Project title:	Development of the entomogenous fungus, *Metarhizium anisopliae*, for control of vine weevil and thrips in horticultural growing media
Project number	DEFRA Horticulture Link Code HL 0171
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Project leader	Dr Tariq M. Butt Reader Department of Biological Sciences University of Wales, Swansea SA2 8PP
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Location	University of Wales, Swansea
Project coordinator:	Dr. Paul Sopp Fargro Ltd. Toddington Lane Littlehampton West Sussex BN17 7PP
Date commenced:	1st April 2005
Expected Completion Date	31st March 2008
Key words	*Metarhizium*, Black vine weevil, western flower thrips, sublethal doses, reduced chemical insecticides, fipronil, imidacloprid, growth media, peat, coir, bark, composted green waste

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Table 1 Primary milestones and progress made in first 24 months

Milestone	Title	Target Date	Progress to date
	Start project	1.4.05	
1.1	Physical-chemical properties of growing media characterised	15.7.05	Accomplished
2.1	Synergy between *M. anisopliae* and sublethal dose of imidacloprid/fipronil established	15.9.05	Accomplished
2.2	Efficacy of *M. anisopliae* for control of black vine weevil (BVW) larvae and western flower thrips (WFT) pupae in different media determined – *M. anisopliae* enhancing medium identified	15.12.05	Accomplished
3.1	*M. anisopliae* compatibility with cold tolerant entomopathogenic nematodes determined	31.4.06	Accomplished
4.0	Influence of media porosity on loss of conidia determined	15.8.05	Accomplished
6.1	Microbial activity in different media established	15.10.06	Studies in progress
6.2	Profile of microbial community based on molecular and substrate utilisation studies determined	15.10.07	Studies in progress
6.3	Impact of natural microbial population on the efficacy of *M. anisopliae* assessed	15.11.07	Studies in progress
7.1.	Shelf life studies completed and data analysed	1.01.08	Studies in progress
8.1	Grower trials of *M. anisopliae* assessed (linked with workshops)	15.11.07	
	End Project	31.03.08	

Table 2 Secondary milestones and progress made in first 24 months

Milestone	Title	Target Date	Progress to date
1.1	First batch of humic-fulvic substances prepared for use in objective 5.	15.6.05	Accomplished
2.1	Studies on the effect of sublethal doses of imidacloprid and fipronil on pest behaviour and acquisition of *M. anisopliae* conidia completed	1.03.06	Accomplished
2.2	**Manuscript** on synergy between sublethal doses of insecticide and *M. anisopliae* submitted	15.5.06	Accomplished (2 papers published see outputs)
2.3	Efficacy of drench *versus* pre-mixed *M. anisopliae* application methods determined	1.03.06	Accomplished
3.1	Efficacy of two cold tolerant entomopathogenic nematode species (Nemasys vs Entonem) established	1.03.06	Accomplished
4.1	**Manuscript** on the impact of the physical-chemical properties of media on the germination and virulence of *M. anisopliae* submitted	15.9.06	Submitted to Biocontrol (under review)
5.1	Inhibitory/stimulatory effects of media leachates especially fulvic and humic substances on germination and virulence determined	15.01.06	Accomplished
6.1.	Relationship between microbial community profile and *M. anisopliae* efficacy established.	1.10.07	Studies in progress
7.1	**Manuscript** on shelf life (viability and virulence) of *M. anisopliae* in different media submitted	30.03.08	Studies in progress
7.2	**Protocols for** testing impact of physical-chemical and microbial components of horticultural **growing media on fungal BCAs**	30.03.08	Consultation with partners initiated
8.1	**Grower protocols** on different ways of deploying *M. anisopliae* completed. Protocols take into account feedback from participants of grower trials and workshops.	30.03.08	Studies in progress

Grower summary

HL 0171/HNS 133

Development of the entomogenous fungus, *Metarhizium anisopliae*, for control of vine weevil and thrips in horticultural growing media

2nd Annual Report 2007

HL 0171/HNS 133

Development of the entomogenous fungus, *Metarhizium anisopliae*, for control of vine weevil and thrips in horticultural growing media

Headline

The entomogenous fungus *Metarhizium anisopliae* has provided excellent control of black vine weevil larvae in a range of soil-less substrates and can provide more complete control when used in conjunction with chlorpyrifos or predatory nematodes.

Background and expected deliverables

Black vine weevil (BVW) is considered the most important pest of hardy nursery stock (HNS) causing annual losses of around £30 million. Western flower thrips (WFT) are pests of protected plants causing damage directly through feeding and indirectly through the transmission of plant viruses. Both BVW and WFT spend part of their life cycle in growing media. Control of the subterranean stages of these pests would contribute significantly to IPM programmes. This project complements earlier studies funded by HDC and the EU which aim to reduce insecticide inputs, especially for BVW and WFT control. The overall aim of this project is to develop the V275 strain of the entomogenous fungus *M. anisopliae* for the control of BVW larvae and WFT pupae in different plant growing media. It offers a benign alternative to chemical insecticides that are currently under threat of being phased out (e.g. chlorpyriphos) or which are becoming less effective because of increasing resistance in pest populations (WFT is resistant to many pesticides e.g. chlorpyriphos).

The expected project deliverables will include:

- Reduced inputs of chemical insecticides for the control of BVW larvae and WFT pupae.

- Data on the efficacy and robustness of *M. anisopliae* V275 in peat and other soil-less substrates. .

- Elucidation of the interactions between *M. anisopliae* and sub-lethal doses of insecticides.

- Determination of whether *M. anisopliae* is compatible with cold tolerant entomopathogenic nematodes for the control of BVW larvae.

- Grower protocols (advisory leaflet) on the use of *M. anisopliae* for the control of BVW larvae and WFT pupae.

- Standardized protocols for testing the efficacy of microbial pest control agents in plant growing media.

Summary of the project and main conclusion

The main findings of this project are listed below:

- The insect pathogenic fungus *M. anisopliae* V275 was highly effective in controlling black vine weevil (BVW) larvae in a range of different plant species such as *Euonymus fortunei* 'Emerald Gold, *Tellima grandiflora*, *Sedum spurium* 'Ruby Mantle' and *S. telephium* 'Mohrchen' and strawberry (c.v. Elsanta) and growing media (peat, bark, coir, peat blends with 10% and 20% composted green waste (CGW).

- Good control (70-100%) was achieved whether the fungus was applied as a drench or premixed into a range of growing media.

- Excellent (100%) control of BVW larvae was achieved when *M. anisopliae* V275 was used with or without reduced rate of chlorpyriphos (10% of recommended rate) in potted strawberry plants. The control achieved was better than the recommended dose of chlorpyriphos.

- Excellent (100%) control of BVW larvae was achieved when *M. anisopliae* V275 was used in combination with cold tolerant entomopathogenic nematodes. This was verified in strawberry growbag trials conducted at Delflands Nursery. The control achieved was better than using either agent on its own.

- Studies revealed that use of *M. anisopliae* resulted in an increased diversity of microbial populations and had no adverse effect on indigenous populations. Conversely, the microbial populations had no adverse effect on the efficacy of *M. anisopliae.*

Financial benefits

Studies are in progress. A report on the financial benefits will be submitted either at the end of 2nd year or with the final project report.

Action points for growers

M. anisopliae is not yet registered for use as a biocontrol agent and cannot be used by growers in commercial practice. Work is underway to initiate the process of registration.

Science Section

HL 0171/HNS 133

Development of the entomogenous fungus, *Metarhizium anisopliae*, for control of black vine weevil and thrips in horticultural growing media

Objective 2: Control of black vine weevil in different plant species
(additional studies to show robustness of *M. anisopliae* in different plant species)

Introduction

Black vine weevil, *Otiorhynchus sulcatus* (BVW) is a major pest of horticultural and ornamental plants. It is known to feed on over 140 different plant species. During the first phase of this project, we have shown that *M. anisopliae* strain V275 is highly efficacious in the control of BVW larvae in *Euonymus* outdoor pot trials (Shah et al., 2007; 1st annual report HNS 133/HL0171). The aim of this study was to determine if the host plant influenced the efficacy of *M. anisopliae* in controlling BVW larvae.

Material and Methods

Fungal strain, maintenance and mass production

Details of the maintenance and production of *M. anisopliae* V275 are given in Shah et al. (2005).

Source and maintenance of BVW

Vine weevil eggs were obtained from a colony maintained at UWS (1st annual report HNS 133/HL0171).

Plant and growing media

Rooted cuttings of *Euonymus fortunei* 'Emerald Gold" were kindly provided by Johnsons of Whixley, York. Other plant species; *Tellima grandiflora*, *Heuchera micrantha diversifolia* 'Palace Purple', *Galium odoratum*, *Astilbe* 'Bressingham Beauty', *Sedum spurium* 'Ruby Mantle' and *S. telephium* 'Mohrchen' were kindly provided by W. Godfrey

& Sons Ltd, Surrey. The plant growing media used in this study (peat, bark, coir and peat blended with 10% (v/v) or 20% (v/v) composted green waste (CGW) were supplied by Bord Na Mona (Ireland)

Inoculation of plants with BVW eggs

Euonymus was grown in all five growth media whereas the other plant species were grown in peat and bark. Each plant was potted in 0.5l pots and inoculated with 20 melanised BVW eggs – which represents a "worse case scenario".

Application of *M. anisopliae* and insecticides

M. anisopliae was applied as a drench or premixed such that the final concentration of inoculum was 1×10^{10} conidia/l of compost. The chemical insecticides Provado® (Bayer a.i. 5% w/w imidacloprid) and Vi-Nil® (Certis, a.i. 1% fipronil) were used at the recommended rate of 65 ml/l and 1 g/l of growing media, respectively.

Plants were maintained outdoors where the average day and night temperatures ranged between 15-25°C and 10-15°C, respectively. Treatments were arranged in a randomised complete block design with each pot adequately spaced to avoid cross-contamination between treatments. Plants were destructively assessed 6 weeks post egg infestation to determine the efficacy of the above treatments.

Evaluation of assays and analysis of data

Plants were destructively assessed six weeks post egg infestation to determine the efficacy of the above treatments. The data was analysed by one way ANOVA followed by a post hoc test (Duncan test) to identify significant differences among the treatment (SPSS V13). The percent efficacy of any treatment was calculated using the following formula.

$$\frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

Results

BVW control in *E. fortunei*

Significance differences were observed between treatments ($P < 0.001$) and different growth media ($P < 0.001$). Interactions between treatments and growing media were also significant ($P < 0.001$) (Fig 1-5). *M. anisopliae* provided 73-88% (drench) and 71-95% (premixed) control and varied significantly ($P < 0.05$) with drench application only in peat (Fig. 1). In all other growth media, no significant differences in *M. anisopliae* efficacy were observed whether it was applied as a drench or premixed (Figs. 2-5).

Fipronil provided 100% control at the recommended dose. However, imidacloprid when used at the recommended dose gave 56-94% control in the different growth media (Figs. 1-5).

Plant growth media significantly ($P < 0.001$) influenced BVW establishment. In untreated control plants, BVW larval recovery was highest in coir and least in bark (Fig. 6)
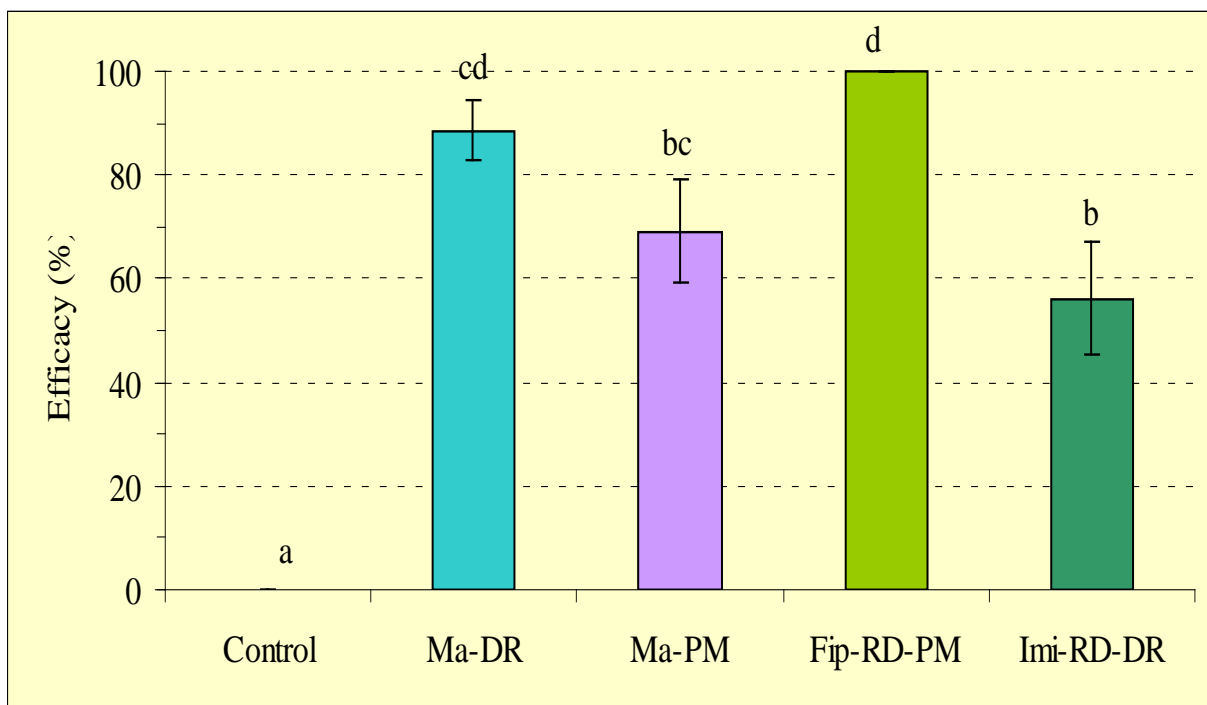

Fig. 1. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat. The treatments included: No treatment (control), *M. anisopliae* applied as drench (Ma-DR), *M. anisopliae* premixed (Ma-PM), Fipronil premixed at the recommended dose

