of infected females was reduced by up to 74% compared with untreated controls. Treated males were able to transfer conidia to untreated females during copulation resulting in high female mortality and reduced fecundity in females, from the 0-2 days old age group. The potential for housefly control using oil based formulations of *M. anisopliae* conidia is discussed.

INTRODUCTION

The housefly (*Musca domestica*) continues to be a serious pest of intensive animal units in the United Kingdom (UK). Continuous applications of insecticide space sprays and toxic baits has led to widespread resistance (Chapman *et al.*, 1993). This increase in resistance together with recent current awareness of the impact of toxic chemicals on the environment has created a need to develop new non-chemical methods for housefly control.

The susceptibility of houseflies to entomopathogenic fungi, particularly the Deuteromycetes, reported by Steinkraus *et al.*, (1990), and more recently Barson *et al.*, (1994), shows that these pathogens have potential as biocontrol agents for this pest. Many attempts to use entomopathogenic fungi for the control of agricultural insect pests in the field have given inconsistent and often poor results (Gillespie 1988). This lack of success has been attributed to the prevailing environmental conditions, in particular, where high relative humidity is considered essential to achieve infectivity (Gillespie, 1988). Conidia of *Metarhizium* spp, when formulated in vegetable oils, are infective at low relative humidities (Bateman *et al.*, 1993) and have superior efficacy against adult houseflies by a factor of two, compared with water based formulations (Barson *et al.*, 1994).

Speed of kill is an important factor when considering entomopathogenic fungi as biocontrol agents for houseflies because a protracted time interval between treatment and kill may allow mature female houseflies time to oviposit. This study describes laboratory tests to investigate the fecundity of gravid female *M. domestica* after treatment with linseed oil suspensions of *M. anisopliae* conidia and whether similarly treated male houseflies have the ability to transmit infection to females during mating.

## MATERIALS AND METHODS

### Insect Strains

A field strain of *M. domestica*, resistant to synthetic pyrethroid and organophosphorous insecticides was used for all experiments.

### Conidial suspensions

A UK isolate of *M. anisopliae* IMI 152487, cultured on Sabouraud dextrose agar (Unipath Ltd), was used in all tests. Conidia were harvested from 11-21 day old cultures by washing the surface of the mycelia with linseed oil (10ml) and agitating with a sterile loop. The density of the conidial suspension was estimated using a Neubauer haemocytometer and subsequently adjusted to $2 \times 10^8$ conidia/ml.

### Assessment of the fecundity of *M. domestica* infected with *M. anisopliae*

Ten male and ten female houseflies (1-3 days old) were treated with 0.5µl of conidial suspension (~$1 \times 10^5$ conidia/fly) applied to the ventral surface of the abdomen. The houseflies were then released into an aluminium cage (30 x 30 x 30 cm). Access was via a 15 cm diameter portal, which was covered in a nylon stocking sleeve to prevent houseflies escaping. Two open sides of each cage were covered with plastic gauze (540 mesh). Sucrose solution (20 ml, 10% wt/$V$) was provided as a food source, absorbed onto cotton wool and placed in a 25 ml beaker. This was replaced at 48 hr intervals.

Adult housefly mortality was assessed at 24 hour intervals for this and succeeding experiments. The dead flies were then removed from each cage, sexed, counted and placed in a petri dish on moist filter paper to determine mycosis. A small plastic pot (120 ml) containing 50 mls of synthetic diet (Kelly *et al.*, 1987) was placed into each cage as an egg laying substrate, after the first 24 h of the test. This was replaced with fresh diet at 24 hour intervals for a further 9 days. Once removed, the diet was incubated for 10 days at 25°C and 85% r.h. to allow the eggs to hatch and develop through to pupae, after which time the pupae were counted. Five replicate cages containing treated houseflies were set up together with five control cages containing flies treated with linseed oil alone. Experiments were repeated for houseflies 4-6 and >6 days old at the start of the tests. All experiments were carried out at 25°C and 85% r.h., with a 12 hour light regime of 22 lux intensity.

### Transfer of *M. anisopliae* conidia between male and female houseflies during mating

Five replicate cages were set up as described above. Male houseflies only were treated with the conidial suspension, ($1 \times 10^5$ conidia/fly). Five control replicates were also set up with male *M. domestica*, treated with linseed oil alone. All female houseflies in both treatment and control replicate cages were untreated. All other experimental procedures are as described above. Experiments were carried out with houseflies 0-2, 1-3 and 4-6 days old at the start of the tests.

Statistical analysis

All mortality data were analysed by analysis of variance. Fecundity data were analysed using the Mann-Whitney test.


RESULTS

The fecundity and mortality of adult *M. domestica* treated with linseed oil suspensions of *M. anisopliae* conidia are shown in Table 1. Control mortality was low for all females, ($\leq$8.0%), but was up to 60% for males from the >6 day-old age group. After seven days exposure significantly more males and females from the 1-3 and 4-6 day-old age groups treated with *M. anisopliae* conidia died, compared with linseed oil treated controls (P<0.01).

TABLE 1. Fecundity (expressed as the number of eggs developing to pupae) and percentage mortality of adult *M. domestica* treated with a linseed oil suspension of *M. anisopliae* conidia.

| Time (days) after treatment | | Percentage Mortality/Fecundity | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| **1-3 day-old adults** | | | | | | | | |
| Treatment. | Females | 0 | 0 | 6 | 36 | 82 | 88 | 94 |
| | Males | 4 | 10 | 28 | 46 | 50 | 68 | 76 |
| | No. of pupae | -* | 1512 | 118 | 790 | 124 | 114 | 0 |
| Controls. | Females | 0 | 0 | 0 | 0 | 2 | 4 | 4 |
| | Males | 0 | 6 | 18 | 28 | 32 | 36 | 36 |
| | No. of pupae | -* | 1196 | 880 | 1638 | 1588 | 1359 | 465 |
| **4-6 day-old adults** | | | | | | | | |
| Treatment. | Females | 0 | 4 | 10 | 62 | 90 | 94 | 96 |
| | Males | 2 | 8 | 18 | 22 | 38 | 54 | 64 |
| | No. of pupae | -* | 727 | 27 | 0 | 128 | 0 | 0 |
| Controls. | Females | 0 | 2 | 2 | 4 | 6 | 8 | 8 |
| | Males | 4 | 12 | 14 | 20 | 26 | 28 | 36 |
| | No. of pupae | -* | 868 | 351 | 246 | 393 | 676 | 164 |
| **>6 day-old adults** | | | | | | | | |
| Treatment. | Females | 0 | 2 | 18 | 94 | 98 | 100 | |
| | Males | 4 | 22 | 44 | 58 | 62 | 76 | |
| | No. of pupae | -* | 947 | 0 | 0 | 0 | 0 | |
| Controls. | Females | 0 | 0 | 0 | 2 | 2 | 4 | |
| | Males | 4 | 26 | 36 | 52 | 54 | 60 | |
| | No. of pupae | -* | 954 | 545 | 581 | 994 | 460 | |

* Egg laying substrate not used during first 24h of test.

TABLE 2. Fecundity (expressed as the number of eggs developing to pupae) and percentage mortality of untreated female *M. domestica* exposed for 10 days to males treated with a linseed oil suspension of *M. anisopliae* conidia.

| | | Percentage Mortality/Fecundity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Time (days) after treatment | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **0-2 day-old adults** | | | | | | | | | | | |
| Treatment. | Untreated females | 2 | 2 | 2 | 8 | 54 | 72 | 82 | 84 | 88 | 88 |
| | Treated males | 2 | 18 | 32 | 40 | 42 | 60 | 68 | 82 | 86 | 94 |
| | No. of pupae | -* | 317 | 2449 | 397 | 457 | 387 | 285 | 159 | 123 | 0 |
| Controls. | Females untreated | 0 | 4 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| | Males oil only | 0 | 8 | 20 | 26 | 28 | 30 | 32 | 34 | 36 | 36 |
| | No. of pupae | -* | 159 | 1768 | 1538 | 2603 | 854 | 1224 | 2266 | 1434 | 1139 |
| **1-3 day-old adults** | | | | | | | | | | | |
| Treatment. | Untreated females | 0 | 0 | 0 | 0 | 6 | 10 | 12 | 12 | 14 | 22 |
| | Treated males | 4 | 30 | 48 | 54 | 62 | 72 | 81 | 88 | 92 | 92 |
| | No. of pupae | -* | 1245 | 734 | 1967 | 559 | 2190 | 110 | 529 | 1710 | 362 |
| Controls. | Females untreated | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Males oil only | 2 | 18 | 32 | 34 | 34 | 34 | 36 | 40 | 40 | 48 |
| | No. of pupae | -* | 1730 | 739 | 2040 | 539 | 1857 | 151 | 1293 | 1889 | 1184 |
| **4-6 day-old adults** | | | | | | | | | | | |
| Treatment. | Untreated females | 0 | 4 | 12 | 16 | 16 | 18 | 18 | 22 | 24 | 26 |
| | Treated males | 8 | 30 | 50 | 54 | 70 | 80 | 84 | 98 | 98 | 98 |
| | No. of pupae | -* | 1361 | 498 | 355 | 235 | 1427 | 0 | 997 | 260 | 698 |
| Controls. | Females untreated | 0 | 0 | 6 | 8 | 8 | 8 | 8 | 12 | 14 | 14 |
| | Males oil only | 4 | 16 | 32 | 36 | 40 | 42 | 50 | 52 | 58 | 60 |
| | No. of pupae | -* | 2034 | 360 | 1127 | 462 | 2281 | 309 | 223 | 654 | 999 |

* Egg laying substrate not used during first 24h of test.

After six days exposure significantly more treated females >6 days old died compared with the female controls (P<0.01) however, there was no significant difference in mortality between treated and control males from this age group. *M. anisopliae* was significantly more pathogenic to treated females compared with treated males for flies 1-3 and 4-6 days old (P<0.05). For flies >6 days old, *M. anisopliae* was more pathogenic to treated females from 4-6 days post treatment (P<0.05). Females >6 days old were more susceptible to infection by *M. anisopliae* than females from the 1-3 or 4-6 day-old age groups (P<0.05). All females >6 days old died 6 days after treatment, whereas, for females 1-3 or 4-6 days old, 94 and 96% had died respectively by the end of the seven day test period.

Fecundity (expressed as the number of eggs developing to pupae) of gravid females from all age groups tested, was significantly lower for females treated with a linseed oil suspension of *M. anisopliae* conidia, compared with controls treated with linseed oil alone (Table 1; P<0.05). However, from the eggs laid on day 2, a large number of pupae were

subsequently produced, with little or no difference in fecundity between treated and control females for all the age groups. The number of pupae produced by treated and control females 4-6 and >6 days old was significantly lower than the number produced by females 1-3 days old (P<0.05). Treated females 4-6 days old produced most pupae (82.4%) from eggs laid on day 2, with no eggs being laid on days 4, 6 and 7. For treated females >6 days old, all pupae were produced from eggs laid on day 2, with no further eggs laid during the experiment.

The results in Table 2 show that male houseflies treated with a linseed oil suspension of *M. anisopliae* conidia, can transfer infection to untreated females, resulting in high female mortality and reduced fecundity. Significantly more treated males, and subsequently infected untreated females from all age groups tested, died compared with control males and females (P<0.01). Mortality was high for treated males and infected females 0-2 days old, being 88 and 94% respectively after 10 days exposure. For adults 1-3 days old, mortality of infected untreated females was significantly greater than the mortality of control females (P<0.01). However, the number of infected females from this age group which died (22% after 10 days) was significantly lower than the number which died (88% in 10 days) from the 0-2 day old age group (P<0.01).

Fecundity of untreated females exposed to treated males was significantly lower than the fecundity of untreated control females but only for the 0-2 days old age group. (P<0.05) (Table 2). There was no significant difference in fecundity between treatments and controls for houseflies 1-3 and 4-6 days old (Table 2).

There was no evidence of mycosis in any untreated control cadavers, whereas mycosis occurred on all dead adults exposed to *M. anisopliae* treatments.

DISCUSSION

Both male and female houseflies were susceptible to infection with a linseed oil suspension of conidia of the entomopathogenic fungus *M. anisopliae*. However a proportion of males were susceptible to linseed oil alone at the application rate of 0.5μl/fly, whereas females appeared to be unaffected by linseed oil applied at the same rate. The high mortality of female *M. domestica* treated with conidia, resulted in a considerable reduction in fecundity, compared with untreated control females. For all age groups of flies tested, this reduction was first observed three days after treatment, when the majority of treated females were still alive. Although the physiological affects of *M. anisopliae* to female *M. domestica* were not investigated here, the reduction of fecundity was probably due to the invasive action of mycelia or the toxic action of fungal metabolites, or a combination of both.

In the infection transfer tests, only females from the 0-2 day-old age group produced fewer progeny than control females. This reduced fecundity was caused by the high level of mortality of the infected untreated females after exposure to treated males. Males were topically treated with conidia applied to the ventral surface of the abdomen, and probably transferred infection to females during copulation. Females usually mate 48-72h after emergence, occasionally after 24h (Sacca, 1964) therefore a high proportion of flies from the 0-2 day-old age group will not have previously mated. In contrast, a low level of female mortality was recorded for houseflies from the other age groups tested, with fecundity similar to the untreated controls. This was probably caused by a lower level of sexual receptivity in

females which mated prior to treatment. Most females are monogamous and will rarely mate more than a few times, mated females rejecting males by 'kicking' the wings of the male (Sacca, 1964), thus reducing physical contact and the likelihood of infection transfer.

In the laboratory tests, conidia were applied only to the ventral surface of the abdomen, resulting in infection and reduced fecundity. However, in a field situation the use of a space spray based on *M. anisopliae* conidia suspended in vegetable (linseed) or other oils may increase the control potential by providing a more even distribution of conidia over the surface of the housefly and within the housefly population.

The suspension of entomopathogenic fungi with lipophilic conidia in vegetable oils, enhances pathogenicity (Barson *et al.*, 1994) and allows germination and infection at low r.h. (Bateman *et al.*, 1993). The results of this and previous studies (Barson *et al.*, 1994) indicate that entomopathogenic fungi have considerable potential for the control of houseflies. Future work will be directed towards the development of an effective delivery system for applying space spray or residual applications of a commercial oil formulation of *M. anisopliae* conidia.

## ACKNOWLEDGEMENTS

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*BEAUVERIA BASSIANA* (ATCC 74040): CONTROL OF INSECT PESTS IN FIELD CROPS AND ORNAMENTALS

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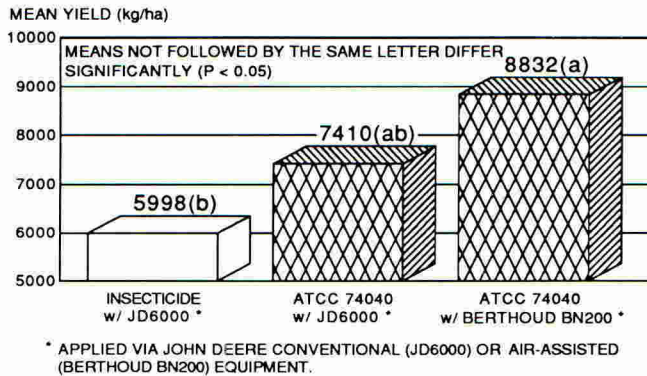
ABSTRACT

Naturalis[®] L is a formulated product containing a naturally-occurring strain (ATCC 74040) of the insect-specific fungus *Beauveria bassiana*. The product has been evaluated in the laboratory, greenhouse, and in the field for seven years.  Originally targeted toward boll weevil (*Anthonomus grandis*) control, the product also showed activity against cotton fleahopper (*Pseudatomoscelis seriatus*), tarnished plant bug (*Lygus lineolaris*), and sweet potato whitefly (*Bemisia tabaci*) in subsequent studies.  In 1991, the product was field tested in order to study its effect on *B. tabaci*.  From 1992 to 1994, studies were expanded to include researchers and commercial cooperators in 10 states.  Results of over 50 trials indicate that in field crops and ornamentals, ATCC 74040 biocontrol products control target insects under a wide variety of production conditions whether applied alone or in combination with conventional insecticides.  Furthermore, it has been established that there are no adverse effects on beneficial insects, fish, honeybees, or mammals.

INTRODUCTION

The product is based upon a naturally-occurring insect-specific strain of *Beauveria bassiana* (ATCC 74040) which has proven to be highly effective in controlling overwintering boll weevil (*A. grandis*) in early-season applications (Knauf, 1991).  This particular strain was isolated from a boll weevil cadaver in 1986 by Dr. James Wright of the USDA-ARS at Weslaco, Texas.  After three years of studies, it became apparent in 1990 that weevils were not the only insects affected by the product.  Cotton fleahopper (*P. seriatus*) and whitefly (*B. tabaci*) were also killed (Wright and Chandler, 1991).  In 1991, the scope of cotton studies was expanded to include two new objectives: use of the product with conventional chemical insecticides in an IPM approach and secondly, the practical production of cotton using only the biorational product for total season insect control.  Meanwhile, research was extended to include replicated plot work in vegetables, ornamentals and other crops in order to reference and identify product activity against *B. tabaci*.  As a result of this extended research, it was learned in 1991 that the product could control *B. tabaci* in vegetable fields where infestations had reached critical levels (Knauf, 1992a).  Studies on cucumber plots using the product and air-assisted application equipment indicated significant yield increases with the formulation compared to the best conventional program (Figure 1).  In addition, 1991 studies included evaluation of some formulation components, effects of different timings of product applications, and the efficacy of various application methods.  Further refinements in product formulations and application methodology continued through 1992 (Wright, 1992; Rektorik and Wright, 1992).  In 1992, research began in California, Arizona, Texas, Louisiana, Mississippi, and Florida and by August included studies of product efficacy on *B. tabaci* infesting greenhouse ornamentals (Knauf, 1992b).  By the end of the year, formulations of the product had been studied under commercial conditions using a variety of facilities, personnel, and production procedures on cotton, vegetables, and ornamentals.  Several commercial crops of cotton and cantaloupes were successfully grown to maturity using only the product for full season insect control.  However, highest yields were seen when the product was used in combination with conventional insecticides in an IPM approach (Wright, 1993).  It was also evident that beneficial insect populations were not adversely affected where the product was

used. This has been confirmed by numerous EPA-mandated studies which show that the product does not accumulate in the environment, is harmless to fish and honeybees, and is very safe for applicators (Krygsman, 1994).

FIGURE 1. Effect of B. bassiana (ATCC 74040) on cucumber yield in B. tabaci-infested fields.

MEAN YIELD (kg/ha)



* APPLIED VIA JOHN DEERE CONVENTIONAL (JD6000) OR AIR-ASSISTED (BERTHOUD BN200) EQUIPMENT.

## MATERIALS AND METHODS

In 1993, issuance of an Experimental Use Permit made it possible to involve researchers and commercial cooperators on a broader scale. Specific details of the experimental designs and test methodologies cannot practically be covered here due to numerous variations in study structure, treatments, and test parameters, but may be obtained by approaching the individual workers cited in "References" for further information and copies of their studies.

## RESULTS OF THE 1993-1994 RESEARCH PROGRAM

### Cotton

It is evident that, depending on location and prevailing practices, the product can be used either alone or in an IPM program under a wide range of environments and under varied cultural conditions to control various cotton insects.

Schuster and Schuster(1994) studied the effect of the product on insects in cotton grown in the Texas blacklands and concluded that the product was effective for season-long control of insects in cotton whether combined with *Bacillus thuringiensis* (*B.t.*) or used with low-rate selective insecticides and *B.t.* in an IPM program. Generally, control was equal to that achieved with the conventional insecticide control. Yield did not differ significantly regardless of the treatment regime used (Table 1).

Microclimate is very important to survival of fungi in a desert environment. Two 1993 studies investigated the effect of two irrigation programs on the efficacy of the product against whitefly. Akey and Henneberry (1994) sought to evaluate whitefly control with the product and insecticides in furrow and sub-drip irrigated cotton. The researchers concluded that the product was as effective as bifenthrin and zetacypermethrin in controlling whitefly eggs and nymphs in this arid Southwest production environment. Yield was identical with all three

TABLE 1. Effect of ATCC 74040 and conventional insecticides on cotton insect control and yield protection in Central Texas

| Control System | Plants (ha) | Plant Height (cm) | Seed Cotton (kg/ha) |
|---|---|---|---|
| Insecticides | 159,315 | 67.1 | 1070 |
| IPM + ATCC 74040 early season | 156,228 | 62.5 | 1105 |
| ATCC 74040 + Fermone B. t. full season | 153,165 | 63.5 | 1018 |
| LSD | ns | ns | ns |

TABLE 2. Effect of various insecticides and ATCC 74040 on B. tabaci nymphs and cotton yield protection.

| Treatment | Large Nymphs/cm$^2$ ($P < 0.001$) | Yield, Bales/ha ($P < 0.05$) |
|---|---|---|
| ATCC 74040 | 6.6 (a) | > 6.9 (a) |
| Bifenthrin | 5.9 (a) | > 6.9 (a) |
| Zeta-Cypermethrin | 6.9 (a) | > 6.9 (a) |
| Best Ag Practice at Sundance | 5.9 (a) | > 6.9 (a) |
| Untreated Check at Sundance | 41.3 (b) | < 5.2 (b) |

treatments (Table 2). The reports of Kennedy (1994) and Wright (1994) also confirm the efficacy of the product in a diversity of crop production conditions.

The field research program disclosed that there are two vital prerequisites for successful control with the product: it must be used in a prophylactic approach which involves product application according to the recommended intervals, and control is in direct proportion to coverage. Therefore, best possible coverage strategies must be used.

In lieu of a preventative approach and during severe infestations, a conventional insecticide may be added to the product to increase immediate efficacy, as the product requires longer to achieve its maximum effect compared to conventional insecticides (Yuster, 1994). Conversely, the product may also have additive efficacy when mixed with conventional insecticides. Burris (1994) found that amendment of malathion and esfenvalerate with the product can significantly reduce damage caused by *A. grandis* and *L. lineolaris* when these conventional insecticides used alone were ineffective.

Ornamentals

Studies utilizing the ornamental formulation of the product were extended to shade houses in Texas and Florida with *B. tabaci* infestations. The ornamental product controlled whitefly eggs, immatures, and adults under a wide spectrum of commercial conditions.

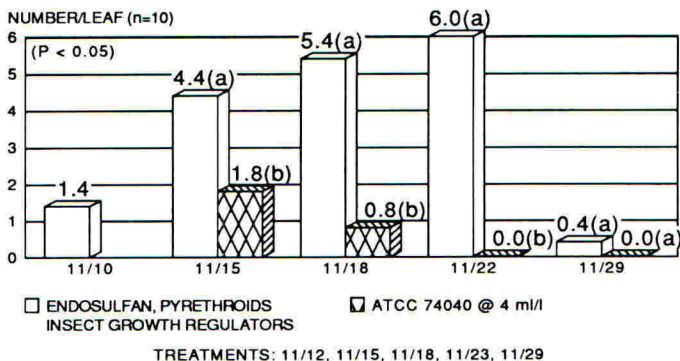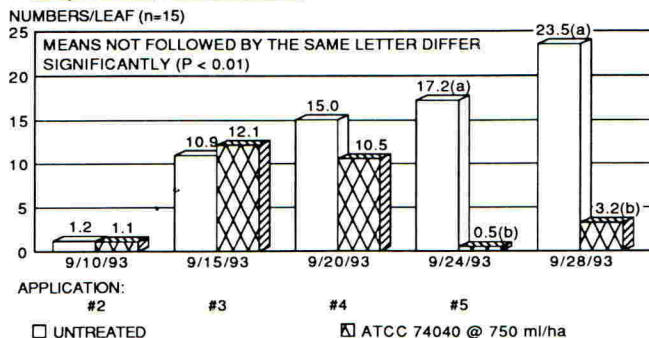FIGURE 2. Control of B. tabaci adults on poinsettia with ATCC 74040. Air-assisted application.