(Fip-RD-PM) and Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR). Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$).
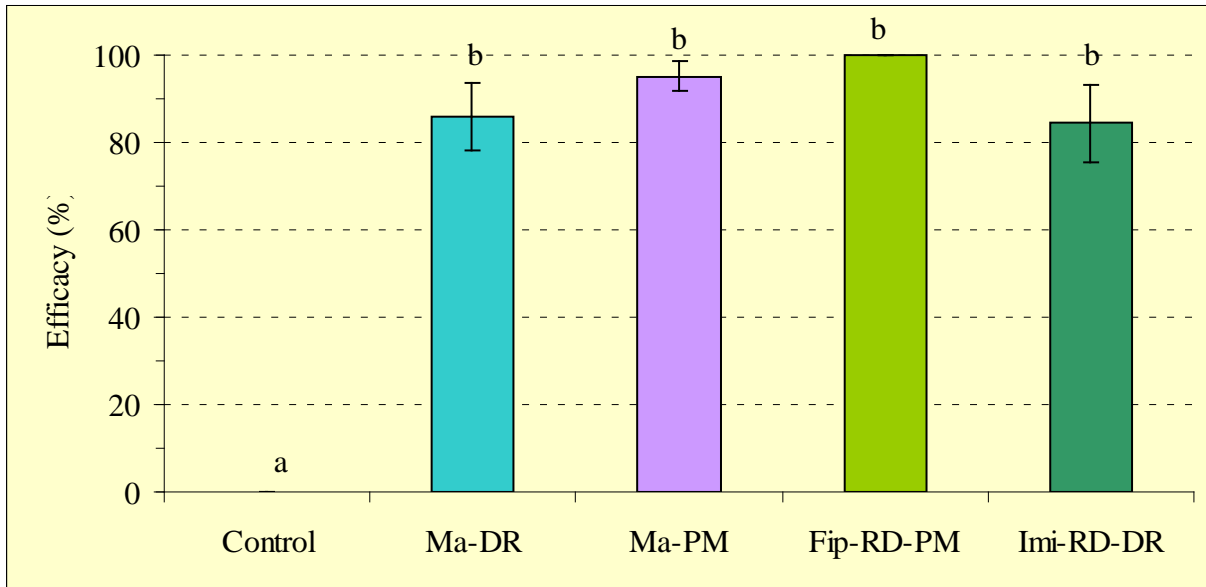
Fig. 2. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in bark. Treatments are the same as those described in Fig 1.
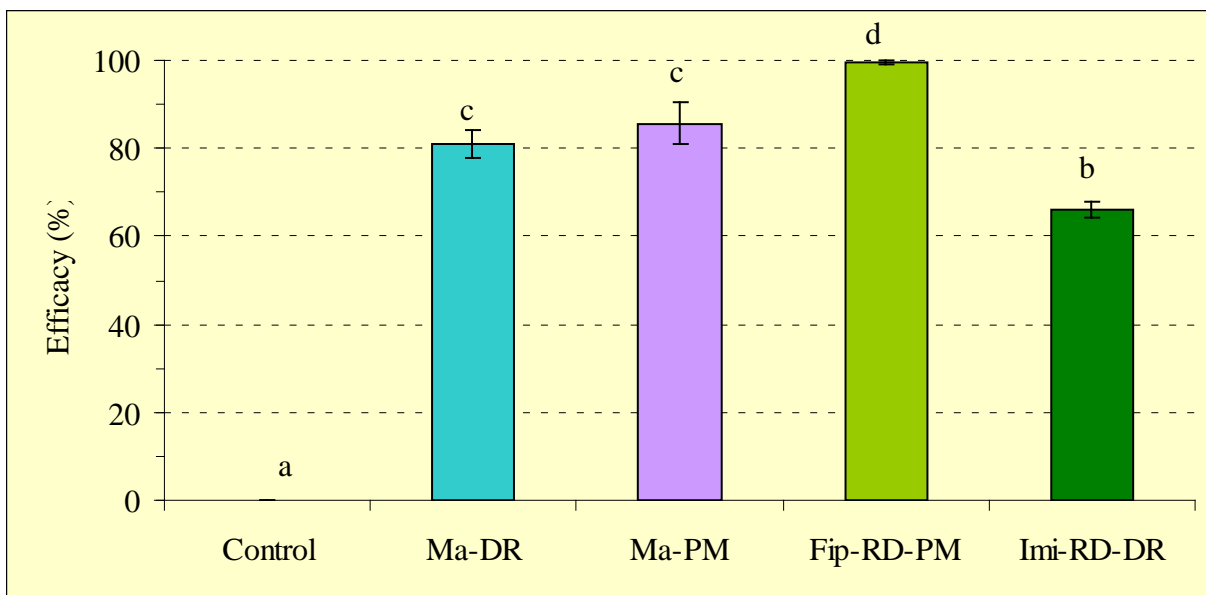

Fig. 3. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in coir. Treatments are the same as those described in Fig 1.
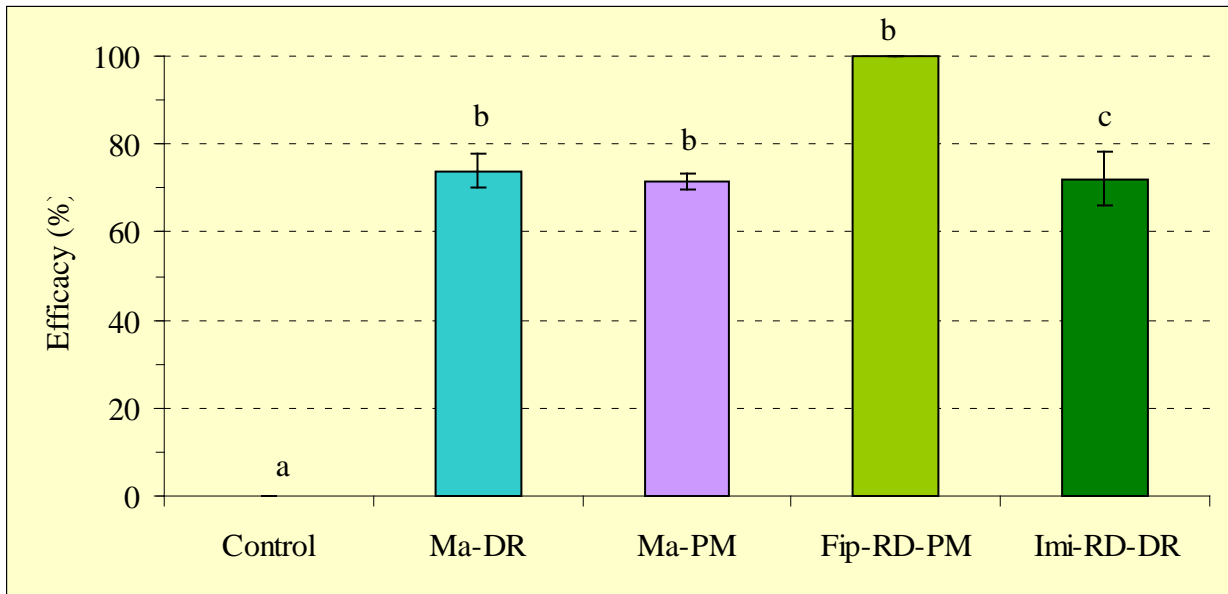
Fig. 4. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat blended with 10% CGW. Treatments are the same as those described in Fig 1.
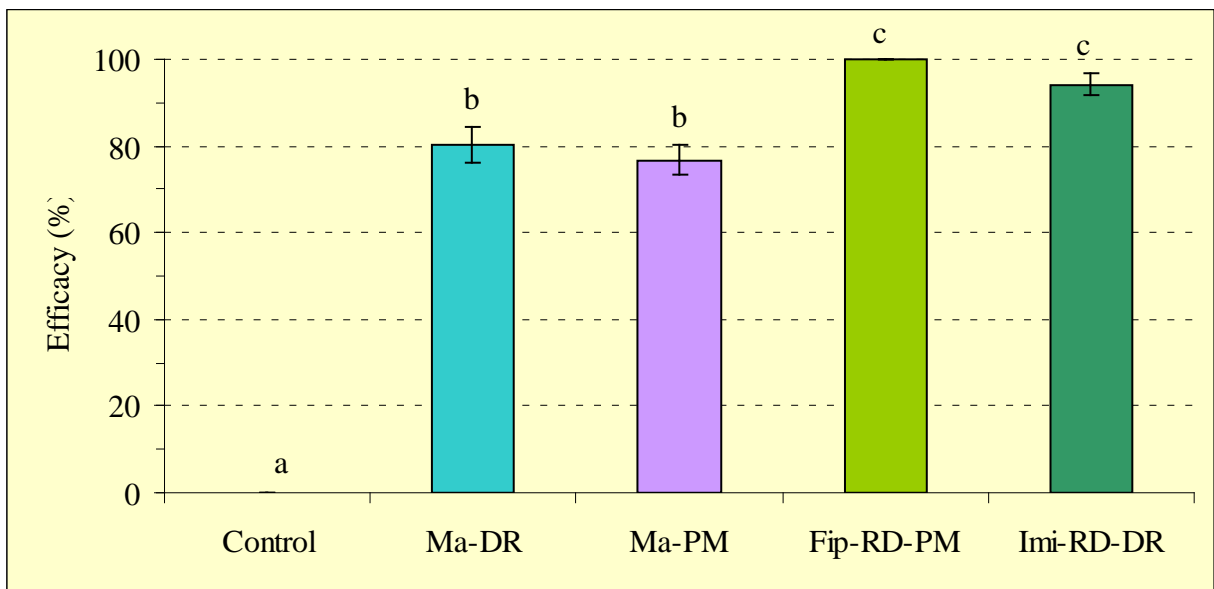

Fig. 5. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat blended with 20% CGW. Treatments are the same as those described in Fig 1.
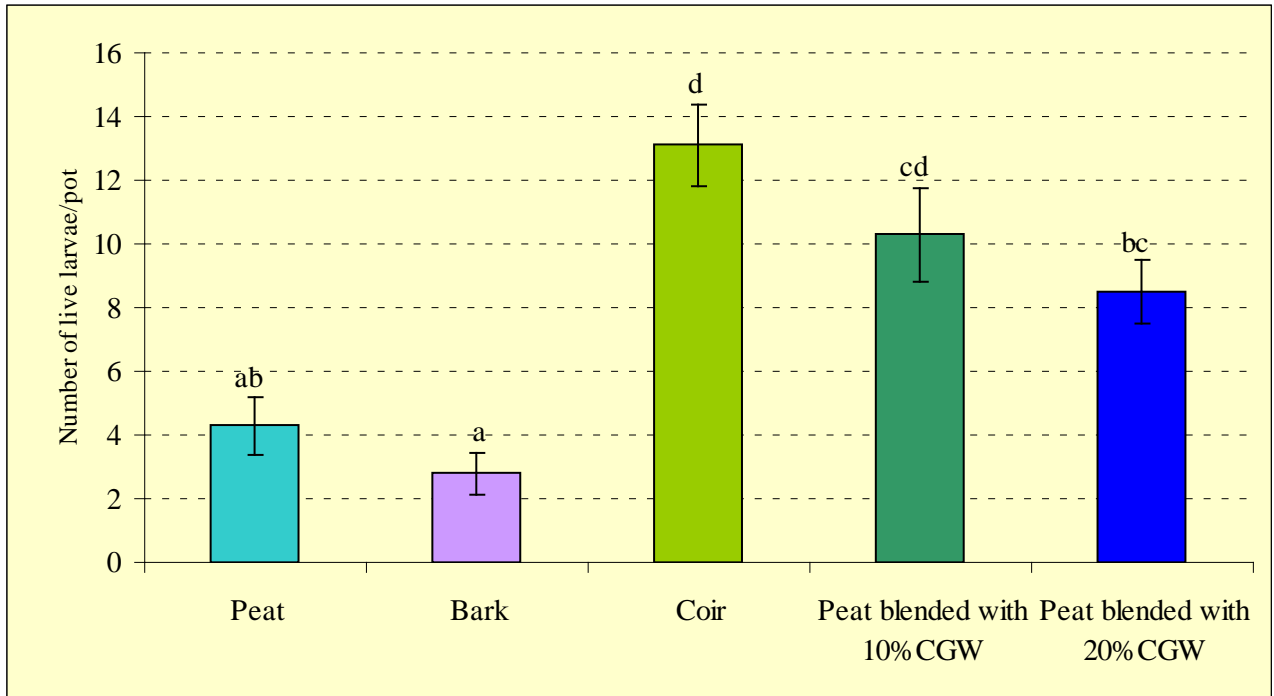
Fig. 6. Establishment of BVW larvae in different plant growth media. Each pot was infested with 20 melanised BVW eggs and destructively assessed 6 weeks post-infestation.

BVW control in *Tellima grandiflora*

Significant differences were observed between treatments ($P < 0.001$) in peat. There was no significant difference in *M. anisopliae* efficacy whether it was applied as drench or premixed. Drench applications gave more than 95% control and varied non-significantly with fipronil and imidacloprid. Both insecticides provided 100% control of BVW (Fig. 7).
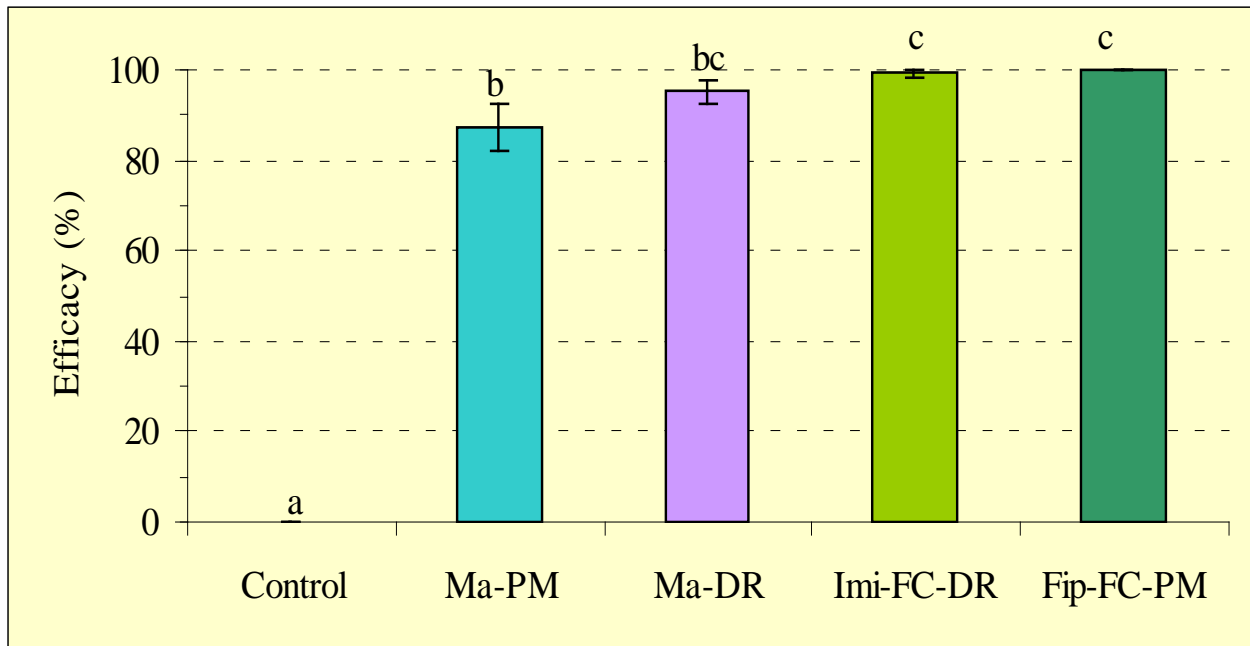
Fig. 7. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *T. grandiflora* potted in peat. The treatments are listed below. No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed at the recommended dose (Fip-RD-PM). Bars displaying same letter vary non-significantly (Tukey's test, *P* < 0.05).

Plants treated with *M. anisopliae* or insecticides appeared healthy and had an extensive root system (Fig. 8) while untreated plants were stunted and had poorly developed root systems (Fig. 9).
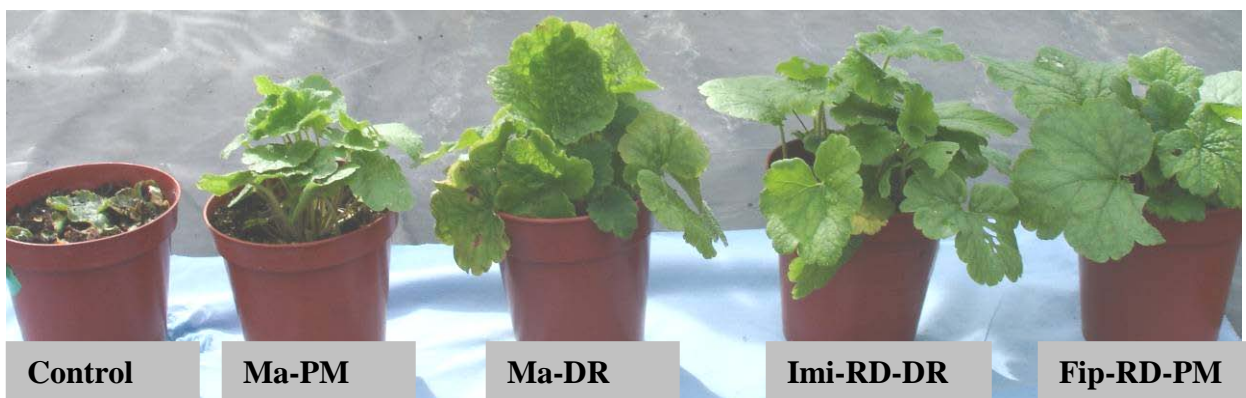

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Fig. 8. Comparison of the plant growth of *T. grandiflora* exposed to the following treatments: No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed into the peat at the recommended dose (Fip-RD-PM). Each pot was infested with 20 BVW eggs and destructively assessed 6 weeks post infestation.
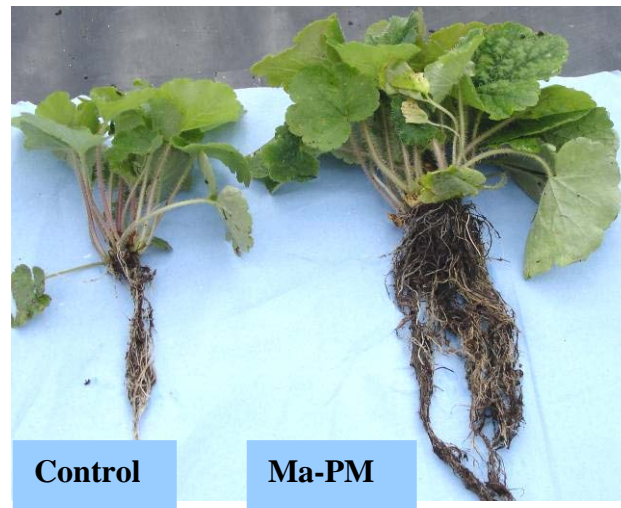

Fig. 9. Comparison of the root system of *T. grandiflora* untreated (control) *M. anisopliae* premixed (Ma-PM) in peat.

BVW control in *Sedum spurium* 'Ruby Mantle'

Significant differences were observed among treatments ($P < 0.001$) in peat. Drench application of *M. anisopliae* provided 100% control. However, premixed application of fungus gave only about 80% control and varied significantly ($P < 0.001$) with drench application. Both insecticides provided 100% control of BVW (Fig. 10).
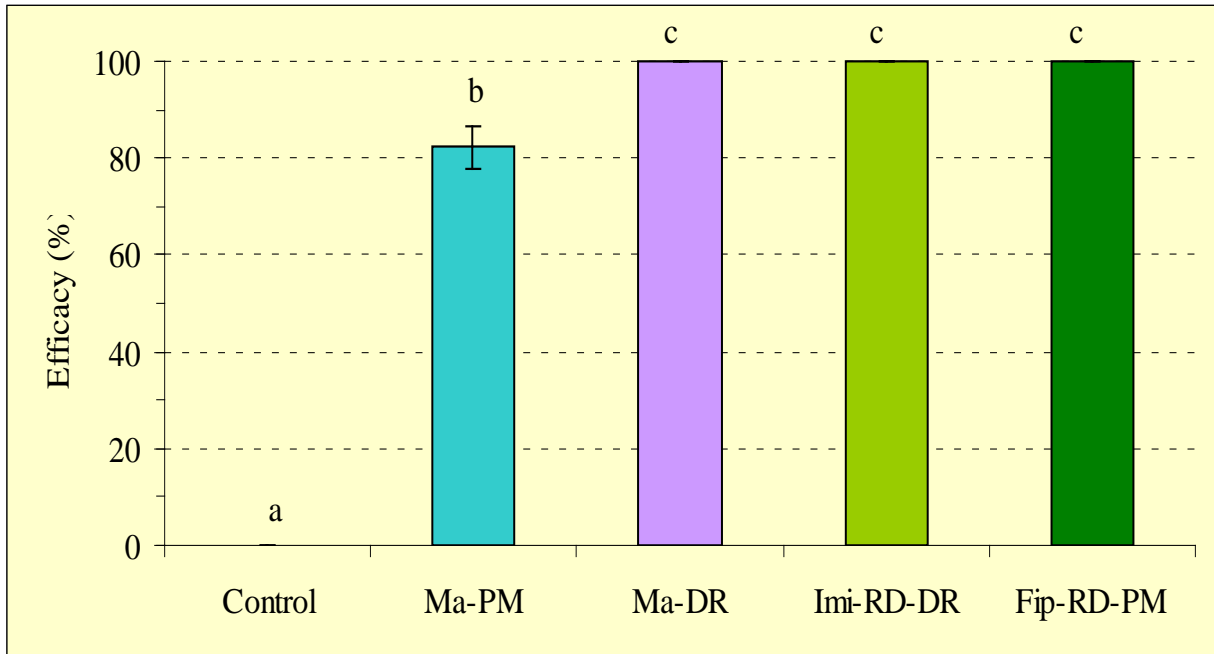

Fig. 10. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *S. spurium* 'Ruby Mantle' potted in peat. The treatments are listed below. No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed at the recommended dose (Fip-RD-PM). Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$).

Plants treated with *M. anisopliae* or insecticides appeared healthy while untreated plants appeared stunted (Fig. 11).

Fig. 11. Comparison of the plant growth of *S. spurium* 'Ruby Mantle' potted in peat exposed to the following treatments: No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed at the recommended dose (Fip-RD-PM). There was one *S. spurium* plant per pot which was infested with 20 BVW eggs and destructively assessed 6 weeks post infestation.

High temperatures and limited rainfall resulted in rapid drying of the bark media, therefore, no larval establishment was observed in *T. grandiflora* and *S. spurium* potted in this medium. Similarly, we did not found any larvae in *S. telephium* 'Mohrchen' and *H. micrantha diversifolia* 'Palace Purple' potted in peat and bark growth media.

Discussion

This study clearly demonstrates that *M. anisopliae* V275 is robust since it gives good control of BVW larvae in a range of commercial horticultural growing media and is not affected by plant species. The level of control provided by *M. anisopliae* was similar to fipronil but higher than imidacloprid in *Euonymus*. In other plant species, such as *T. grandiflora* and *S. spurium*, imidacloprid gave relatively better protection and varied non-significantly with *M. anisopliae* or fipronil. These findings indicate that plant species may influence imidacloprid efficacy, however, *M. anisopliae* and fipronil efficacy is independent of growth media and/or plant species. These findings corroborate earlier work by Shah et al. (2007) who showed excellent control of BVW with *M. anisopliae* V275 used alone or in combination with low doses of fipronil or imidacloprid in different growing media.

This study confirmed two other earlier findings: (1) *M. anisopliae* efficacy was statistically independent of the application method even though drench applications appear to give marginally better control of BVW larvae. (2) The plant growth medium influenced BVW establishment. Coir provided the most favourable environment whereas the bark medium provided the least favourable environment for establishment of BVW larvae.

Objective 2: Efficacy of *M. anisopliae* to control black vine weevil in potted strawberries

Introduction

Having established that *M. anisopliae* V275 was effective in controlling BVW larvae in a range of HNS (see above objective and Shah et al., 2007) we extended our studies to strawberries. More specifically we wanted to: (i) compare the efficacy of V275 with the chemical insecticide Cyren (chlorpyrifos) and (ii) determine if *M. anisopliae* worked synergistically with reduced rates of chlorpyrifos.

Material and Methods

Plants

Young strawberry plants (c.v. Elsanta) kindly provided by Hargreaves Nursery, Plants Ltd, Lincolnshire, UK were transplanted into 0.5 l pots filled with peat (Bord Na Mona, Ireland).

Application of *M. anisopliae* and chlorpyrifos

M. anisopliae V275 applied as a drench or premixed at the rate of 1×10^{10} conidia/l of growth medium. V275, was used with or without a reduced rate (1/10 dilution) of the insecticide Cyren (46% w/w chlorpyrifos; Cheminova, Denmark). Chlorpyrifos was also used alone at the recommended and reduced rates. Untreated control plants were treated with water only. Each plant was infested with 20 BVW eggs which were gently placed around the base of the plant.

Trials were conducted in an unheated glasshouse between September-October 2006 when the average day and night temperatures ranged between 18-25ºC and 15-20ºC, respectively. Each treatment was replicated 20 times, however, the experiment was done once only. Treatments were arranged in a randomised complete block design with each pot adequately spaced to avoid cross-contamination among treatments.

Plants were destructively assessed 6 weeks post egg infestation to determine the efficacy of the above treatments.

Results

All treatments caused significantly higher BVW control than the untreated control ($P <$ 0.001). *M. anisopliae* V275 provided 97 and 100% control of BVW larvae whether it was premixed or applied as a drench (Fig. 12). There were no significant differences when *M. anisopliae* was applied alone or in combination with the reduced rate of chlorpyrifos. Chlorpyrifos gave 95% control at the recommended rate but 52% control at the reduced rate (Fig. 12).
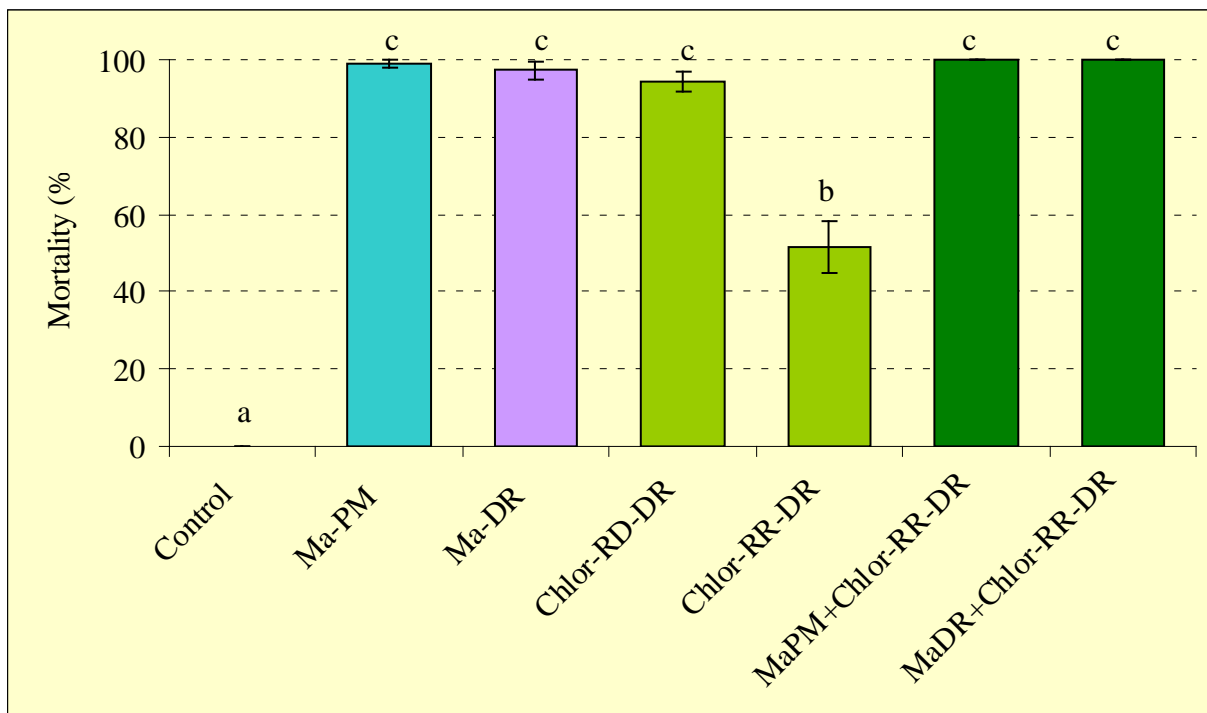

Fig. 12. Comparison of the efficacy of *M. anisopliae* V275 and chlorpyrifos for BVW control in strawberries. The treatments included: *M. anisopliae* premixed into the compost (MaPM) or applied as a drench (MaDR). Chlorpyrifos applied as a drench at the recommended dose (Chlor-RD-DR) or reduced rate (Chlor-RR-DR). *M. anisopliae* premixed into growth media and reduced rate of chlorpyrifos applied as a drench

(MaPM+Chlor-RR-DR), *M. anisopliae* and reduced rate of chlorpyrifos applied as a drench (MaDR+Chlor-RR-DR). There was one strawberry plant per pot which was infested with 20 BVW eggs and destructively assessed 6 weeks post infestation. Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$). Data shown are corrected for control mortality.

Plants treated with *M. anisopliae* V275 or chlorpyrifos grew vigorously while untreated plants were stunted and had poorly developed root systems (Fig. 13).

Fig. 13. Comparison of the root system of strawberry plants exposed to the following treatments: From left: untreated control, *M. anisopliae* premixed into the compost (Ma-PM) or applied as a drench (Ma-DR). Chlorpyrifos applied as a drench at the recommended dose (Chlor-RD-DR) or reduced rate (Chlor-RR-DR). *M. anisopliae* premixed into growing media and reduced rate of chlorpyrifos applied as a drench (Ma-PM+Chlor-RR-DR), *M. anisopliae* and reduced rate of chlorpyrifos applied as a drench (Ma-DR+Chlor-RR-DR). There was one strawberry plant per pot which was infested with 20 BVW eggs and destructively assessed 6 weeks post infestation.

Conclusions

Our results shows that *M. anisopliae* V275 can reduce inputs of commercially available insecticides for control of BVW larvae in strawberry when used alone or in combination with reduced rate of insecticides (e.g. Chlorpyrifos). The latter approach gives immediate protection because the chemical insecticides stop insects from feeding and

gives the slower acting fungus more time to kill its host. Furthermore, this strategy gives control similar to the recommended dose of the chemical insecticide.

Objective 6: Elucidate the impact of microorganisms in growing media on the efficacy of *M. anisopliae*

Introduction

This objective takes into account that microbial activity will vary with different media and will have a profound effect on peat blends e.g. immature compost when mixed with peat can result in an accelerated breakdown of the peat. This will affect both plant growth and the efficacy of fungal BCAs like *M. anisopliae*. Very little is known about the interactions between naturally occurring microbes with entomogenous fungi. By monitoring microbial activity and the microbial community profile and comparing data on efficacy (objective 2), conidial leaching (objective 4), humic-fulvic substances (objective 5), and shelf life (objective 7) it will be possible to define conditions under which *M. anisopliae* will give optimal control and when it will be least effective. It may reveal which microbes predominate when *M. anisopliae* is least efficacious. This will be the first study to assess the impact of potential microbial antagonists on *M. anisopliae* and will help towards the delivery of testing protocols for BCAs. Objective 6 complements objectives 2, 4, 5 and 7.

Materials and Methods

Preparation of plant growth media for microbial community analysis

M. anisopliae was premixed in all five plant growth media to the final concentration of 1 x 10^{10} conidia/l of media. Media without *M. anisopliae* was used as control. There were four treatments for each media as described below.