NUMBER/LEAF (n=10)

6.0(a)
5.4(a)
4.4(a)
1.8(b)
1.4
0.8(b)
0.0(b) 0.4(a) 0.0(a)

(P < 0.05)

11/10    11/15    11/18    11/22    11/29

☐ ENDOSULFAN, PYRETHROIDS          ☑ ATCC 74040 @ 4 ml/l
  INSECT GROWTH REGULATORS

TREATMENTS: 11/12, 11/15, 11/18, 11/23, 11/29


FIGURE 3. Control of B. tabaci immatures on Euphorbia accrurensis with ATCC 74040

NUMBERS/LEAF (n=15)

MEANS NOT FOLLOWED BY THE SAME LETTER DIFFER SIGNIFICANTLY (P < 0.01)

23.5(a)
17.2(a)
15.0
12.1
10.9    10.5
1.2  1.1
0.5(b)
3.2(b)

9/10/93    9/15/93    9/20/93    9/24/93    9/28/93

APPLICATION:
#2        #3        #4        #5

☐ UNTREATED          ◩ ATCC 74040 @ 750 ml/ha


Figures 2 and 3 are typical of the results observed. Product performance was best where superior spray coverage strategies were used. The product was applied alone or combined with conventional insecticides. In either case, thorough coverage which treats leaf undersides was essential for best control of B. tabaci. Where coverage is thorough and application timing was correct, excellent control was achieved with the product (Knauf and Wright, 1993a).

## Tomatoes

Eight studies were initiated on commercial tomato farms in Florida. A variety of application equipment, strategies and conventional chemical combinations were utilized to control B. tabaci using the product. Without exception, the product worked successfully in all trials implemented. These field studies demonstrated that the product, when applied using best-coverage techniques, controls whitefly as well as the conventional insecticides currently in use in commercial production programs on the cooperating farms in these trials. When combined with conventional insecticides, the product provided significant enhancement in control compared to insecticides used alone (Knauf and Wright 1993b). Summary results from selected studies may be seen in Figures 4 and 5.

FIGURE 4. Control of **B. tabaci** immatures on tomatoes with ATCC 74040 with 8-row electrostatic equipment.
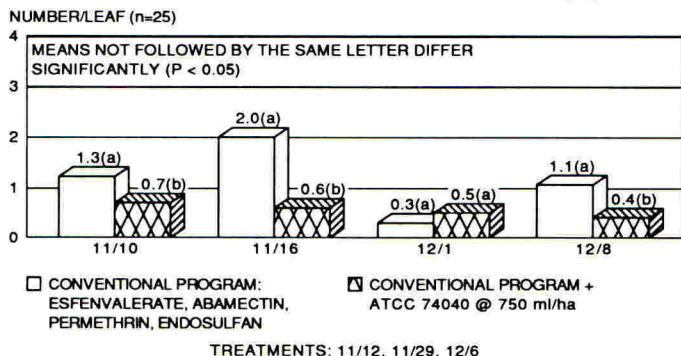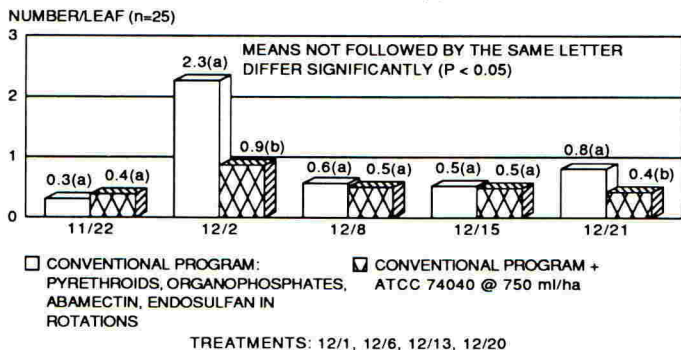
NUMBER/LEAF (n=25)



MEANS NOT FOLLOWED BY THE SAME LETTER DIFFER SIGNIFICANTLY (P < 0.05)

☐ CONVENTIONAL PROGRAM: ESFENVALERATE, ABAMECTIN, PERMETHRIN, ENDOSULFAN

◪ CONVENTIONAL PROGRAM + ATCC 74040 @ 750 ml/ha

TREATMENTS: 11/12, 11/29, 12/6

FIGURE 5. Control of **B. tabaci** adults on tomatoes with ATCC 74040. Air-assisted application.

NUMBER/LEAF (n=25)



MEANS NOT FOLLOWED BY THE SAME LETTER DIFFER SIGNIFICANTLY (P < 0.05)

☐ CONVENTIONAL PROGRAM: PYRETHROIDS, ORGANOPHOSPHATES, ABAMECTIN, ENDOSULFAN IN ROTATIONS

◪ CONVENTIONAL PROGRAM + ATCC 74040 @ 750 ml/ha

TREATMENTS: 12/1, 12/6, 12/13, 12/20

International Activities

The product is being tested in many countries besides the United States. It has provided control of cotton boll weevil equivalent to high rates of methyl parathion in Nicaragua. It has controlled whitefly in Turkey, Mexico, Egypt, Paraguay, and Central America in a variety of crops. The product is receiving expedited registration status in many countries due to its lack of toxicity to humans, mammals, fish, and beneficial insects (Kennedy, 1994).

DISCUSSION

The product has proven to be a viable biocontrol product on certain insects where it can be applied on a timely basis using application technology that insures good coverage. When combined with *Bacillus thuringiensis* products for later season insect control, it provides an effective cotton insect control strategy that does not disturb the integrity of the environment.

This may be the only insect control program suitable for use in some environmentally-sensitive areas. As concerns for the environment continue to grow, regulation may usher in a time when biorational insect control will be the only available strategy. Many governments are emphasizing safer alternatives to conventional insecticides. This product is considered such an alternative. It is effective and it does not impact test animals, aquatic

organisms, beneficial insects, or applicators. It does not persist in the environment for more than 72 hours. Therefore, it is a promising candidate for the new "safer" pesticide category.

Although the product may be used alone to control *B. tabaci* in ornamentals and vegetables, its effect can be enhanced by application with conventional insecticides. In fact, the best use of the product, against *L. lineolaris* and *P. seriatus* appears to be as a component in an IPM program which utilizes strategically-timed and reduced-rate applications of conventional insecticides in tandem with biorationals. Future work will further refine and reference the effect of fungal biorationals on a wider range of insects which damage economically important crops.

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*TRICHODERMA HARZIANUM* T39 INTEGRATED WITH FUNGICIDES: IMPROVED BIOCONTROL OF GREY MOULD

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## ABSTRACT

Trichodex (*Trichoderma harzianum* T39) controlled *Botrytis* diseases in greenhouse crops effectively and in vineyards when applied alone or in combination with chemical fungicides. Alternation with chemical fungicides resulted in as good disease suppression as that achieved by the fungicide alone and more consistent than that by the biocontrol alone. A decision support system, Botman (*Botrytis* manager), was developed for integration of chemical and biological controls. Decisions on whether to apply biological or chemical measures are taken before each spray according to past weather and a four-day weather forecast. When weather is extremely unfavourable to *B. cinerea* development, then spraying is not recommended at all; when weather is extremely favorable to *B. cinerea* development (*e.g.*, rainy weather) then a chemical fungicide is suggested; in all other cases, *T. harzianum* T39 is recommended. This approach was tested on tomatoes and strawberries, with appreciable success and a minimal number of chemical sprays.

## INTRODUCTION

*Botrytis cinerea* is a ubiquitous pathogen which causes severe losses in many fruit, vegetable and ornamental crops (Schwinn, 1992) and which can be especially important in greenhouse production. The pathogen infects leaves, stems, flowers and fruits of various crops. The factors influencing the occurrence and severity of the various symptoms of grey mould are not all well understood. The complexity of the diseases which it causes makes *Botrytis* an important pathogen in many countries. Epidemics occur in cool and humid conditions, which favour attack and may also predispose the host to infection (Jarvis, 1980). High relative humidity in the greenhouse and free moisture on plant surfaces are considered the most important environmental factors which influence infection by *B. cinerea* (Elad, 1989; Jarvis, 1980). Control of the disease is a continuing challenge for plant protection specialists. Resistance of the pathogen to benzimidazoles has developed in all areas of intensive use. In the late 1970s and early 1980s, dicarboximides replaced benzimidazoles in most situations. Although their initial high efficacy has been decreased due to the development of resistance (Elad *et al.*, 1992; Pommer and Lorenz, 1982), they are still officially recommended for use. Among inhibitors of ergosterol biosynthesis (EBI), some are effective against *B. cinerea*. Tebuconazole and fenbuconazole can be used alone or in mixtures, but a case of reduced sensitivity of the pathogen to the two EBI fungicides, was reported recently (Elad, 1992). Multi-site fungicides are still effective after many years of use. Of these, dichlofluanid, thiram, captan, and chlorothalonil are the most common. They are less effective than the specific fungicides employed against grey mould, but are commonly used in anti-resistance strategies.

One of the alternative methods for control of *B. cinerea* is by means of biocontrol agents (BCAs). Infection by necrotrophic pathogens, including *B. cinerea*, can be reduced by pre-inoculation of the phylloplane with epiphytic fungi, bacteria or yeasts (Blakeman and Fokkema, 1982). Adequate control of *B. cinerea* by *Trichoderma harzianum* has been reported for grape (Dubos, 1984), apple (Tronsmo and Ystaas, 1980) and strawberry (Tronsmo and Dennis, 1977). Recently, an isolate of *T. harzianum* (T39) isolated in Elad's laboratory, effectively controlled *Botrytis* diseases in greenhouse crops and grapes (Elad, 1994; Elad *et al.*, 1993). A commercial preparation developed from T39 (Trichodex, 25P, Makhteshim Ltd., Beer Sheva, Israel) has been registered in Israel and other countries. It has been found to be effective in most of the geographical regions in the northern and southern hemispheres. In this paper we describe the integration of biological and chemical methods for the suppression of *B. cinerea*.

## EFFECT OF ABIOTIC CONDITIONS ON THE BIOCONTROL AGENTS AND THEIR INTEGRATION WITH CHEMICAL FUNGICIDES

Biocontrol organism will not persist and be active unless it is adapted to the plant environment. Moreover, to be successful in controlling the pathogen, the introduced BCA must compete with other microorganisms and establish an active population on the phylloplane. The effect of microclimatic conditions on the survival of populations of the BCA was studied on leaves of tomato, pepper and geranium plants incubated under various conditions. Treatments involved high (>90%) or low (75-85%) r.h. and relatively high ($25\pm3°C$) or low ($15\pm3°C$) temperatures. Survival was greater under the higher temperatures and r.h. (Elad and Kirshner, 1993). Significant control of grey mould of cucumber was correlated with high r.h. (80-97%) but not with conditions of air saturation and dew deposition. Optimal temperatures for biocontrol were found to be around 20-25°C (Elad *et al.*, 1993).

Integration of chemical and non-chemical controls is an essential goal which is important for several reasons. It may provide an opportunity to reduce fungicide use and the reliance of growers on chemicals; it may enable us to cope more effectively with pathogen populations resistant to common fungicides and, in many cases, it will result in superior disease suppression, thus minimizing yield loss. In most studies reported so far, integration of no more than two different controls was examined. Integration of biological and cultural controls against *B. cinerea* is currently being studied in northern Italy (Gullino *et al.*, 1993), and in Israel and the Netherlands (Elad, Shtienberg and Dik, unpublished). Some examples exist of integration of each of these methods with chemical control. Integration of biological and chemical control in disease suppression in the *B. cinerea* pathosystems has been investigated in some studies. In most trials conducted so far, the BCAs were treated as a biological fungicide, *i.e.*, they were applied at predetermined fixed intervals. These spraying schedules were arranged either on a weekly basis (in greenhouses) or according the developmental stage of the crop (in vineyards). Results of several experiments conducted in Israel are shown in Table 1. In greenhouse tomatoes, alternation of *T. harzianum* with the standard chemical fungicide was as good as each of the treatments alone. In greenhouse cucumbers and in grape vineyards the alternation treatment resulted in similar effects.

A summary of 86 experiments in vineyards and 64 experiments in greenhouses (cucumber, tomato and strawberry), carried out around the world during 1988-94, is given in Table 2. In each experiment, treatments were compared with untreated controls. Some experiments included more than one comparison. The biocontrol preparation was tested alone,

or was compared with the standard chemical fungicides, and with combinations of the biocontrol and chemical fungicides. The combinations consisted of either tank mix or weekly alternation of treatments. Tank mixing was generally effective but it is not considered desirable because it does not achieve reduced use of chemicals. Alternation of biocontrol treatment with the chemical treatments on a calendar basis was more effective than applying the biocontrol alone, on all four crops. Furthermore, this treatment was more consistant than the other treatments, as demonstrated in the results of experiments with tomato diseases (Table 3). The variation in percentage disease control was lower in the alternation treatments than in the treatments with *T. harzianum* or the fungicides alone.