1. Plant growth media alone

2. Plant growth media + *Euonymus*

3. Plant growth media + *M. anisopliae*

4. Plant growth media + *M. anisopliae* and *Euonymus*

Each treatment was replicated five times in 0.5l pots. Pots were maintained outdoor and a composite sample was taken from each treatment at 0, 1, and 6 months post inoculation. Studies will be terminated at 12 months post inoculation. Composite samples were then processed for different studies to understand the structure and profile of microbial community and its influence on *M. anisopliae* efficacy.

Task 6.1 Determination of microbial biomass and activity in different plant growth media

Microbial biomass was determined by substrate-induced respiration (SIR) (Anderson and Domsch 1978) studies. Briefly, 30g of medium was blended with 2% glucose (glucose-monohydrate, Merck, 104074) in test tubes and connected to an automated system for assessing continuous soil respiration (Heinemeyer et al. 1989). Studies were done at 22 ± 0.5°C and a gas flow setting of 200 ± 10/ml. Carbon dioxide production was measured hourly over 43 h by infra-red gas analysis (Type 225 MK3, Analytical Development Company). Microbial biomass was calculated following Anderson and Domsch (1978).

Microbial activity in growth media was measured by the fluorescein diacetate (FDA) hydrolysis assay (Schnurer and Rosswall, 1982). Briefly, FDA was dissolved in acetone (2 mg/ml) and 0.5 ml of this stock solution added to 1g of growth medium suspended in 50 ml of 60 mM phosphate buffer (pH 7.6) in a 125-ml Erlenmeyer flask. Thus the FDA working solution was 20 µg/ml. The flask was incubated for 1 h at 27°C in a GallenKamp orbital incubator (Sanyo) at 200 rpm, then 50 ml of absolute acetone added to stop further FDA hydrolysis. Soil was removed from the suspension by centrifugation for 5 min at 6,000 rpm followed by filtration through Whatman no. 1 filter paper. This produced a clear solution with a low-background absorbance. The amount of FDA hydrolyzed was measured at A490 in a FluoStar spectrophotometer (BMG labtechnologies).

Results

SIR studies showed that substrates inoculated with *M. anisopliae* showed increased levels of microbial biomass (up to 31.9 %) compared to corresponding substrates without fungus. Initial microbial biomass was higher in coir and peat blends with CGW and least in peat medium. FDA test showed high levels of microbial activity in all the samples, however, statistical analysis showed no significant difference in samples with or without *M. anisopliae*. Similarly, microbial activity varied non-significantly among different plant growth media.

Task 6.2 Structure of microbial populations

Structure of microbial population was determined by exploitation of physiological and molecular difference among microbes using two complementary techniques.

A. Community level physiological profiling (CLPP)

Introduction

This method is based on the microbes ability to utilise different carbon substrates. The substrate utilisation approach, using the Biolog MicroPlate Assay, facilitates a community level physiological profile. This is a rapid, high throughput method that provides a "metabolic fingerprint" of the microbial community over time and has been used widely by soil ecologists (Gamo and Shoji, 1999; Balser et al., 2002; Classen et al., 2003)

Materials and Methods

Samples were extracted in quadruplicate following Hopkins et al. (1991) and Monidini and Insam (2003) with some modifications: Fresh media samples (1 g dry weight) were blended with 20 ml of 0.1 % (w/v) sodium cholate solution (Sigma, C1254), 8.5 g cation exchange resin (Dowex 88, Sigma-Aldrich, 436682), and 30 glass beads (2 mm). The suspension was shaken head-over-head for 2 h at 4°C and centrifuged (Sorvall SS34 rotor, 2200 rpm) for 2 min. The supernatant was decanted in a flask. The pellet was re-suspended in 10 ml of 0.1 M Tris-buffer (pH 7.4), shaken and centrifuged as before and the supernatant and the extract of the first step pooled. To separate media particles, this suspension was filtered through a cotton column (Newmeyer 1990). All extracts were diluted tenfold in sterile ¼ strength Ringer solution (Merck, 10113) to reach an optical density (O.D.) of 0.2 absorption units (595 nm) for the darkest solution. All solutions, reagents and glassware were sterilised at 121 °C for 20 min prior to the extraction steps. Biolog Ecoplates containing 31 different carbon compounds and a control well (repeated three times on a 96-well plate) were inoculated with 130 μL of diluted extracts and incubated at 25°C. Colour formation at 595 nm was measured every 8 hours for 7 days (Anthos-Zenyth 3100 Microplate Reader). Raw O.D. data were corrected by subtracting the absorbance values of respective control wells. Following Lindstrom et al. (1998) and Mondini & Insam (2003), three kinetic parameters were estimated by fitting the curve of corrected O.D. vs. time to a density dependent logistic growth equation:

$$y = OD_{595nm} = \frac{K}{(1 + e^{-R \cdot (t - S)})}$$

Where K is the maximum that the test well O.D. curve reaches, R determines the exponential rate of O.D. change, t is the time following inoculation of the microplates and S is the time when the reversal point of the exponential portion of the curve is reached. Parameters were estimated using the software OriginPro (version 7G SR4). Exponential Rates R resulting from curve fits with $\chi^2 < 0.05$ and being normally distributed (95% CI) were used for principal component analysis (PCA) (Statistica, version 7.1). Following these criteria, about 7 % of exponential rates were discarded due to bad curve fit. In case of a particular bad fit, all parameters of the respective carbon source were omitted for further analysis.

Results and Discussion

Community level physiological profiling

Comparison of all treatments within any one plant growth medium (0, 1 and 6 month's samples with or without *M. anisopliae* and/or plant) showed a stable utilisation of 31 carbon substrates in the Biolog kit (Fig. 14). The only anomaly was the untreated (no plant, no V275) bark at 1 month which differed from the other treatments in carbon substrate utilisation (Fig. 15). Both the activity and range of carbon substrate utilised was higher in the 6 months than 0 and 1 month samples suggesting increased microbial activity and diversification. All the media had similar carbon substrate utilisation profiles at 0 and 1 months (Figs. 16- 19). However, at 6 months two distinct groups were distinguished: Group1 consisting of bark and peat blends with 10% and 20% CGW and Group 2 consisting of peat and coir (Figs 20-21). These observations suggest distinct microbial communities in these two groups of media which are not apparent until 6 months into the study.

Incorporation of *M. anisopliae* into composts had no negative impact on the physiological ability of the community to utilise the 31 carbon sources examined.
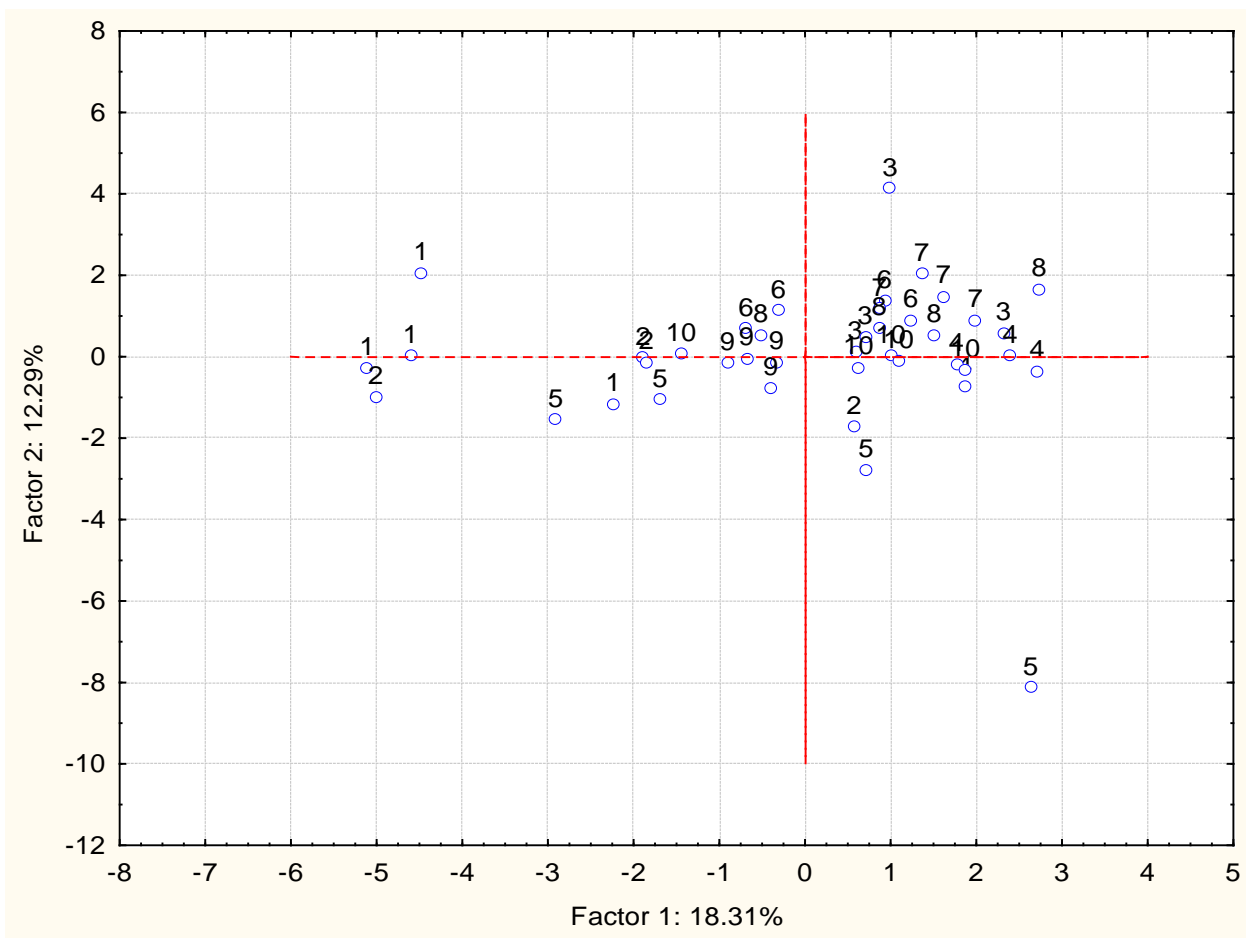
Figure 14. PCA scaling plot of peat samples based on a correlation matrix of association between carbon-substratre utilisation rates. Numbers (dots) in graph corresponds to following treatments:

1 Peat - (0-month)
2 Peat + *M. anisopliae*- (0-month)
3 Peat – (1-month)
4 Peat + *M. anisopliae* - (1-month)
5 Peat + Plant- (1-month)
6 Peat + *M. anisopliae* + Plant (1-month)
7 Peat (6-months)
8 Peat + *M. anisopliae* (6-months)
9 Peat + Plant – (6-months)
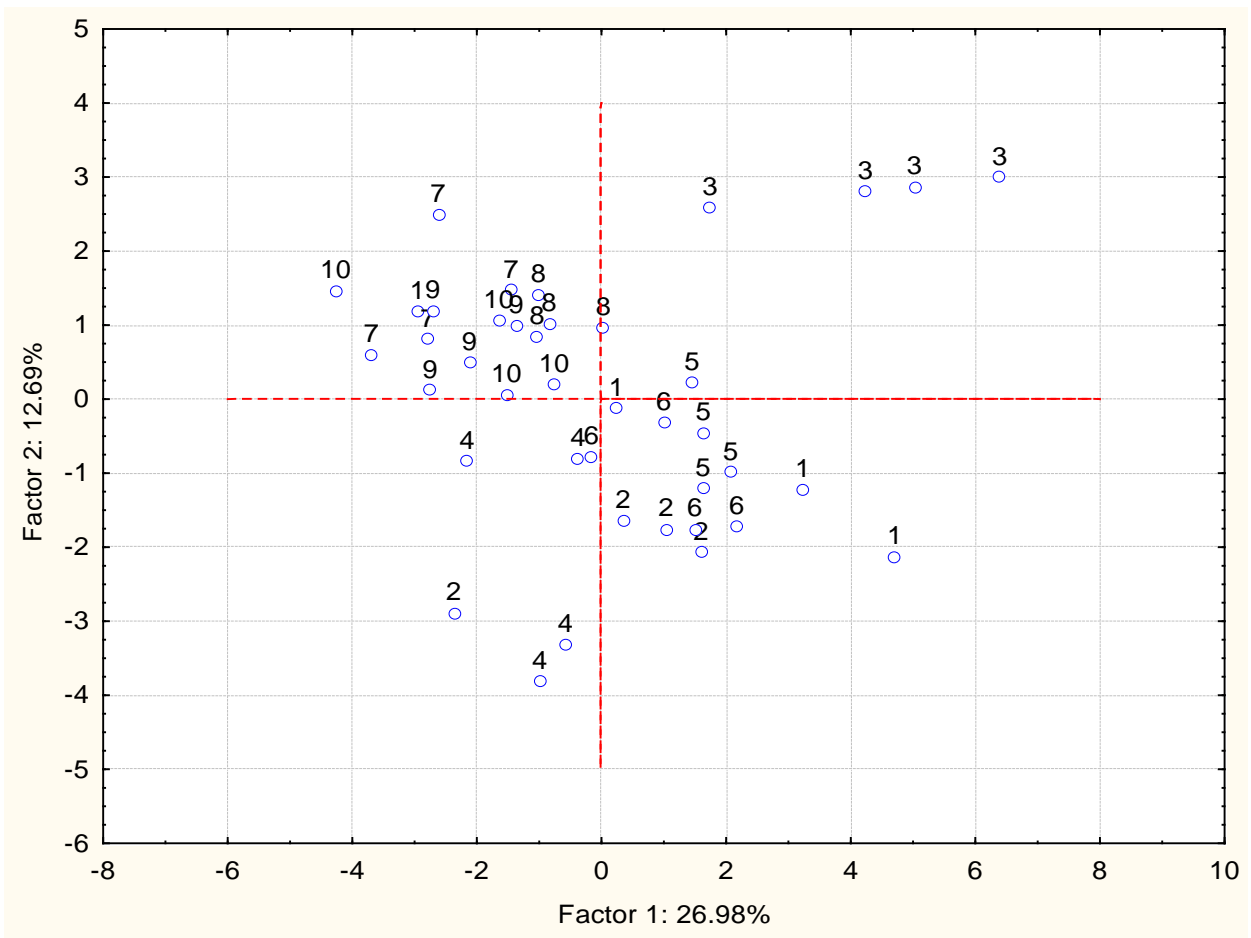10 Peat + *M. anisopliae* + Plant (6-months)

Figure 15. PCA scaling plot of peat samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph corresponds to following treatments:

1 Bark - (0-month)
2 Bark + *M. anisopliae*- (0-month)
3 Bark – (1-month)
4 Bark + *M. anisopliae* - (1-month)
5 Bark + Plant- (1-month)
6 Bark + *M. anisopliae* + Plant (1-month)
7 Bark (6-months)
8 Bark + *M. anisopliae* (6-months)
9 Bark + Plant – (6-months)
10 Bark + *M. anisopliae* + Plant (6-months)

Fig 16. PCA scaling plot of 0-month untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph corresponds to following treatments:

1-Peat blend with 10 % CGW

3-Peat blend with 20 % CGW

5- Bark

7- Peat

9 - Coir

Fig 17. Vector loadings of carbon substrate utilisation rates of 0 month-old untreated compost samples.
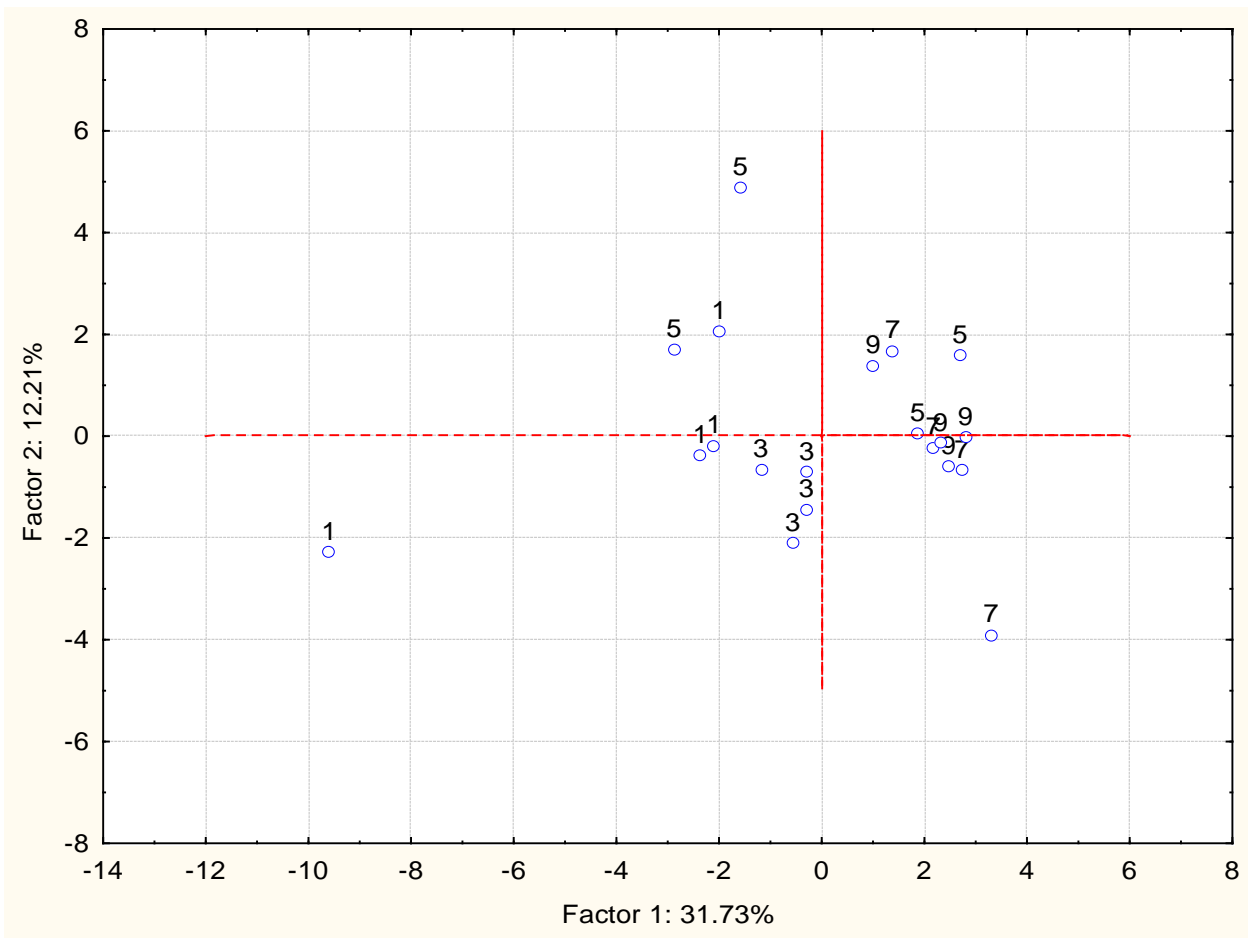
Fig 18. PCA scaling plot of 1-month untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates.

Numbers (dots) in graph corresponds to following treatments:

1- Bark

5 -Peat

9 - Coir

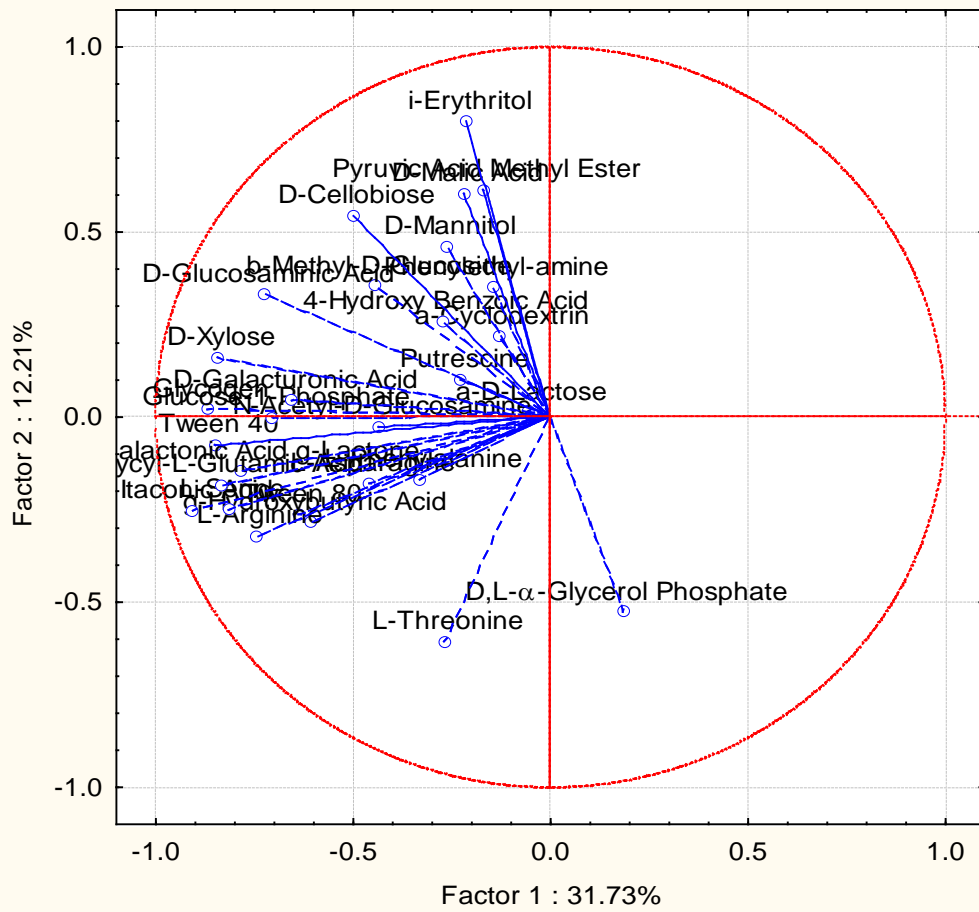13-Peat blend with 10 % CGW

20-Peat blend with 20 % CGW

Fig 19. Vector loadings of carbon substrate utilisation rates of 1 month-old untreated compost samples.
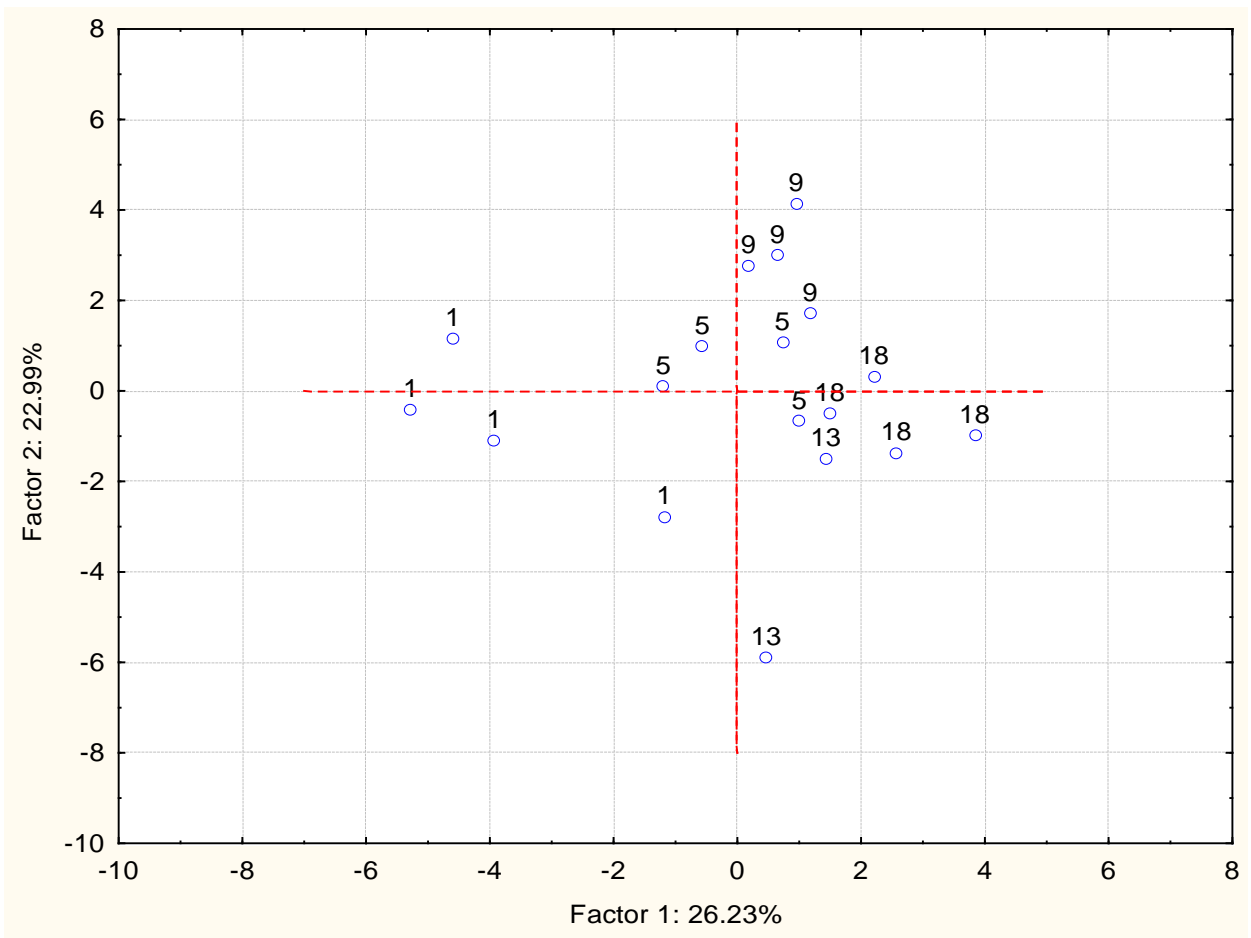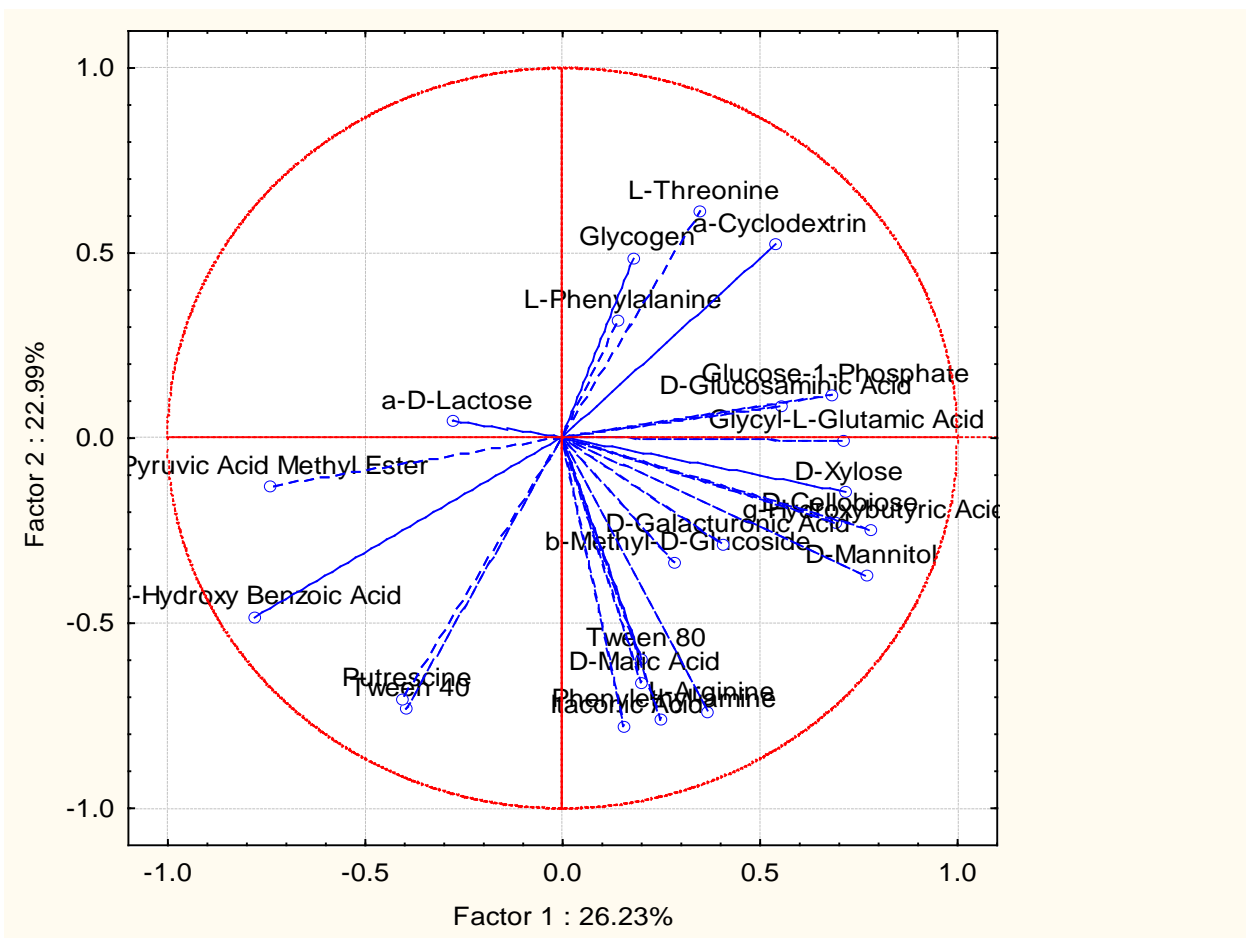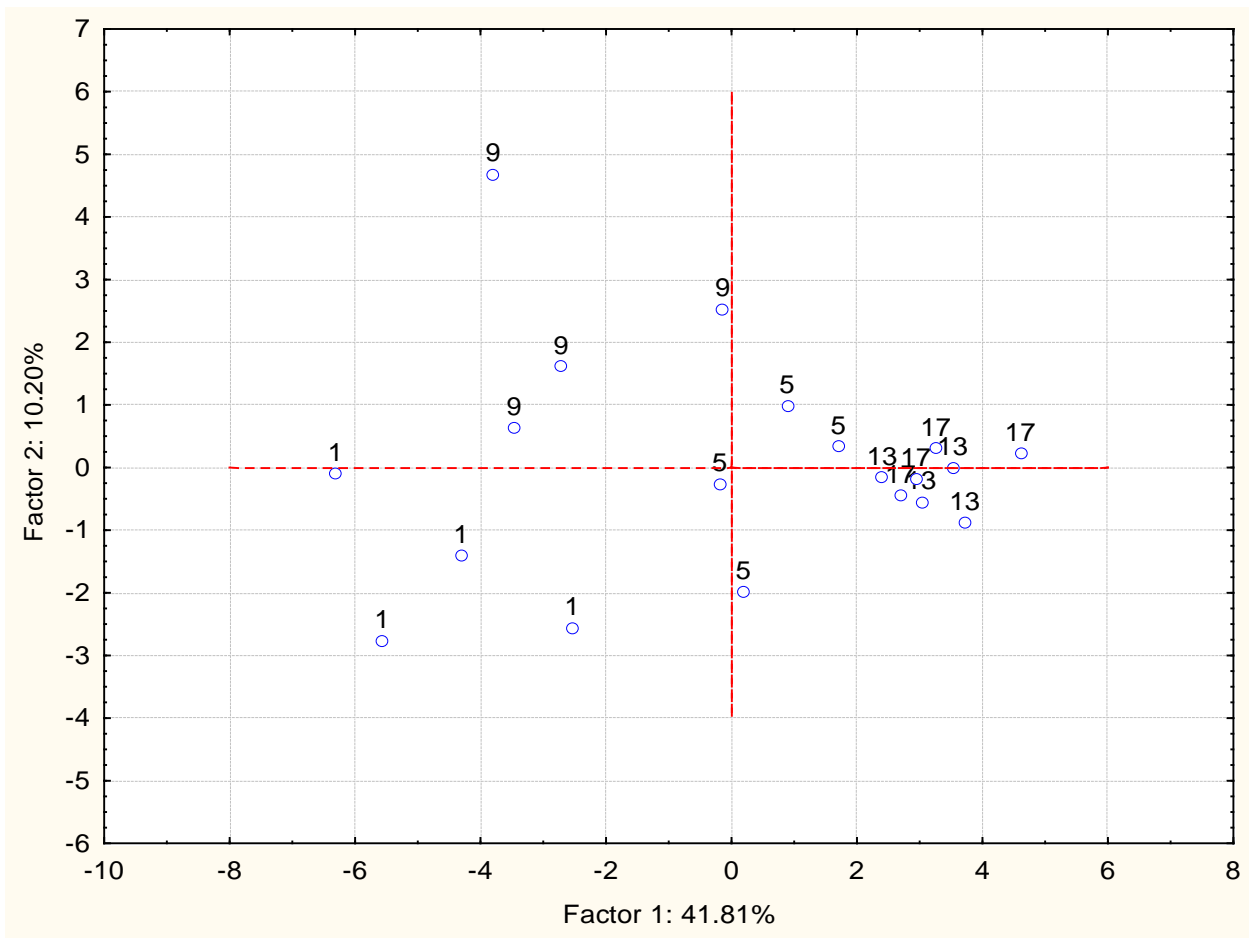
Fig 20. PCA scaling plot of 6-months untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph corresponds to following treatments:

1-Peat blend with 20 % CGW

5-Bark

9-Peat blend with 10 % CGW

13-Coir

17-Peat

Fig 21. Vector loadings of carbon source utilisation rates of 6 month-old untreated compost samples.

B. Molecular approaches i.e. (automated ribosomal intergenic spacer analysis - ARISA) to determine microbial population in different plant growth media

Introduction

Molecular techniques are culture independent, and their application to the study of microbial ecology allows a more thorough understanding of the complexities of the microbial community composition and its effects in soils (Ranjard et al., 2001). The bacterial ARISA (automated ribosomal intergenic spacer analysis) technique is based on the analysis of intergenic 16S-23S internally transcribed spacer sequences (ITS1) within the ribosomal RNA operon, This is a highly variable non-coding sequence present in all bacteria whose size variants can be analyzed using PCR and capillary electrophoresis. Fungal ARISA exploits an equivalent eukaryotic length polymorphism in the nuclear ribosomal DNA (rDNA) region that contains the two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2).

Materials and Methods

DNA isolation

Total DNA from plant growth media was isolated using Ultraclean soil extraction kits (MO BIO Laboratories Inc. as per manufacture's instructions. Quantity and quality of extracted DNA was checked By NanoDrop spectrophotometer.

Primers for fungal and bacterial ARISA

Primers representing the consensus sequences found at the 3' end of the 18S gene (primer 2234C, 5'-GTTTCCGTAGGTGAACCTGC-3') and at the 5' end of the 28S gene (primer 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') were used for fungal ARISA (Ranjard et al., (2001). Primers for bacterial ARISA were ITSF/ITSReub as detailed in Cardinale et al., (2004). The 5'and 3' ends of primers ITSF (GTCGTAACAAGGTAGCCGTA) and ITSReub (GCCAAGGCATCCACC) are complementary to positions 1423 and 1443 of the 16S rRNA respectively. The Fungal-ARISA 2234C primer was labelled with 6-Fam (Sigma) and the Bacterial-ARISA ITSReub primer was labelled with VIC (Applied Biosystems).

PCR conditions

PCR conditions were the same for both amplifications of fungal and bacterial target gene. Briefly, each PCR contained 1x Buffer, 1.5mM Mg^{2+}, 0.25mM dNTPs, 1x Additive, 0.08mM of each primer, 0.025u/ul Taq (GoTaq, Promega) and 0.5ug/ul DNA.

PCRs were carried out in a PTC -200 thermal cycler (MJ Research). Cycling was 94°C 3 min followed by 33 cycles of 94°C 45 sec, 55°C 1 min , 72°C 2 min, then 72°C for 7 min.
Fungal positive control DNA was from *M. anisopliae* 275. Bacterial control DNA was *Streptomyces coelicolor* and bacterial biofilm DNA (kindly provided by Simon Gregory, UWS). 40ul PCRs were done for each sample and 5ul run on a 1.5% agarose gel 115v 30 min to check the amplification had worked. The remainder of the sample was aliquoted into a 96 well microtitre plates and sent to Lark Cogenics for electrophoresis on an ABI 3730 sequencer and sizing relative to a LIZ1200 size standard.

Peaks with a height above 300fu (1% max) and area greater than 2500fu2 (0.5% max) were analysed. Peak sizes were rounded to the nearest base pair (bp), and single peaks

(seen in only one sample) were excluded from the analysis. Peak heights for each sample were arranged into a table and plotted in Excel for comparison

Results and Discussion

For all growth media tested, the number of fungal species detected was initially low, but species number increased with time, and for each medium, the soil community diversified with time (Figs. 22-26).

Initial diversity was similar in bark and coir but lowest in peat, increasing markedly with the amount of composted garden waste (CGW) added. Bands seen in the initial samples disappeared (e.g. coir) or were reduced (e.g. bark) in later samples, and many new bands appeared.

Each medium appeared to have a distinctive profile. Peat had a band at 554 bp in the initial samples, seen in all 0 and 1 month samples containing peat, and only elsewhere in coir samples containing *M. anisopliae* or a plant. After 1 month, a band appeared at 333bp, which was only seen in samples containing peat. In bark, distinctive bands at 633, 645, and 658 bp were seen in the initial samples, although two of these were also seen in one sample of coir (Figs. 23-24). These were reduced in size over time as diversity increased. In addition, a band appeared at 627bp after 1 month, which was distinctive for bark. In coir, after 1 month, bands at 143, 157,178, and 436bp appeared in all samples, and the 178bp band was distinctive for coir. A 500bp band was seen in media containing CGW and some bark samples and a 497bp band appeared at 1 month peculiar to media containing CGW (Figs. 25-26).

The *M. anisopliae* control gave a band at 541bp, which was seen in all the initial samples where *M. anisopliae* was added. In peat and bark, this band disappeared at 1 month. In coir it persisted in the 1 month samples, but was absent at 6 months. The band was still apparent in samples containing CGW at 6 months.

In summary, the band profiles show that the fungal communities are different in different growth media, and increased in complexity as a succession of fungi appeared over time.
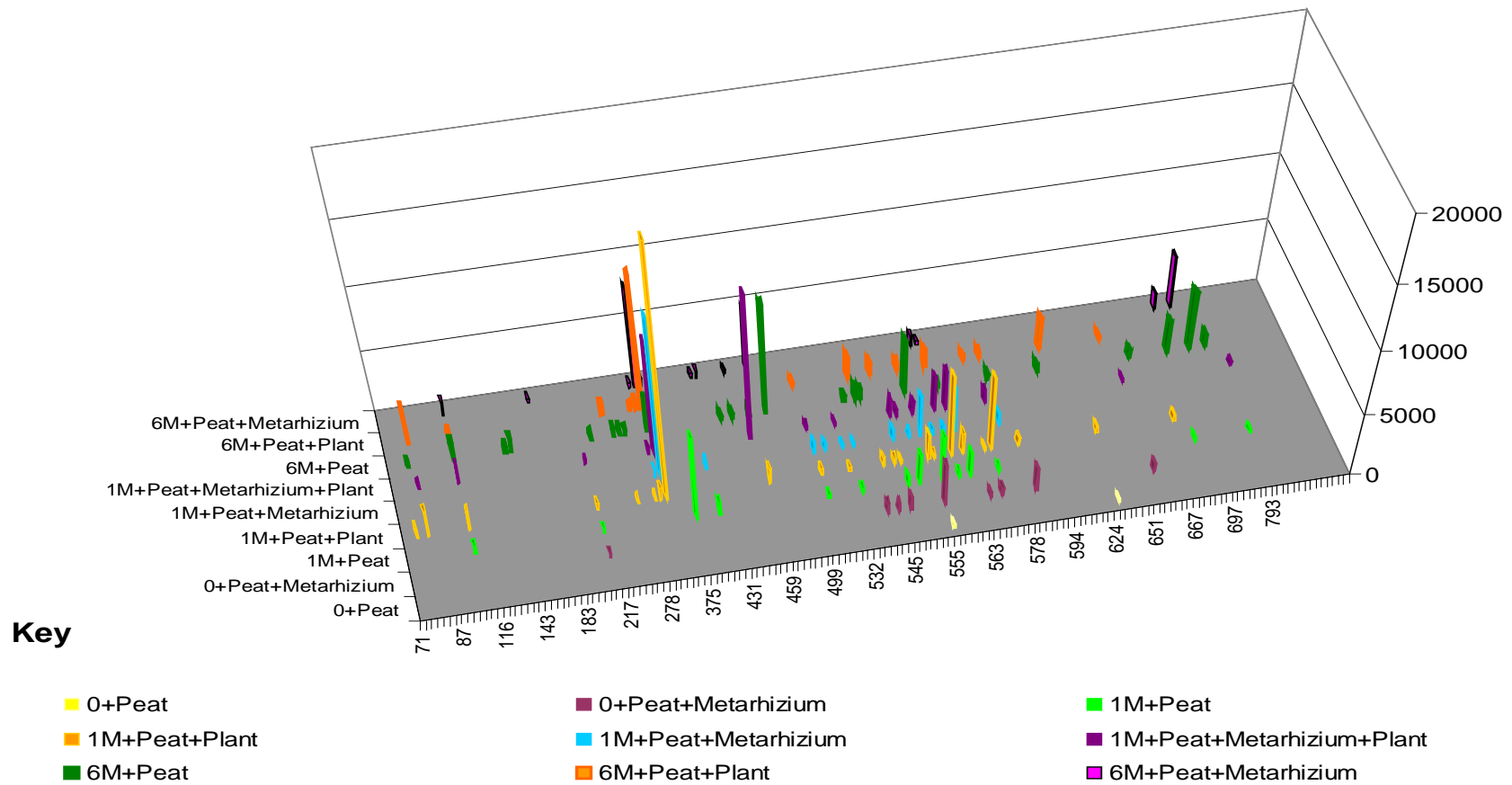
Key

- 0+Peat
- 0+Peat+Metarhizium
- 1M+Peat
- 1M+Peat+Plant
- 1M+Peat+Metarhizium
- 1M+Peat+Metarhizium+Plant
- 6M+Peat
- 6M+Peat+Plant
- 6M+Peat+Metarhizium

Fig. 22 Fungal ARISA profile of peat at different time periods (0, 1, and 6 months) with or without plant or *M. anisopliae*
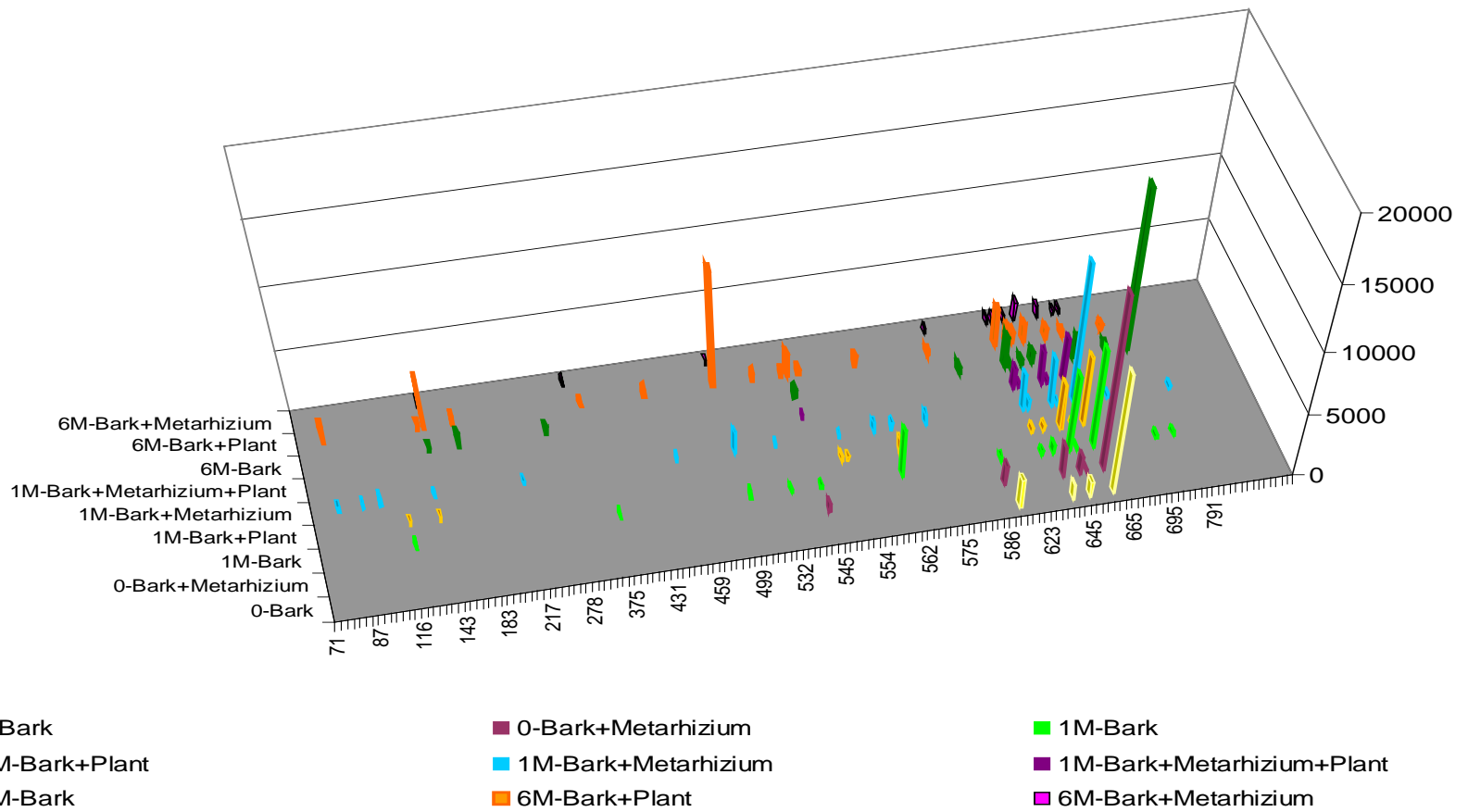
Key