TABLE 1. Biological (*Trichoderma harzianum* T39) and integrated biological-chemical control of grey mould in Israel. Treatments were applied on a calendar basis. Results are the number of infection sites/10 plants.

A. Tomato in greenhouses treated on a weekly basis

| Treatment, and dose (g/l) | 1 - Givat-Koah Fruit infection at 31.III.89 | 2 - Binyamina Leaf infection at 8.III.90 | 3 - Bet Hanan Stem infection at 13.III.91 |
|---|---|---|---|
| Untreated control | 48 a[1] | 43 a | 18 a |
| *T. harzianum* T39 (1.0) | 21 b | 15 b | 5 b |
| Iprodione (0.5) | 8 bc | 18 b | 5 b |
| Iprodione/*T. harzianum* | 2 c | 16 b | 5 b |

B. Cucumber in greenhouses

| Treatment and dose (g/l) | Spraying schedule | 1 - Jat Fruit infection at 8.II.93 | 2 - Bet Shikma Stem infection at 15.III.93 |
|---|---|---|---|
| Untreated control | - | 102 a[1] | 45 a |
| *T. harzianum* T39 (1.0) | weekly | 64 b | nt |
| Iprodione (0.5) | weekly | 53 bc | 17 c |
| Iprodione (0.5) | biweekly | 67 b | 38 bc |
| Iprodione/*T. harzianum* | weekly | 51 bc | 22 bc |
| Prochloraz+folpet (0.4+1.8) | weekly | 30 c | nt[2] |
| Prochloraz+folpet (0.4+1.8) | biweekly | nt | 21 bc |
| Prochloraz+folpet/*T. harzianum* | weekly | 42 bc | 15 c |

C. Grapes in vinyards treated on a weekly basis

| Treatment and dose (g/l) | 1- Ortal at 30.IX.90 | 2- Ofra at 30.IX.90 | 3- Ortal at 25.XIII.91 | 4- Ofra at 19.X.91 |
|---|---|---|---|---|
| Untreated control | 148 a[1] | 23 a | 131 a | 142 a |
| *T. harzianum* T39 (1.0) | 62 b | 4 b | 83 b | 79 ab |
| Iprodione (0.5) | 91 ab | 6 b | 100 ab | 43 b |
| Iprodione/*T. harzianum* | nt[2] | nt | nt | 23 b |

[1] Numbers in each column followed by different letters differ significantly ($P \leq 0.05$) using the SNK test.    [2] nt = not tested.

TABLE 2. Efficacy of control achieved by the biocontrol preparation (*Trichoderma harzianum* T39) and by its combination with chemical fungicides in experiments carried out world wide under commercial conditions[1]

|  | Standard chemical | Trichodex alone | Alternation (Trichodex/standard) | Tank mix |
|---|---|---|---|---|
| Total no. of comparisons in <u>vineyards</u> | 123 | 77 | 142 | 35 |
| Experiments with effective control (%)[2] | 63 | 43 | 70 | 87 |
| Average disease reduction (%) | 59 | 44 | 65 | 87 |
| | | | | |
| Total no. of comparisons in <u>greenhouses</u> | 64 | 49 | 63 | 26 |
| Experiments with effective control[2] | 66 | 45 | 87 | 77 |
| Average disease reduction (%) | 55 | 46 | 62 | 58 |

[1] Experiments were carried out in Austria, Australia, Bulgaria, Chile, Croatia, France, Germany, Great Britain, Greece, Hungary, Israel, Italy, Poland, Romania, Slovenia, South Africa and Switzerland. [2] Effective control was defined as situation where control was more than 50% compared with the non-treated plots.

TABLE 3. Control of grey mould on various parts of greenhouse-grown tomatoes. Results are expressed as a percentage of disease incidence in nontreated plots. Treatments consisted of a weekly application of fungicides (tebuconazole+dichlofluanid or iprodione) or alternation of *Trichoderma harzianum* T39 and the fungicides in four experiments.

| Symptom | Percentage disease reduction ($\pm$S.E.) | | |
|---|---|---|---|
| | Trichodex alone | Chemical fungicides | Alternation |
| Leaf mould | 40 $\pm$ 16 | 28 $\pm$ 22 | 48 $\pm$ 11 |
| Stem infection | 68 $\pm$ 15 | 58 $\pm$ 17 | 54 $\pm$ 7 |
| Fruit rot | 30 $\pm$ 27 | 51 $\pm$ 25 | 47 $\pm$ 19 |
| Ghost spots | 14 $\pm$ 37 | 45 $\pm$ 38 | 35 $\pm$ 27 |

In some cases disease suppression achieved by the alternation treatment was insufficient. This happened whenever the BCA was applied in weather highly stimulative to grey mould epidemics (Elad and Shtienberg, 1994, Shtienberg *et al.*, 1994). A biocontrol preparation differs from a chemical fungicide in that it contains a living organism that is affected by the environment (as is the pathogen). A schematic presentation of this phenomenon is given in Table 4. The activity of the chemical fungicides is generally less affected by climatic conditions than that of the BCA. Therefore, chemicals and BCAs should be integrated according to microclimatic conditions, to ensure the optimum activity of the latter. Following this concept, the integration of biological and chemical controls aided by the use of a forecaster to predict *Botrytis* outbreaks, was examined recently by our group. The overall goal is to

develop an integrated control program in which BCA is the most important tool and chemical control is to be implemented only occasionally, as necessary. A decision support system named Botman (*Botrytis* manager) was developed for integration of chemical and biological controls. Decisions on whether to apply biological or chemical measures are taken before each spray according to past weather (*i.e.*, the data recorded during the preceding 7 days) and a 4-day weather forecast. The National Weather Forecast Service does not provide predictions for the microclimate within greenhouses and therefore, a conversion of the outside forecast to the greenhouse environment should be employed. The decision follows these lines: when weather is extremely unfavourable to *B. cinerea* development (*e.g.*, hot, dry weather), then spraying is not to be recommended at all; when weather is extremely favourable to *B. cinerea* development (*e.g.*, humid, rainy weather), then a chemical fungicide is suggested; in all other cases, a BCA is recommended. This approach was tested for tomatoes and strawberries for two growing seasons, with appreciable success. In two trials on tomatoes, chemicals were used three out of eight and four out of 13 sprays, respectively (the remaining sprays were *T. harzianum*), and disease suppression was as efficient as in plots treated with fungicides only. On strawberry, the chemical fungicide was applied in only one out of six sprays in each of the two experiments, and in both experiments, control of flower, fruit and leaf infection was significant. We expect Botman to enable growers to limit the application of chemicals and rely more on biological control measures.

TABLE 4. Schematic presentation of the influence of the suitability of the environment for *Botrytis cinerea* on control efficacy of a fungicide and a biocontrol agent during a 10-stage season. A. Expected control efficacy of the chemical and biological controls; B. Four possible spraying schemes.

| | Time or stage of crop | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Suitability of the environment to *Botrytis*[1]: | - | + | - | + | + + + | + | + | + + + | + | - |
| **A. Expected control efficacy (High, Moderate, Low or Not needed)** | | | | | | | | | | |
| Biocontrol | N | H | N | H | L | H | H | L | H | N |
| Chemical fungicide | N | H | N | H | M | H | H | M | H | N |
| **B. Spraying schemes (Chemical Fungicide, Biocontrol)** | | | | | | | | | | |
| Exclusively chemical fungicides | F | F | F | F | F | F | F | F | F | F |
| Exclusively biocontrol | B | B | B | B | B | B | B | B | B | B |
| Alternation on calendar basis | F | B | F | B | F | B | F | B | F | B |
| According to BOTMAN | - | B | - | B | F | B | B | F | B | - |

[1] The number of "+" signs indicates how favourable the conditions were for *Botrytis* development; "-" represents conditions limiting *Botrytis* development.

## ACKNOWLEDGEMENTS

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USE OF ANTAGONISTIC FUNGI TO CONTROL *ARMILLARIA* ROOT ROT

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ABSTRACT

Effective treatment of trees and shrubs attacked by species of *Armillaria* depends on detecting infection as early as possible. A number of chemical and biological control methods have been evaluated. Chemical control is difficult as this fungus often has an extensive mycelium that penetrates through wood, under bark and deeply through soil protected in the form of rhizomorphs. Biological control is possible using some of the pathogens that attack the cultivated mushroom (*Agaricus bisporus*). Among the most effective are strains of *Trichoderma harzianum*. The prospects for an integrated management strategy that combines diagnosis and control of virulent species of *Armillaria* in forests or cultivated in plantations, orchards and amenity horticulture are discussed.

INTRODUCTION

Few, if any, trees and shrubs are immune to infection by virulent species of *Armillaria* (Raabe, 1962), losses can be substantial in forestry plantations, orchards, as well as gardens and amenity plantings (Fox, 1990). If there is sufficient damage to the roots of trees, they can become susceptible to windthrow during storms and with it the threat of serious damage to people and property, particularly in garden and amenity areas.

For safety reasons, as well as increasing the chances of successful treatment, any infection must be controlled before it is too late (Turner and Fox, 1988; Fox *et al.*, 1991). Yet at the same time the considerable expense and labour of control measures will be wasted if the rot is caused by a harmless saprophyte (Fox, 1990), so diagnosis needs to be accurate and reliable. If only the symptoms on the tree are used to detect honey fungus, there are likely to be problems as nearly all of the earliest signs of infection, such as the death of leaves, can easily be mistaken for the symptoms of a number of other causes. Although rotten wood can be detected by dogs trained to bark when they smell decay (Swedjemark, 1989), at this late stage it can be difficult, if not impossible, to prevent the disease from becoming so deeply established that it cannot be controlled (Bray, 1970; Pawsey & Rahman, 1976; Filip and Roth, 1977).

In most cases the most reliable method of detecting presence of honey fungus at a sufficiently early stage for effective treatment is to find rhizomorphs. Although these often develop around dead hosts as a smothering network (Hood & Sandberg, 1987), unless they are found penetrating under the bark they really only indicate that

the host may be at risk from infection. Rhizomorphs are not always evident in an infection, as some species of *Armillaria*, such as *Armillaria ostoyae*, which cause significant losses of several economically important conifers, rarely produce them and *Armillaria mellea* frequently infects by root to root contact without rhizomorphs. Without the fruiting bodies, that may only be present in some years and then only for a few days in autumn, it is often very difficult to detect the presence of honey fungus in an old dead stump which no longer has living foliage to exhibit symptoms and the pathogen has become deep seated (Shigo & Tippett, 1981; Fox, 1990).

Once the presence of *Armillaria* root rot has been established beyond reasonable doubt, there are a number of problems associated with treatment that are peculiar to honey fungus.

For many years the advice given when *Armillaria* has been detected is to remove all infected tree stumps and roots, as well as any others that could subsequently become infected in the area (Greig and Strouts, 1983). Stump removal may involve winches and excavators or the stump may be chipped using a machine specifically designed for this purpose. Unfortunately, many clonal colonies of *Armillaria* spp. are so extensive (Smith *et al.*, 1992), that it would be extremely laborious to remove all the sources of inoculum in roots buried in the soil.

CONTROL MEASURES

Detection and Identification

*Armillaria* can be detected by baiting with strawberry plants (Fox & Popoola, 1990), logs (Mallett & Hiratsuka, 1985) or potato tubers (Gregory, 1984) but conventional identification techniques usually rely on trying to culture onto agar some of the mycelium present in the tissue which is decayed. This can be difficult, taking days or even weeks to complete and as *Armillaria* is relatively slow growing it is highly prone to contamination problems.

Neither contamination nor the presence of soil or wood affects methods based on immunology or nucleic acid hybridization for the rapid, reliable and accurate diagnosis of soil-borne pathogens (Fox, 1993; Duncan and Torrance, 1992). Although methods based on immunology have already been produced (Fox & Hahne, 1989; Priestley *et al.*, 1994), methods based on nucleic acid hybridization are also being developed (Fox *et al.*, 1993).

Fungicides

Mixtures of cresylic acids have been marketed in Britain, but there is a need for more effective non-phytotoxic chemicals as protectants or eradicants (Turner & Fox, 1988). Several chemicals, representing three groups of fungicides, including three ergosterol biosynthesis inhibitors, a guanide and a phenolic fungicide were selected for further investigation after extensive laboratory tests (Turner & Fox, 1988). However, subsequent field experiments have also shown that none of these chemicals

or the standard cresylic acid, were effective at eradicating mycelium from an established infection (West *et al.*, 1993) due to their inability to reach the *Armillaria* mycelium which is protected inside its wood substrate. Phytotoxicity can be a problem during the growing season, but can be reduced if treatments are applied to trees and shrubs during the dormant season.