- 0-Bark
- 1M-Bark+Plant
- 6M-Bark
- 0-Bark+Metarhizium
- 1M-Bark+Metarhizium
- 6M-Bark+Plant
- 1M-Bark
- 1M-Bark+Metarhizium+Plant
- 6M-Bark+Metarhizium

Fig. 23 Fungal ARISA profile of bark at different time periods (0, 1, and 6 months) with or without plant or *M. anisopliae*
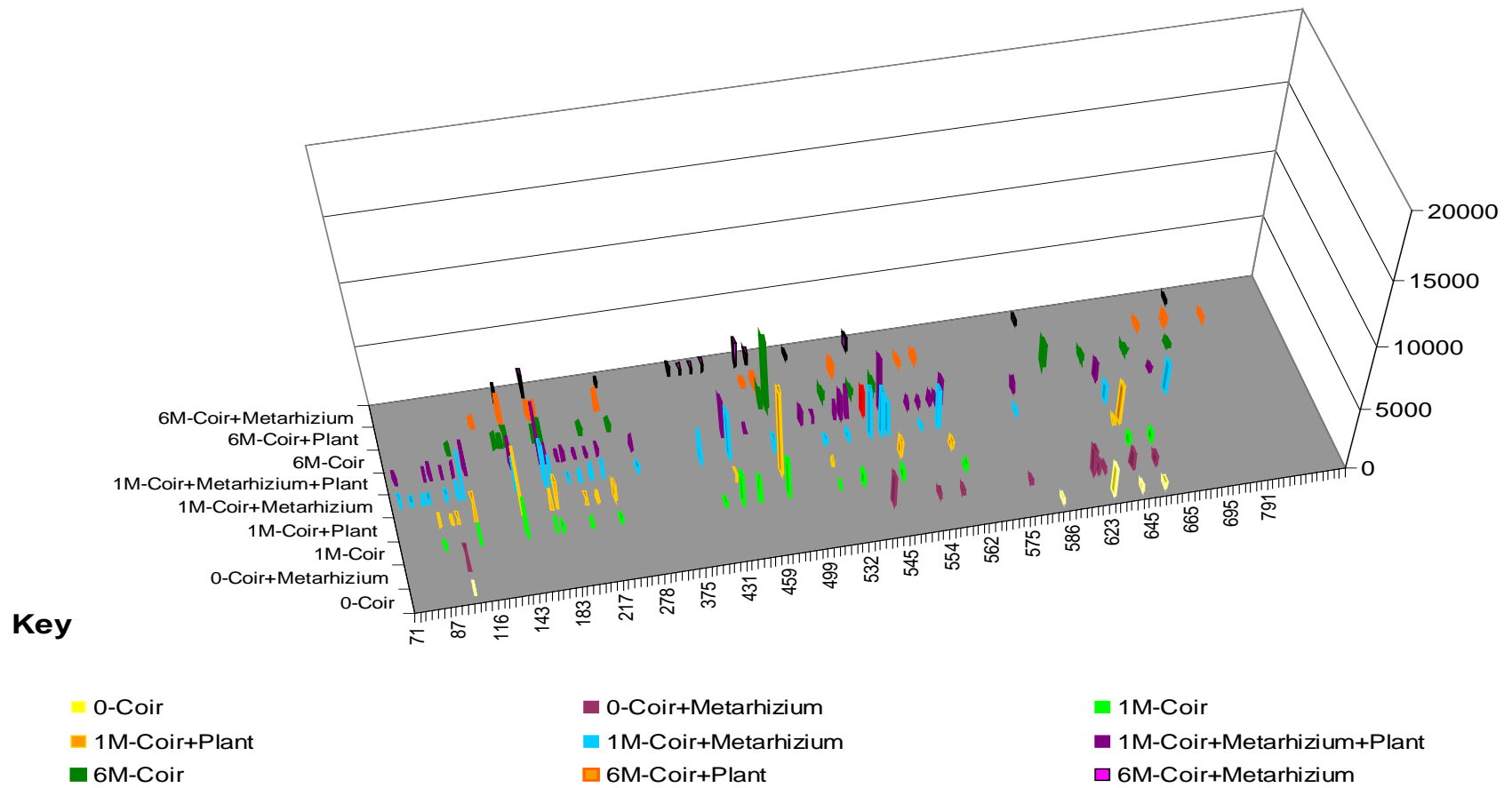
39

Key

▪ 0-Coir	▪ 0-Coir+Metarhizium	▪ 1M-Coir
▪ 1M-Coir+Plant	▪ 1M-Coir+Metarhizium	▪ 1M-Coir+Metarhizium+Plant
▪ 6M-Coir	▪ 6M-Coir+Plant	▪ 6M-Coir+Metarhizium

Fig. 24 Fungal ARISA profile of coir at different time periods (0, 1, and 6 months) with or without plant or *M. anisopliae*

Key

- 0-Peat+10%CGW
- 0-Peat+10%CGW+Metarhizium
- 1M-Peat+10%CGW
- 1M-Peat+10%CGW+Plant
- 1M-Peat+10%CGW+Metarhizium
- 1M-Peat+10%CGW+Metarhizium+Plant
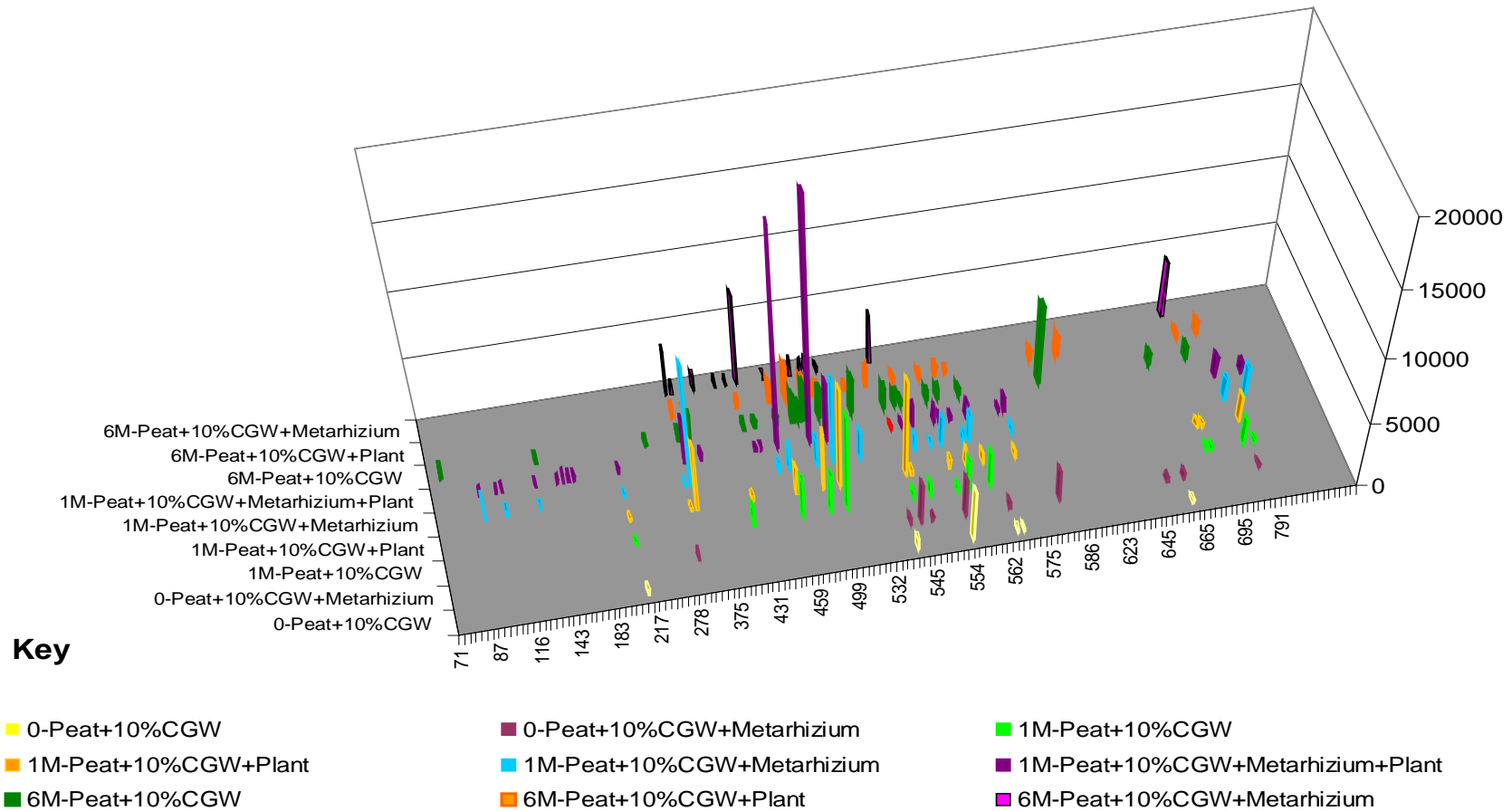- 6M-Peat+10%CGW
- 6M-Peat+10%CGW+Plant
- 6M-Peat+10%CGW+Metarhizium

Fig. 25 Fungal ARISA profile of peat blended with 10%CGW at different time periods (0, 1, and 6 months) with or without plant or *M. anisopliae*
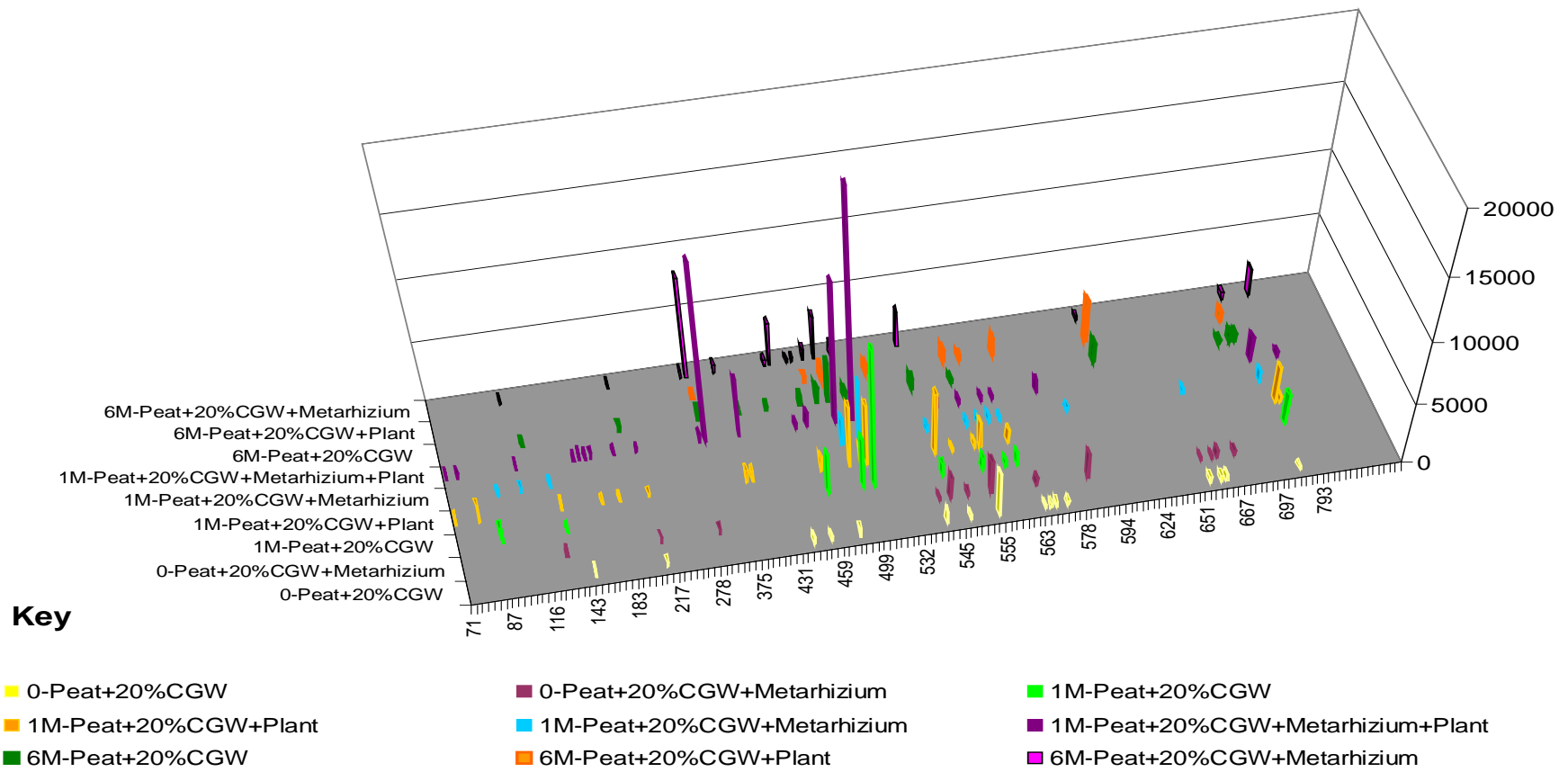
Key

- ■ 0-Peat+20%CGW
- ■ 0-Peat+20%CGW+Metarhizium
- ■ 1M-Peat+20%CGW
- ■ 1M-Peat+20%CGW+Plant
- ■ 1M-Peat+20%CGW+Metarhizium
- ■ 1M-Peat+20%CGW+Metarhizium+Plant
- ■ 6M-Peat+20%CGW
- ■ 6M-Peat+20%CGW+Plant
- ■ 6M-Peat+20%CGW+Metarhizium

Fig. 26 Fungal ARISA profile of peat blended with 20% CGW at different time periods (0, 1, and 6 months) with or without plant or *M. anisopliae*

Objective 7. Determine shelf life of *M. anisopliae* in different media (UWS, Yrs 1-3)

Studies are in progress. No significant differences in *M. anisopliae* efficacy or persistence were observed 6 months post storage under different conditions.

Objective 8: Grower trials to demonstrate the efficacy and robustness of *M. anisopliae* in selected media

Task 8.1: Compatibility of *M. anisopliae* and cold tolerant entomopathogenic nematode (*Steinernema kraussei*) to control black vine weevil in strawberries growbags

Introduction

M. anisopliae shows considerable potential for the control of BVW larvae but an inherent weakness of this fungus is that it is slow acting, particularly at low temperatures. However, the potential exists to use *M. anisopliae* with cold tolerant entomopathogenic nematodes (CTEN). The objective of this trial was to: (1) demonstrate that *M. anisopliae* was compatible with CTEN, and (2) that these two biocontrol agents could be used together to eradicate over-wintering BVW larvae. Note that earlier studies focused on use of *M. anisopliae* as a prophylactic i.e. kill young larvae as soon as they hatch from eggs.

Material and Methods

Cold tolerant entomopathogenic nematodes

Nemasys L containing the CTEN, *S. kraussei* was kindly provided by Becker Underwood, UK and used according to the manufacture's instructions.

Trial at Delflands Nursery

Young strawberry plants (c.v. Elsanta) kindly provided by Hargreaves Nursery, Plants Ltd, Lincolnshire, UK were initially transplanted in peat in 250 ml pots on 20th October 2006. Each pot was inoculated with 5 BVW larvae (late 2nd and early 3rd-instar) obtained from

the UWS colony on the 3rd November 2006. The pots were kept in the glasshouse (at 20 $\pm$ 2°C: 14:10 h light: dark photoperiod) for 1 week to allow the larvae to "settle in". The infested strawberry plants were then transplanted into growbags containing peat kindly provided by Bord Na Mona, Ireland.