The ergosterol biosynthesis inhibitor, fenpropidin is fungistatic to *Armillaria* at low concentrations and did show some activity in field conditions. This chemical reduces the growth of rhizomorphs into treated soil and protect treated wood. However, protection with fenpropidin alone is not sufficient to halt infection, so other control strategies remain under investigation. All the fungicides based on phenyl phenol and cresylic acid demonstrated an unusual phenomenon. The mycelium inside wood treated with these phenolic fungicides was eventually stimulated to grow into the fungicide solution (*in vitro*), or treated soil (*in vivo*).

It is possible that *A. mellea* can detoxify this group of fungicides using the same enzymes that are used by all white rot fungi in wood to break down lignin and detoxifies its toxic phenolic intermediates. As an increase in fungicide concentration eventually led to an increase in growth of mycelium, it is probable that the fungus derived energy from metabolising the phenolic fungicides, but further work is required to understand the mechanism of this stimulation.

Microbiological control

Some fungi which naturally occur in field soil can antagonise *Armillaria* after it has been weakened by a fungicidal soil treatment that alone is non-lethal (Bliss, 1951; Ohr & Munnecke, 1974). Apart from these examples, previous attempts to reduce the severity of root rotting have involved other microbial antagonists that naturally occur in wood as a protectant treatment. These fungi have been introduced into tree stumps ahead of infection by *Armillaria* species (Rishbeth, 1976; Pearce and Malajczuk, 1990).

In the investigations at Reading, pathogens of cultivated *Agaricus bisporus* from commercial mushroom houses were obtained from the MAFF ADAS to test as potential antagonists of *Armillaria* species, these included several strains of *Trichoderma harzianum*, *Dactylium dendroides*, *Diehliomyces microsporus* and *Pseudomonas tolaasi*.

The dense mycelium of *Trichoderma harzianum* encrusted the mycelium of all the *Armillaria* isolates where it sporulated, greatly restricting their growth and softening the texture of their hyphae and rhizomorphs compared to that in the untreated controls. *Trichoderma harzianum* was not inhibited by any of the antibiotics produced by the *Armillaria* species.

The cottony white mycelium of *Dactylium dendroides* grew most densely over the *Armillaria* colonies including the rhizomorphs. This restricted the growth of their mycelium, eventually rotting and disintegrating the hyphae after turning them dark brown.

*Pseudomonas tolaasi* did not have such a marked effect even though the *Armillaria* hyphae did avoid the bacterial colonies resulting in an inhibition zone. Nonetheless, the cells did not markedly restrict the growth of the *Armillaria* hyphae when they were mixed with them or they grew on the agar medium around the rhizomorphs.

After the treatment with the antagonists, the viability of the treated *Armillaria* isolates was tested by inoculating some mycelium onto four fresh 3% malt extract agar plates which were incubated at 25°C for a week. Although the mycelium from *Armillaria* treated with *Trichoderma harzianum* and *Dactylium dendroides* was no longer viable, that treated with *Diehliomyces microsporus* was still viable even though it supported a very sparse growth of this antagonistic fungus. Inoculation with *P. tolaasi* did not affect the viability of the *Armillaria* isolates despite the bacterial colonies which grew out of the mycelium. The dry weights of the mycelium of the *Armillaria* isolates show significant differences between the effects of the antagonists. All those treated with the antagonists were significantly less than the control with *D. dendroides* and *T. harzianum* showing the greatest effect, followed in descending order by *D. microsporus* and the two *P. tolaasi* isolates (Fox *et al.*, 1991). Further tests have been carried out *in vivo* by incorporating mushroom compost infected with the most effective antagonists into the soil around potted strawberry plants which have been inoculated with *A. mellea* infected hazel billets.

Possibility for Integrated control

Most saprophytic fungi grow very fast in comparison with *Armillaria* species even when unaffected by fungicides. Nonetheless, it is feasible that better results might result from the integration of the control of *Armillaria* root rot of trees and shrubs by combining the most effective antagonistic micro-organisms with novel chemical fungicide drenches (Fox, 1990). One combination that might be effective is the combination of a protectant fungicide with an eradicant antagonist. In this case, the fungicide could pretreat the soil before planting while the antagonist could be used to eradicate inoculum already in the soil. This would require skilled timing. However, the present range of chemicals is both insufficiently effective and non-selective, as fungitoxic activity affects antagonists as well as *Armillaria*. Therefore, the integration of biological control with physical and environmental methods of control appears more promising and is currently being investigated. Some other diseases can be controlled by adjusting the environment, but the effect of the environmental factors which determine the infective activity of *Armillaria* are still rather poorly understood. The environmental factors which are likely to be most important are probably waterlogging, shading, drought, defoliation, advanced age and declining vigour of the host plant, damage due to other agents (pollution, insects, fungi, etc.) and a high population density of trees. These factors are undoubtedly complicated in their action and probably interact together to some extent but Popoola & Fox (1989) successfully managed to protect several hosts against *Armillaria* infections by reducing their water stress. The effect of modifying the environment of the host will also effect the performance of the biological control agents and is being investigated.

Even though genetic host resistance is not very great in any species of tree or shrub, there may also be some scope for including it in an integrated control strategy by replanting previously infected areas with trees which are considered less susceptible to *Armillaria* infection (Greig & Strouts, 1983), possibly in a mulch containing the biological control agent.

When trying to control honey fungus, it should never be forgotten that if a large clone of *Armillaria* is involved, it may consist of a huge network of rhizomorphs connecting mycelium well established in numerous massive rotting stumps and the roots of nearby trees (Smith *et al.*, 1992). Therefore, when only a small extremity of a large colonial network is treated, it may only be feasible to fend off a local attack, by either continual or repeated application of the control agent, rather than to expect rapid complete eradication.

## ACKNOWLEDGEMENTS

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BIOCONTROL OF WILLOW RUSTS WITH THE HYPERPARASITE *SPHAERELLOPSIS FILUM*

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ABSTRACT

Willow short rotation coppice is being developed as a source of renewable energy. Rusts (*Melampsora* spp.) are the most important diseases of this crop, reducing biomass yield by as much as 40%. Several species and many rust pathotypes infect willow cultivars in short rotation coppice. Application of fungicide to willow coppice is technically difficult and uneconomic. We are developing a rust management strategy which combines planting mixtures of willow cultivars susceptible to different rust pathotypes with biocontrol by the fungal hyperparasite *Sphaerellopsis filum*. In a field study we found that colonisation of a stem-infecting rust by *S. filum* was high compared with that of a leaf-infecting rust. Two biological factors were responsible: *S. filum* overwintered on stem-infecting rust but not on leaf-infecting rust and *S. filum* dispersed on to stem-infecting rust more readily than to leaf-infecting rust. Consequently, biocontrol will be enhanced if willow cultivars susceptible to stem-infecting rust are included in mixtures. Field studies are underway to develop this promising rust management strategy.

INTRODUCTION

Rust (*Melampsora* spp.) can reduce biomass yield of willow (*Salix*) grown in short rotation coppice (SRC) as a source of renewable energy by up to 40% (Parker *et al.*, 1993). There are six species of rust prevalent on UK willow (Pei *et al.*, 1993). Of these *Melampsora epitea*, *Melampsora capraearum* and *Melampsora ribesii-viminalis* infect the shrub willow cultivars (sub-genus *Vetrix*) commonly grown in SRC. These three rust species can be further sub-divided into pathotypes by their ability to infect different willow cultivars (Pei & Royle, 1993). Thus, a large number of rust:willow pathosystems, i.e. different willow cultivars infected by specific rust pathotypes, are found in SRC.

Rust:willow pathosystems can be grouped by the position of rust infection on the host. The uredinial stage of most rust pathotypes only occurs on the lower surface of the leaf lamina. These pathosystems involve leaf-infecting rusts. However, the uredinial stage of a few pathotypes may occur on both surfaces of the leaf lamina, the leaf midrib and petiole and the young stem (Pei *et al.*, 1994). These pathosystems involve stem-infecting rusts. Leaf-infecting rusts are heteroecious, with sexual stages on the alternate hosts *Larix* or *Ribes*. Stem-infecting rusts are autoecious, existing only as the uredinial stage, and overwinter in willow stem cankers or in bud cushions. Therefore, active uredinial leaf-infecting rust is present in willow SRC from about June until leaf fall, while active stem-infecting rust is present from about April until leaf fall and dormant stem uredinia remain over the winter.

Application of fungicide to willow SRC to control rust is technically difficult and uneconomic. However, endemic populations of the fungal hyperparasite *Sphaerellopsis filum* (synonym *Darluca filum*) affect the development of rust epidemics on willow SRC (Hunter & Pei, unpublished). This hyperparasite is distributed world-wide on hundreds of rust species on different host plants, including several species of *Melampsora* rust on different willows (Kranz & Brandenburger, 1981). *S. filum* colonises all the rust:willow pathosystems commonly found in SRC (Morris & Royle, 1993). Biocontrol with *S. filum*, therefore, may offer an alternative method of rust management in willow SRC.

*Sphaerellopsis filum* reduces the rate of rust development by suppressing the production of rust urediniospores. This is achieved by colonisation of rust uredinia. Sporulating rust mycelium becomes covered with masses of conspicuous black pycnidia (Eriksson, 1966). The pycnidia of *S. filum* produce cirrhi of spindle-shaped bicellular spores in a mucous coating. These cirrhi break down on contact with water and, as in other pycnidial fungi, the pycnidiospores are probably dispersed by water-borne mechanisms (Kranz, 1981).

We are developing a rust management strategy which combines planting mixtures of willow cultivars with different responses to rust pathotypes with biocontrol by *Sphaerellopsis filum*. Mixtures of cultivars appear to delay dispersal of rust inoculum to susceptible willow and so also reduce the rate of rust development. For the rust management strategy to work effectively we need to include rust:willow pathosystems in cultivar mixtures which will encourage endemic populations of *S. filum*. In this study we assessed colonisation of leaf-infecting and stem-infecting rust by endemic populations of *S. filum* in an established SRC plantation in order to determine the factors which may limit efficient biocontrol in the field.


METHODS

The development of endemic rust and *S. filum* populations were studied in an established willow planting near Long Ashton Research Station in the second and third years of crop growth from July 1992 to May 1993. Two rust:willow pathosystems were used: 1) *Salix viminalis* cv. Bowles Hybrid infected by both stem-infecting rust (pathotype SIF) and leaf-infecting rust (pathotype LET1) and 2) *Salix burjatica* cv. Korso infected by the leaf-infecting rust (pathotype LR1).

From July to October 1992, monthly samples were taken of every 10th leaf on 10 randomly selected shoots in a plot of 16 plants randomly selected in each of 4 blocks of the two willow cultivars (Fig. 1). The number of *S. filum* pycnidia in 20 randomly selected rust uredinia on the lower surface of the lamina of each leaf was recorded with the aid of a binocular microscope (x60). Sporulation of each uredinium was also noted (+ or -). Leaf midrib uredinia on Bowles Hybrid were separately assessed in the same way.

Counts of up to 10 pycnidia were recorded separately but higher numbers were recorded as >10. Values between 4 and 10 were sparse, so for analysis, counts were assigned to six ordinal categories: 0, 1, 2, 3-4, 5-8 and 9+. A logistic regression model based on cumulative response probabilities (McCullagh, 1980) was fitted to the ordinal data for pycnidial counts:

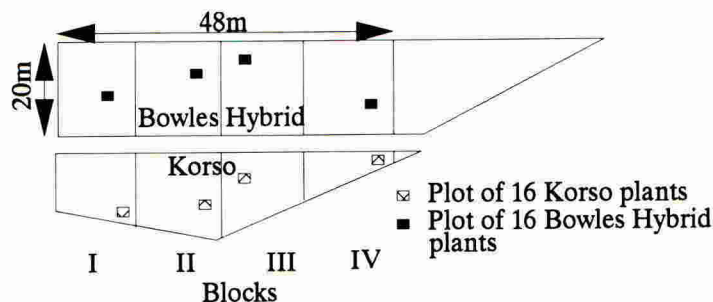$$\log\{\gamma_j(X)/(1-\gamma_j(X))\} = \theta_j - \beta^T X \qquad j=1\ldots5$$

Figure 1. The experimental design imposed on part of an established SRC planting of the cultivars *S. viminalis* cv. Bowles Hybrid and *S. burjatica* cv. Korso.

This assumes an underlying pycnidial density variable Z, a score of j corresponding to $\theta_{j-1} < Z < \theta_j$. The $\theta_j$ values are the estimated cut-points between the ordinal categories for the underlying distribution with $\gamma_j(X)$ the cumulative probability J.

Also, each month, the number of stem uredinia in the area down to 1.0cm below every 10th leaf was recorded on Bowles Hybrid. The presence or absence of *S. filum* pycnidia and of sporulation in each stem uredinium was recorded with the aid of a hand lens (x20).

Overwintering of rust and *S. filum* on Bowles Hybrid stems produced in 1992 was assessed at the end of April 1993. Presence or absence was recorded for rust and *S. filum* on emerging shoots at every leaf on 30 overwintered stems randomly sampled from each of 4 blocks (Fig. 1). The presence of *S. filum* pycnidiospores in free-hand sections of overwintered stem uredinia was checked with the aid of a light microscope (x400) using cotton blue in lactophenol as a stain.

RESULTS

From July to October 1992, the pycnidial density of *S. filum* was higher in the uredinia of stem-infecting rust on the leaf midrib than in uredinia of either stem-infecting or leaf infecting rusts on the leaf lamina (Fig. 2). Thus, by October, the maximum pycnidial density on the leaf midrib reached 9 per uredinium but on the leaf lamina only around 1 per uredinium. Also, if only leaf-infecting rust was present, the pycnidial density in uredinia on the leaf lamina was initially lower than the density in uredinia on the leaf lamina where uredinia of stem-infecting rust were also present on the leaf midrib. By October, the density in all uredinia on the leaf lamina had become the same.

Incidence of *S. filum* was greater and increased more rapidly in uredinia of stem-infecting rust on the stem and leaf midrib than in uredinia of either stem-infecting or leaf-infecting rust on the leaf lamina (Fig. 3). Thus, by October, the incidence of *S. filum* reached a maximum of over 80% in uredinia of stem-infecting rust on the stem and leaf midrib but only around 50% in uredinia of either stem-infecting or leaf-infecting rust on the leaf lamina.

In April 1993, inoculum of *S. filum* was seen in sections through uredinia from all overwintered stems. Further, there was a low incidence of *S. filum* on stem-infecting rust that
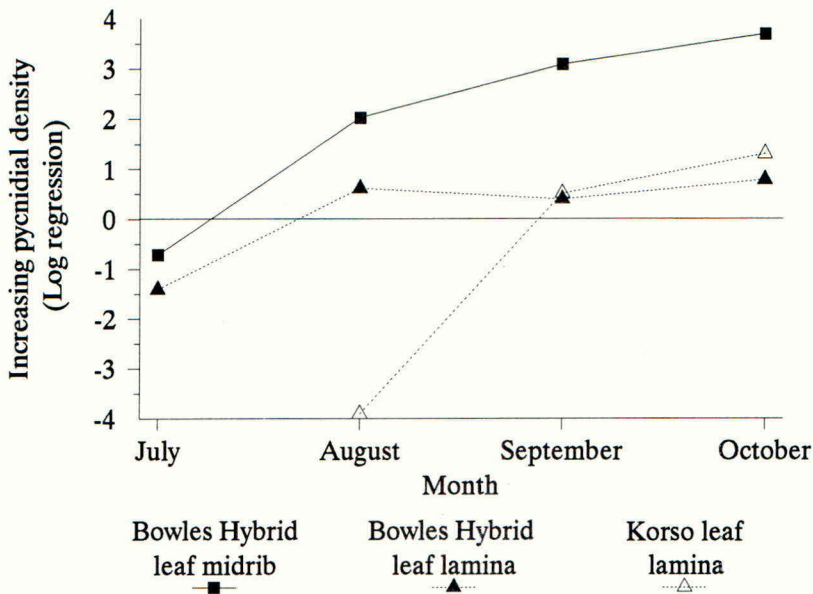
Figure 2. The density of *S. filum* pycnidia in uredinia on the leaf lamina and midrib of *S. viminalis* cv. Bowles Hybrid, and in uredinia on the leaf lamina of *S. burjatica* cv. Korso, July - October 1992.
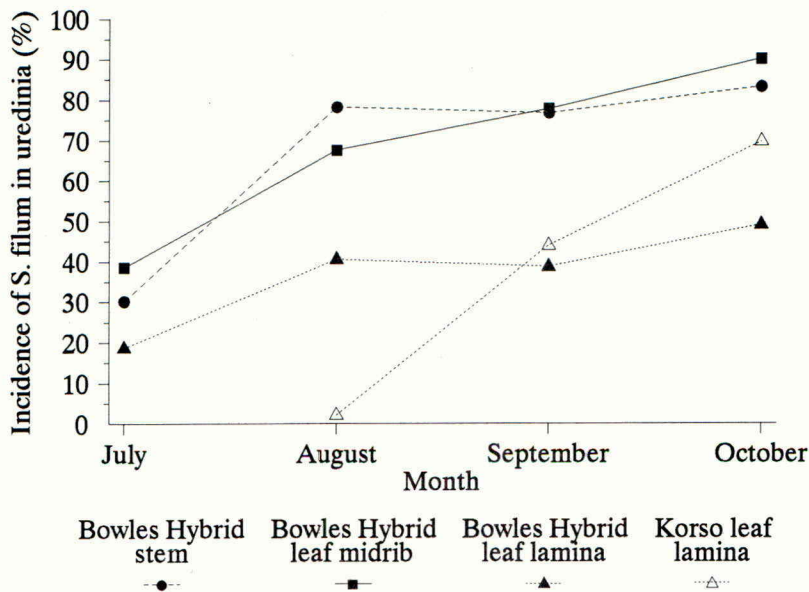


Figure 3. The incidence of *S. filum* in uredinia on the leaf lamina, midrib and stem of *S. viminalis* cv. Bowles Hybrid and in uredinia on the leaf lamina of *S. burjatica* cv. Korso, July - October 1992.

had only begun to infect the emerging shoots around a month earlier (Fig. 4). *S. filum* had colonised rust up to more than 80 leaves from the base of the overwintered stems, which was the zone with the greatest incidence of stem-infecting rust.
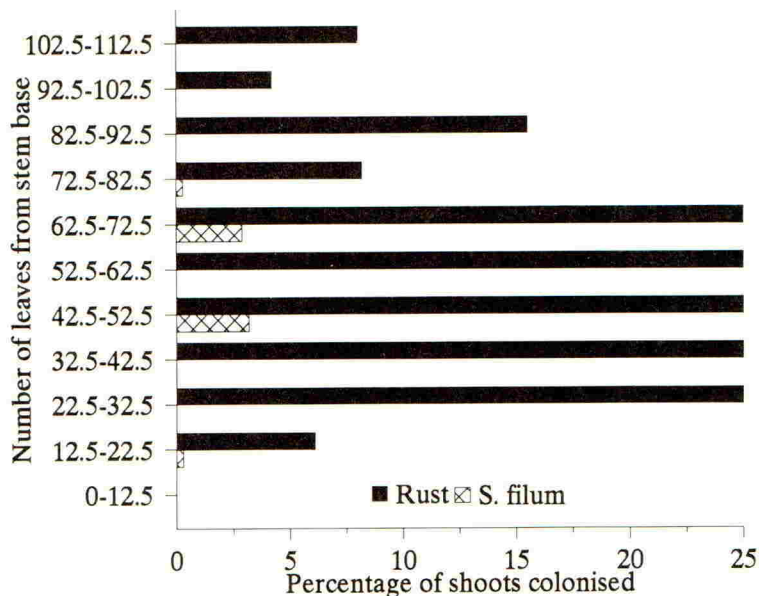


Figure 4. The distribution of *S. filum* on stem-infecting rust on *S. viminalis* cv. Bowles Hybrid in April 1993.

DISCUSSION

The rust hyperparasite *S. filum*, a potential biocontrol agent, was endemic in the established willow plantation studied. However, the colonisation of rust by *S. filum* was quantitatively affected by the rust:willow pathosystem. Stem-infecting rust was colonised earlier and there was more rapid development of *S. filum* than on leaf-infecting rust (Figs. 2 & 3). A long period (7 months) of stem-infecting rust activity on its perennial host plant satisfies the criterion of sucessful biocontrol by a hyperparasite (Kranz, 1981). Therefore, the inclusion of willow cultivars which are susceptible to stem-infecting rust in cultivar mixtures is likely to increase biocontrol by endemic *S. filum* and contribute to rust management in SRC.

The data showed three differences in the interactions of *S. filum* with stem-infecting and leaf-infecting rust. First, the ability of *S. filum* to produce inoculum increased more rapidly on stem-infecting rust since colonisation of uredinia on the leaf midrib yielded more pycnidia than colonisation of uredinia on the leaf lamina (Fig. 2). Second, in agreement with Kranz (1981), inoculum of the hyperparasite appeared to be dispersed by water-borne mechanisms since the incidence of *S. filum* in uredinia of stem-infecting rust on the stem and leaf midrib was greater than in uredinia of leaf-infecting rust on the underside of the leaf lamina (Fig. 3). Environmental conditions have a large effect on hyperparasitic colonisation (Kranz, 1981)

and could also explain this pattern of incidence. However, it is difficult to envisage that the environments of uredinia on the lamina and midrib of the same leaves were sufficiently different to account for it. Third, over-wintering of *S. filum* on stems in dormant uredinia of stem-infecting rust, which did not occur with leaf-infecting rust, enabled its establishment at the onset of the stem-infecting rust epidemic in March/April (Fig. 4). Further, *S. filum* remained distributed in the leaf canopy through overwintering on the stem thus aiding dispersal in spring. Essentially, this colonisation study showed that the development of endemic *S. filum* in a willow plantation was limited by inoculum availability and dispersal. There is very little published information on these aspects of hyperparasite biology in the literature (Kranz, 1981).

The effect of different mixtures on the dispersal of *S. filum* from stem-infecting rust to leaf-infecting rust is currently being studied. Further, while we used *S. viminalis* cv. Bowles Hybrid infected by the stem-infecting pathotype as the model stem-infecting rust in this study, other willow cultivars commonly grown in SRC are also susceptible to different forms of stem-infecting rust. Further studies are therefore in progress to compare the development of *S. filum* on pathosystems with different stem-infecting rusts.

## ACKNOWLEDGEMENTS

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LYTIC ENZYMES IN THE BIOLOGICAL CONTROL OF *FUSARIUM* WILT OF TOMATO

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ABSTRACT

Different fungi producing lytic enzymes and their enzymatic complexes were tested for their ability to control *Fusarium* wilt of tomato in the greenhouse. Significant reductions were observed in disease incidence, disease severity and AUDPC. The most effective antagonists were: *Aspergillus nidulans*, *Penicillium oxalicum* and a mixture of *Fusarium subglutinans* and *Fusarium moniliforme*. The most active enzymatic complexes were obtained from: *Aspergillus nidulans*, *Mucor rouxii*, *Penicillium oxalicum* and *Fusarium oxysporum* f.sp. *lycopersici*.

INTRODUCTION

*Fusarium* wilt of tomato is caused by *Fusarium oxysporum* f.sp. *lycopersici*. The disease is usually controlled by resistant cultivars but the appearance of new races of the pathogen that overcome resistance (Tello & Lacasa, 1988) makes a search for new control methods necessary. Biological control can be an alternative method for the control of *Fusarium* wilt of tomato.

Mitchell & Alexander (1961) reported control of *Fusarium* wilt by bacterial strains producing lytic enzymes. Cell walls of *F. oxysporum* f.sp. *lycopersici* race 2 were degraded *in vitro* by lytic enzymes produced by *Mucor rouxii*, *Aspergillus nidulans*, *Penicillium oxalicum* and *F. oxysporum* f.sp.*lycopersici* race 2 (Alfonso *et al.*, 1992a).

In this paper we report the results a study of the control of *fusarium* wilt of tomato by fungi producing lytic enzymes, or by their enzymatic complexes.

MATERIALS AND METHODS

Fungal isolates and enzymatic complex

*F. oxysporum* f.sp. *lycopersici* race 2 (FOL), *Fusarium subglutinans* (FS), *Fusarium moniliforme* (FM), *A. nidulans* (AN), *M. rouxii* (MR) and *P. oxalicum* (PO) were grown on Petri dishes containing PDA or Czapek agar in the dark at 20-25°C for mycelial and conidial production. To obtain the enzymatic complexes, twenty-five-day old filtrate cultures of AN, MR, PO and FOL were concentrated five fold under

vacuum at 30°C and then precipitated with 1% tannic acid and washed by centrifugation (4000xg) with acetone at 0°C until tannic acid was totally eliminated. Precipitates were air-dried and stored at 4°C. The activities were periodically redetermined and found to be stable for several months. Two hundred mg of enzymatic complexes so obtained were included in alginate and/or chitosan pellets.

Greenhouse assays

Two assays were carried out in the greenhouse on tomato plants var. Novy (susceptible to FOL race 2). Seedlings (2-4 true leaf stage) were transplanted into pots containing a sterile or natural mixture of soil-peat-sand (1:1:1 v:v:v). The pots were placed in a greenhouse at 15-30°C. Plants were inoculated with the pathogen and treated with the antagonists or the enzymatic complexes. Pellets of the enzymatic complexes were placed in the pots before transplanting. A randomised block design was set up for the experiment, with 10 blocks and one pot with 5 plants per treatment.

Disease severity was assessed by using a 1-5 scale: 1:(0-24 %) healthy plant, all leaves green; 2:(25-49 %) lower leaves yellow; 3: (50-74 %) lower leaves dead and some upper leaves yellow; 4: (75-99 %) lower leaves dead and upper leaves wilted; 5: (100 %) dead plant. Disease incidence was also determined as the number of diseased plants from which the pathogen was isolated. All the plants were placed in humid chamber at the end of the experiment and the presence/absence of the pathogen was recorded after 48 h incubation. The area under the disease progression curve (AUDPC) was also calculated for each treatment.

Trial 1
Roots were submerged before transplanting in a suspension of $10^6$ microconidia of FOL/ml for 30 minutes. To obtain the microconidial suspension, flasks (250 ml) containing 150 ml of sterile Czapek-Dox (Difco) were inoculated with three mycelial plugs of FOL from 7 d old Czapek-Dox agar (Difco) cultures. After 5 days of incubation in a rotatory shaker (150 rpm and room temperature), the culture was filtered through sterile glass wool and the suspension was diluted to $10^6$ spores/ml. Treatments with fungal antagonists were applied by watering with 10 ml/plant of a spore suspension ($10^9$ spores/ml) in aqueous Tween 80 (0.1%). The following treatments were applied after transplanting: 1) AN enzymatic complex on chitosan, 2) AN enzymatic complex on alginate, 3) MR enzymatic complex on chitosan, 4) MR enzymatic complex on alginate, 5) chitosan 6) alginate pellets, 7) suspension spores of AN, 8) suspension spores of MR, 9) no treatment and 10) no treatment and no inoculation with FOL. Disease development was assessed for 30 days after transplanting.

Trial 2
To obtain chlamidospores in soil, pots were watered with 60 ml of a suspension of FOL ($10^6$ microconidia/ml) 30 days before transplanting (De Cal *et al.*, unpublished). Only alginate pellets were used as carriers of the enzymatic complexes. Treatments with antagonists were applied before transplanting as a root bath in an aqueous spore suspension ($10^9$ spores/ml) with 0.1% Tween 80. The following treatments were applied: 1) FOL enzymatic complex, 2) PO enzymatic complex, 3)

AN enzymatic complex, 4) 1+2+3, 5) alginate pellets, 6) suspension spores of PO, 7) suspension spores of PO+AN, 8) suspension spores of FS+FM, 9) no treatment and 10) no treatment and no inoculation with FOL. Disease development was assessed for 67 days after transplanting.

## Statistical analysis

Disease severity data were arcsin transformed. These data and AUDPC data were then analyzed using analysis of variance. When significant effects were observed, comparisons of means were carried out using the Least Significant Difference (LSD) test. Disease incidence data and data indicating presence/absence of the pathogen were analyzed by a chi square test. In all cases p=0.05.

## RESULTS

The inoculum type of FOL used in the two assays was different. The induced disease severity in Trial 1 was higher than in Trial 2, while AUDPC values were lower in trial 1 than Trial 2 (Fig.1). In both assays, uninoculated and untreated plants gave levels of disease severity of about 25%, but disease incidence was lower than 10% and the pathogen was isolated from these plants only in trial 2 (Table 1 and 2).
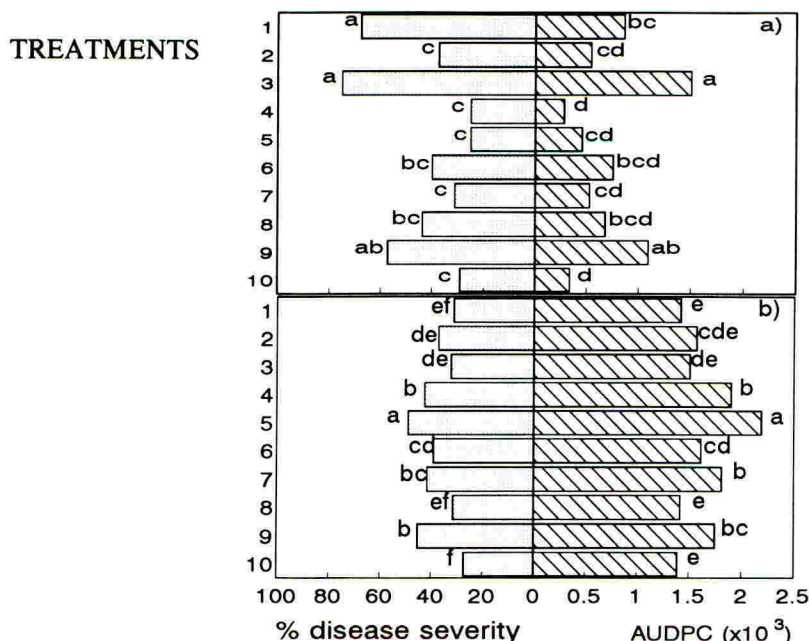


Figure 1. Effect of treatments in trial 1 (a) and 2 (b) on percentage disease severity (▦) and AUDPC (▧). Bars for each parameter with the same letter indicate no significant differences (p≤0.05).

TABLE 1. Effect of treatments with antagonists or enzymatic complexes on disease incidence and isolation of *Fusarium oxysporum* f.sp. *lycopersici (FOL)* 67 days after inoculation with the pathogen (trial 1)[*]

| TREATMENT | DISEASE INCIDENCE (%) | | ISOLATION OF FOL (%) | |
|---|---|---|---|---|
| 1 | 25.00 | b | 20.00 | ab |
| 2 | 16.66 | b | 16.66 | ab |
| 3 | 66.6 | b | 66.6 | b |
| 4 | 0.00 | a | 0.00 | a |
| 5 | 0.00 | a | 0.00 | a |
| 6 | 40.00 | b | 33.30 | ab |
| 7 | 25.00 | b | 55.50 | ab |
| 8 | 100.00 | b | 60.00 | b |
| 9 | 71.42 | b | 88.88 | b |
| 10 | 0.00 | a | 0.00 | a |

[*] Data are means of 50 replicates. Data followed by the same letter in each column are not significantly different (chi square test, p=0.05).

TABLE 2. Effect of treatments with antagonists or enzymatic complexes on disease incidence and isolation of *Fusarium oxysporum* f.sp. *lycopersici (FOL)* 67 days after inoculation with the pathogen (trial 2)[*]

| TREATMENT | DISEASE INCIDENCE (%) | | ISOLATION OF FOL (%) | |
|---|---|---|---|---|
| 1 | 14.70 | ab | 45.45 | b |
| 2 | 32.35 | b | 64.70 | b |
| 3 | 24.24 | b | 45.45 | b |
| 4 | 40.52 | b | 52.94 | b |
| 5 | 64.70 | c | 67.64 | b |
| 6 | 50.00 | b | 54.83 | b |
| 7 | 41.17 | b | 50.00 | b |
| 8 | 27.02 | b | - | - |
| 9 | 58.82 | c | 70.58 | b |
| 10 | 7.69 | a | 25.64 | a |

[*] Data are means of 50 replicates. Data followed by the same letter in each column are not significantly different (chi square test, p=0.05).

In trial 1 a significant reduction in disease severity and AUDPC was observed in treatments with chitosan (56 and 59% reduction respectively), spore suspensions of AN (45 and 52%) and enzymatic complexes of MR (56 and 75%) and AN (35 and 50%) in alginate (Fig.1a). Only treatments with chitosan and the enzymatic complex of MR on alginate produced a significant reduction in disease incidence and isolation of the pathogen (Table 1). Data from these treatments were not significantly different from those observed in uninoculated and untreated plants.

In trial 2 treatments with spore suspensions of PO, FS+FM and the enzymatic complexes of FOL, PO and AN reduced disease severity (Fig.1b). Disease reduction produced by PO (14% reduction) and the enzymatic complexes of PO (18% reduction) and AN (28% reduction) was similar, while FS+FM treatment and the enzymatic complex of FOL resulted in disease severity similar to that recorded in the uninoculated and untreated plants. However, only treatments with FS+FM and the enzymatic complexes of AN and FOL gave lower AUDPC data than the inoculated and untreated control. Disease incidence was reduced by all the treatments in this assay, except the treatment with alginate pellets (Table 2). The enzymatic complex of FOL produced a reduction in disease incidence to the control level. However, none of these treatments resulted in a reduction of the presence of FOL in the plants at the end of the experiment.

DISCUSSION

The enzymatic complexes of MR, AN, PO and FOL in alginate pellets reduced *fusarium* wilt of tomato in the greenhouse. These results do not agree with those reported by other authors (Sneh *et al.*, 1984), who consider lytic enzymes are not important factors in the biocontrol of plant diseases. Resistance of *Fusarium* to lysis has been considered to be a consequence of the high chitin, hexosaminases and protein content in the cell wall (Sivan & Chet, 1989). In our experiments the enzymes in the assayed complexes were: chitinase, chitosanase, $\beta$-1,3-glucanase, pectinase, xylanase, galactamase and $\beta$-N-acetylglucosaminidase (Alfonso *et al.*, 1992b; Nuero *et al.*, 1993). These enzymes can partially attack cell walls of FOL, which are composed of three different polysacharide fractions: 52% $\beta$-1,3-glucan-chitin, 15% $\beta$-gluco-galacto-mannan y 1.3% $\beta$-1,3-glucan (Santamaria *et al.*, 1994). The cell wall fraction ($\beta$-gluco-galacto-mannan) present in FOL and other species of the same genus constitute an important factor in their resistance to the lysis (Sivan & Chet, 1989). This resistance could explain the presence of the pathogen in the plants at the end of our experiments, although the enzymatic treatments had diminished its effects on tomato plants. The treatment including enzymes of AN, PO and FOL only reduced disease incidence at the end of the experiment (30% reduction). This can be explained by a lower content in each specific enzymatic activity.

PO, AN and FS+FM reduced *fusarium* wilt of tomato in the greenhouse. Kaiser & Hannan (1984) using an isolate of *P.oxalicum*, controlled damping-off of pea to levels similar to those obtained with captan. Non-pathogenic *Fusarium* spp. have been used to control diseases caused. by different *formae specialis* of *F. oxysporum* (Lemanceau & Alabouvette, 1993). Amongst the different proposed modes of action,

competition for nutrients seems to be the main one. Our study indicates that lytic enzymes can play an important role in the biocontrol of plant pathogens by nonpathogenic fusarium and other antagonists.

## ACKNOWLEDGEMENTS

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## CONTROL OF THE PINE PROCESSIONARY MOTH (*THAUMETOPOEA PITYOCAMPA*) BY *BACISSUS THURINGIENSIS*-BASED COMMERCIAL PRODUCTS

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ABSTRACT

The efficacy of three bioinsecticides based on *Bacillus thuringiensis* var *kurstaki* was compared to that of the insect growth regulator (IGR) diflubenzuron for control of the pine processionary moth, *Thaumetopoea pityocampa*. Doses used were those recommended by the manufacturers for aerial ultra low volume applications. In the first two instars 100% mortality was attained in about two weeks with all bioinsecticides and the IGR. However, in L3 and L4 the effects of the bioinsecticides were different from those of the IGR, but 100% efficacy was attained after four weeks (longer for the IGR). Insects on pines treated with bioinsecticides stopped eating after a few days, while those on pines treated with the IGR exhibited a normal behaviour in all respects until their death (some weeks later). Thus, the bioinsecticides tested afforded significantly greater protection to the pine trees than the IGR.

INTRODUCTION

The processionary moth *(Thaumetopoea pityocampa)* (Lepidoptera: *Thaumetopoeidae*) is a pest of pine forests in Mediterranean countries (Sanchis *et al.*, 1990) of major economic significance (Robredo, 1980). It attacks primarily the genus *Pinus*, with *Pinus radiata* the preferred species (Avtzis, 1986). As the insect feeds on pine needles, it stunts the growth of trees, significantly decreasing annual wood production. In addition, larvae shed stinging hairs which cause allergic reactions that can prevent the use of recreational areas. During years with favourable climatic conditions, over 1/3 of the total conifer area can be infested.

In the Basque Country (Spain) 42% of the forest area consists of *P. radiata*. The processionary moth is controlled with aereal applications of diflubenzuron, an insect development regulator. After years of use, some negative effects are becoming apparent, due primarily to its lack of specificity and its persistance.

Most bioinsecticides are based on the toxin of the entomopathogenic bacterium *Bacillus thuringiensis* (Miller *et al.*, 1983), and only affect certain orders and families of insects (Currier & Gawron-Burke, 1990). Their efficacy against other forest pests, such as *Lymantria dispar,* has been demonstrated in earlier studies. This study was undertaken to determine the efficacy of some commercially available bioinsecticides to control *T. pityocampa* and to compare their effect with that of diflubenzuron, in an attempt to reduce the use of this chemical.

## MATERIALS AND METHODS

All insecticides used in this study were provided by the manufacturers. The main characteristics of these products, the doses applied and larval instars tested are summarized in Table 1. In this report these products will be referred to by the capital letter indicated in parenthesis (Table 1). All products were applied with a manual sprayer BIOBIT TMXL (courtesy of Novo-Nordisk).

Eggs and larvae were field-collected and maintained outdoors on pine branches, in cages (0.6 m x 1.0 m) constructed with fine mesh netting.

TABLE 1. Characteristics of the insecticides used and doses applied.

| INSECTICIDE | TOXIC[a] POTENCY BIU/l | DOSE l/ha | LARVAL INSTAR |
|---|---|---|---|
| FORAY 48B (A) | 12.7 | 1.00 1.60 2.00 3.00 | L1,L2,L3 L1,L2,L3,L4 L3(g)[b] L4 |
| DIPEL 8L (B) | 17.6 | 0.80 1.25 1.56 2.30 | L1,L2,L3 L1,L2,L3,L4 L3(g)[b] L4 |
| CONDOR (C) | 25.3 | 0.50 0.80 1.00 1.50 | L1,L2(g)[b] L1,L2,L3,L4 L2,L3 L4 |
| DIFLUBENZURON (D) | | 0.15 | L1,L2,L3,L4 |

[a]: Billions of International Units as stated by the manufacturers.
[b]: Value used only in greenhouse experiments.

Experiments conducted in an insectary

*P. radiata* trees (3 - 4 years old) were individually planted in pots (32 cm) and maintained in an insectary (8 x 16 m) protected from the rain, but at local ambient

temperatures (25°C to -4°C). For each set of experiments, 5 trees were treated with each of the doses indicated, with 5 untreated trees as a control. Twenty-five caterpillars of the same instar were placed on each tree, which was then individually covered with a fine mesh cloth bag to prevent migration of caterpillars. At 7 day intervals the number of live insects on each tree was recorded. The behaviour of insects on treated and untreated trees was recorded daily. After each experiment treated trees were moved outdoors and not used again until the following year.

Experiments conducted in the greenhouse

Branches (10 cm) from treated trees were layed on moist soil in glass jars (500 ml) and covered with fine mesh netting. Twenty larvae of the same instar were introduced into each jar. All jars, including untreated controls, were maintained at a temperature of between 9 and 16°C. Numbers of live insects were recorded as described above. The weight of the branches was recorded at the beginning and end of each experiment.

Statistical analysis

Results are expressed as percent efficacy after Schneider-Orelli (Ciba-Geigy S.A., 1981), calculated from mortality data using Bliss´s formula (Sokal & Rohlf, 1969). Mean separation was calculated when the variance analysis was statistically significant (Student t test, $p < 0.05$).


RESULTS AND DISCUSSION

Efficacy based on mortality data

In the insectary, (Fig. 1a) all bioinsecticides applied to 1st instar larvae gave a higher percentage efficacy than diflubenzuron at 7 days after treatment, although 100% efficacy was attained with all products after 14 days. In experiments conducted during the previous year, the difference in percentage efficacy between the bioinsecticides and diflubenzuron was more pronounced at the first sampling date (80% and 40% efficacy, respectively).

With 2nd instar larvae (Fig. 1b), all bioinsecticides gave higher efficacies than diflubenzuron at the first sampling date. One hundred percent efficacy was obtained with diflubenzuron at 14 days after treatment, and with all bioinsecticides at 21 days after treatment. Results from the previous year showed higher efficacies were obtained for bioinsecticides at the first 2 sampling dates, with all products resulting in 100% efficacy at 21 days. These differences could be due to the fact that the caterpillars used in the second year were in a late L2 stage, thus being more susceptible to diflubenzuron.

The effect of the bioinsecticides was seen more clearly with 3rd and 4th instars (Fig. 1c and 1d). Higher efficacies were consistently obtained with the bioinsecticides from the 1st week after treatment. These differences were statistically significant at 14 and 21 days after treatment. With L4 instars 100% efficacy was obtained at 6 weeks after treatment, while only 40% efficacy was obtained with diflubenzuron at this time.
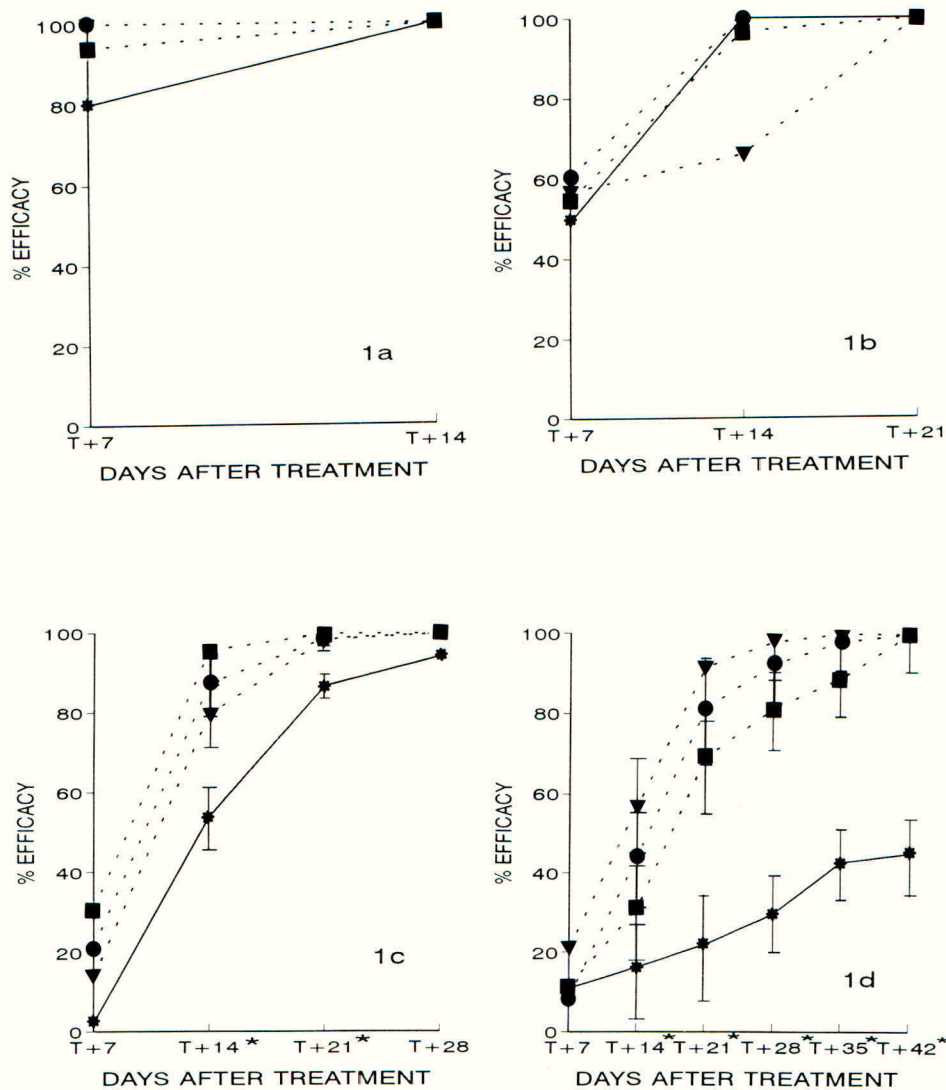
Figure 1. Percent efficacy obtained with bioinsecticides (....) A (■), B (▼) and C (●) and diflubenzuron (✳) on larvae of the processionary moth, *Thaumetopoea pityocampa*: a) 1st instars; b) 2nd instars; c) 3rd instars; and d) 4th instars. Vertical bars indicate LSD and * indicates statistically significant differences (p < 0.05).

Experiments conducted in the greenhouse gave similar results to those described above for the first 3 instars. The duration of the 4th larval stage was considerably shorter, and differences between the effects of bioinsecticides and those of diflubenzuron were less pronounced. This could be due to the higher temperature at which these experiments were conducted.

The results presented in this paper clearly demonstrate that the processionary moth can be effectively controlled by the three bioinsecticides tested. Differences in efficacies among these products (at the same toxic level) were not statistically significant. Higher doses at the same larval stage resulted in higher efficacies in shorter times. As larvae develop from the 2nd to the 4th instar, 100% mortality is attained only after longer times, for the same toxic level (Fig. 1). Thus, increasing the doses applied appears to be necessary if total control is to be obtained earlier in the instar.

The differences observed between the bioinsecticides and diflubenzuron in L3 and L4 (Fig. 1c and 1d) could be due to their different mode of action, which affects larvae at different points within each instar. In L1 and L2 (Fig. 1a and 1b) these differences are not observed because these stages are of a considerably shorter duration.

Behavioural Changes

In the insectary, insects on bioinsecticide treated pines did not exhibit gregarious behaviour, nor did they eat or construct their protective bag. Thus, bioinsecticides appear to destabilize the colony.

To quantify changes in eating habits, and determine the quantity of pine needles consumed, branches used in the greenhouse tests were weighed at the beginning and at the end of each experiment. The largest differences were recorded for L4 (Table 2), due to the voracity of these larvae. The differences in the weight of branches after bioinsecticide and diflubenzuron treatments were statistically significant. Insects on branches treated with bioinsecticides (particularly A and B) did not defoliate them and stopped eating shortly after

TABLE 2. Differences in the weight of branches treated with bioinsecticides and diflubenzuron at 28 days after treatment.

| INSECTICIDE | TOXIC POTENCY (BIU/ha) | DOSE (l/ha) | MEAN (g) AT 28 DAYS AFTER TREATMENT | STANDARD DEVIATION |
|---|---|---|---|---|
| A | 20.3 | 1.60 | 0.89 a | 0.35 |
| A | 38.1 | 3.00 | 0.86 a | 0.11 |
| B | 20.3 | 1.25 | 1.03 a | 0.29 |
| B | 38.1 | 2.30 | 1.18 a | 0.33 |
| C | 20.3 | 0.80 | 1.25 ab | 0.37 |
| C | 38.1 | 1.50 | 2.03 bc | 1.08 |
| D | -- | 0.15 | 1.97 bc | 0.64 |
| CONTROL | -- | -- | 2.56 c | 0.42 |

Values followed by the same letter are not statistically significant at the 95% level (21 d.f. and LSD = 0.780).

being placed on them. However, those on branches treated with diflubenzuron exhibited similar behaviour to that of control insects until their death. Thus bioinsecticides prevent tree defoliation by interferring with the insects feeding behaviour.

Thus the results presented in this paper clearly show that the bioinsecticides tested yielded percent efficiacies comparable to, or better than, those of diflubenzuron. In addition, they afforded greater protection to the trees by effectively preventing defoliation. Thus, these products are an excellent alternative to the more frequently used diflubenzuron.

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