Treatments were applied as a drench on the 9th November 2006 in 1 L of water per plant using a watering can. Treatments included: Untreated control (water only), *M. anisopliae* (1 x 10^{10} conidia/l of compost), *S. kraussei* (250,000 nematodes/plants) and a combination of *M. anisopliae* and *S. kraussei*. Each treatment was replicated 5 times (3 plants per growbag). The treatments were set out in a randomized block design with each growbag adequately spaced to avoid cross-contamination among treatments. Growbags were destructively assessed 10 weeks post larval inoculation to determine the efficacy of the above treatments.   During the experimental period, the average air temperature ranged between 2.6°C and 15.2°C.

Results

Significant differences were observed between treatments ($P < 0.001$) for BVW control. There were no significant differences in BVW control between *M. anisopliae* (50%) and CTEN (61%). However, control was significantly higher when *M. anisopliae* was used with CTEN. This observation shows that *M. anisopliae* is compatible with CTEN and that these agents work synergistically in eradicating BVW larvae (Fig. 27). This is the first time such synergy has been reported between these organisms. Plants treated with nematode or fungus appeared healthy and had extensive root systems while untreated plants were stunted and had poorly developed root systems (Fig. 28).
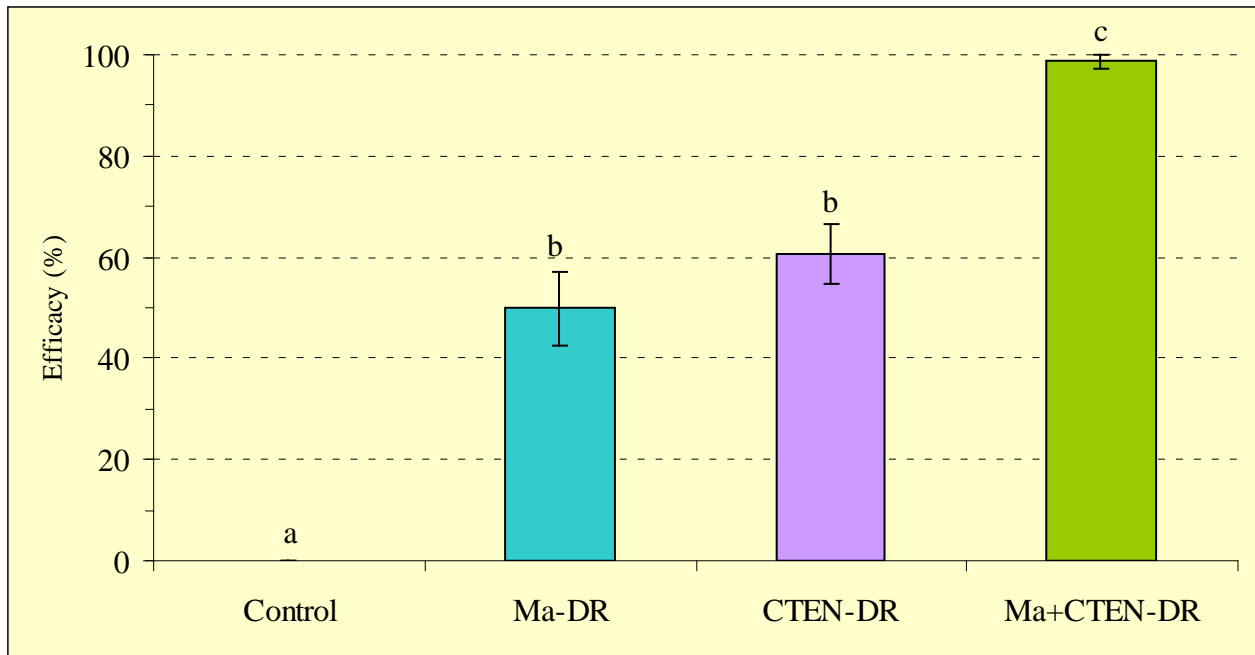
Fig. 27. Efficacy (%) of *M. anisopliae* and cold tolerence nematode (CTEN) against BVW in strawberry growbags. Treatments are listed below. No treatment (control), *M. anisopliae* applied as a drench (MaDR), Nematodes applied as a drench (CTEN-DR), Nematodes and fungus applied as a drench (Ma+CTEN-DR). Each strawberry plant was infested with 5 BVW larvae (late 2nd and early 3rd instar) and destructively assessed 10 weeks post inoculation. Bars displaying same letter vary non-significantly (Tukey's test, *P* < 0.001).
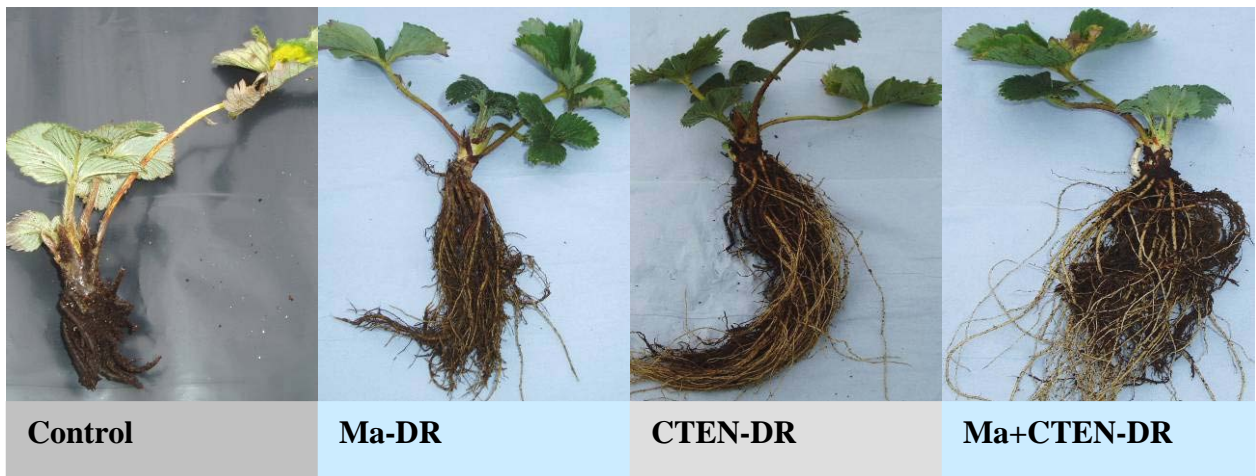

Fig. 28. Comparison of the root system of strawberry plants exposed to the following treatments: No treatment (control), *M. anisopliae* applied as a drench (MaDR),

Nematodes applied as a drench (CTEN-DR), Nematodes and fungus applied as a drench (Ma+CTEN-DR). Each strawberry plant was infested with 5 BVW larvae (late 2nd and early 3rd instar) and destructively assessed 10 weeks post inoculation.

Task 8.2: Efficacy of *M. anisopliae* and low dose insecticide for control of BVW larvae infesting strawberries in growbags

Introduction

M. anisopliae V275 shows much promise for the control of BVW in HNS when used alone or together with low doses of chemical insecticides (Shah et al., 2007). V275 was used as a prophylactic which prevented establishment of BVW larvae on emergence from eggs. The objective of this study was (i) to expand our knowledge in the use of *M. anisopliae* and low dose of insecticides for BVW control in strawberries produced in growbags and, (ii) establish if *M. anisopliae* could be used to eradicate BVW larvae.

Material and Methods

Trial at Wallings Nursery

Young strawberry plant (c.v. Elsanta) kindly provided by Hargreaves Nursery, Plants Ltd, Lincolnshire, UK were transplanted in peat on 20th October 2006 in 250 ml pots. Each pot was inoculated with 5 BVW larvae (2nd and early 3rd-instar) on 3rd November 2006. The pots were kept in the glasshouse (at 20 ± 2°C; 14:10 h light: dark photoperiod) for 1 week to allow the BVW larvae to "settle in". After a further 1 week, the pots were transplanted into growbags as described in Task 8.1.

Treatments were applied as a drench on 10 November 2006 in 1 L of water per plant using a watering can. The treatments included: (i) Untreated control (water only), (ii) *M. anisopliae* (1 x 10^{10} conidia/l of compost), (iii) Imidacloprid applied at the reduced rate (1/100 of recommended rate), and (iv) and combination of *M. anisopliae* with reduced rate of imidacloprid. The treatments were arranged in a randomized block design with each growbag adequately spaced to avoid cross-contamination of treatments. Growbags were destructively assessed 10 weeks post larval inoculation to determine the efficacy of the above treatments. During the experimental period, average air temperatures ranged between were 3.9°C and 23.2°C.

Results and Discussion

Significant differences in BVW control were observed among treatments ($P < 0.001$). Imidacloprid when used at the reduced rate gave only 11% control whereas *M. anisopliae* provided 65% control. No significant differences were observed in *M. anisopliae* efficacy whether it was applied alone or in combination with the reduced rate of imidacloprid (Fig. 29).
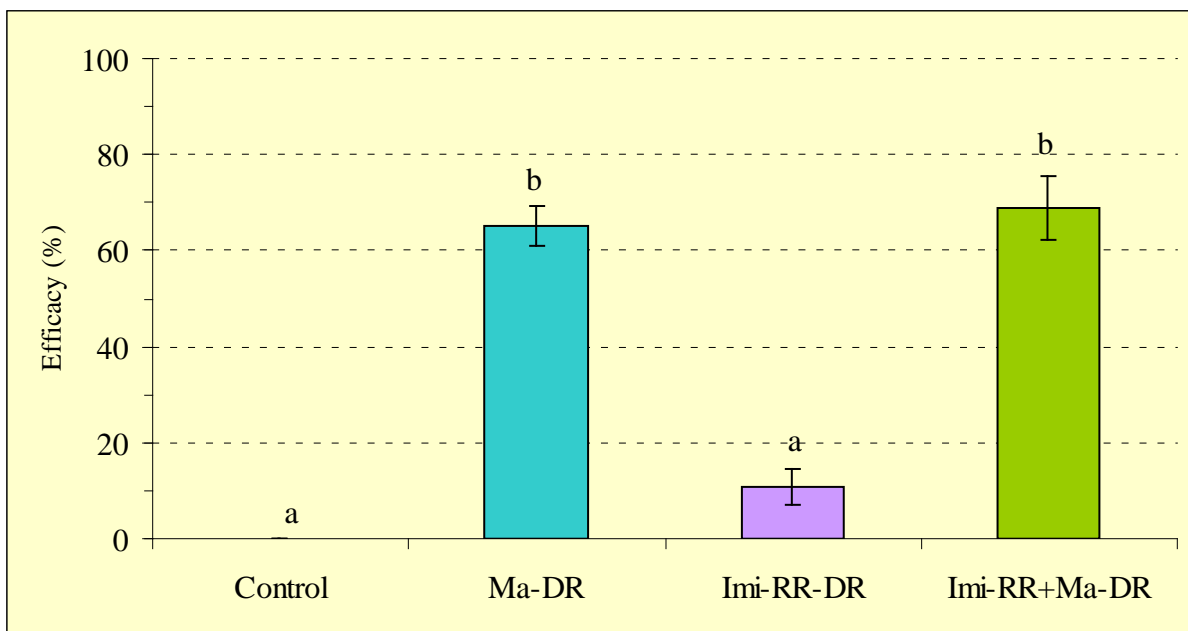

Fig. 29. Efficacy (%) of *M. anisopliae* and insecticide against BVW in strawberry growbags. Treatments are listed below. No treatment (control), *M. anisopliae* applied as a drench (Ma-DR), imidacloprid applied as drench at the reduced rate (Imi-RR-DR), combined application of fungus and reduced rate of insecticide applied as a drench (Imi-RR+Ma-DR). Each strawberry plant was infested with 5 BVW larvae (late 2nd and early 3rd instar) and destructively assessed 10 weeks post inoculation. Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.001$).

Plants treated with *M. anisopliae* grew vigorously while untreated and plants treated with reduced rate of imidacloprid were stunted and had poorly developed root systems (Fig. 30).
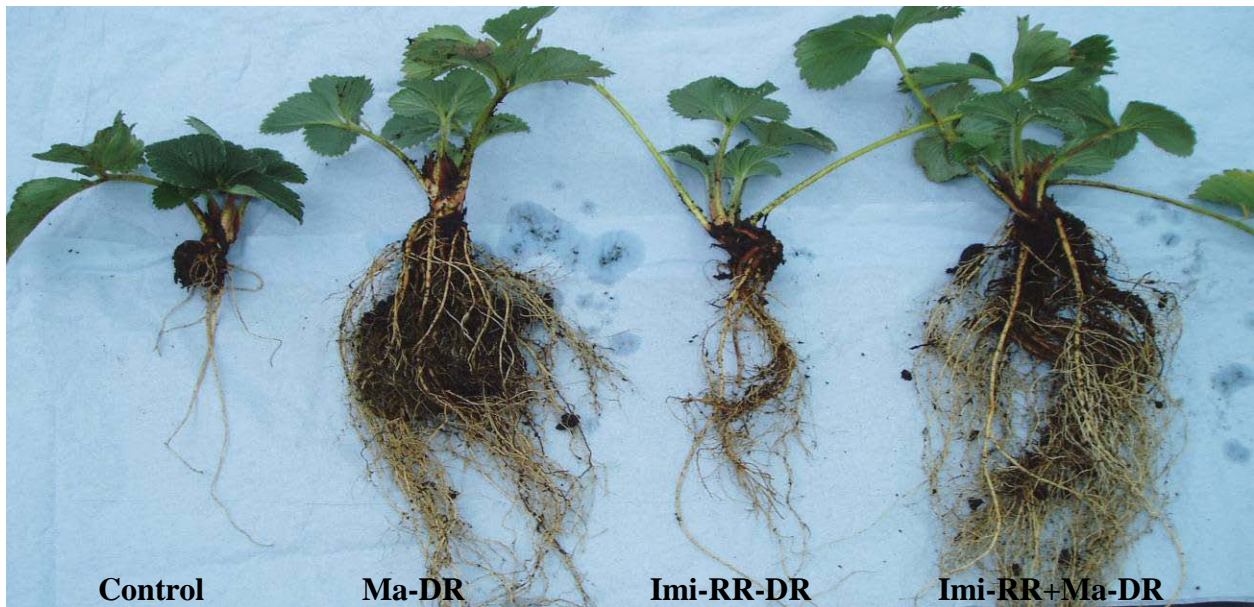
Fig. 30. Comparison of the root system of strawberry plants exposed to the following treatments. From left: No treatment (control), *M. anisopliae* applied as a drench (Ma-DR), Imidacloprid applied as drench at the reduced rate (Imi-RR-DR), combined application of fungus and reduced rate of insecticide applied as a drench (Imi-RR+Ma-DR). Each strawberry plant was infested with 5 BVW larvae (late 2nd and early 3rd instar) and destructively assessed 10 weeks post inoculation.

Technology Transfer

Publications

1. Shah, F.A., Ansari, M.A., Prasad, M. and Butt, T.M. (2007). Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. *Biological Control*, 40**:** 246-252

2. Ansari, M.A., Shah, F.A., Whittaker, M., Prasad, M., Butt, T.M., (2007). Control of western flower thrips (*Frankliniella occidentalis*) pupae with *Metarhizium anisopliae* in peat and peat alternative growing media. *Biological Control*, 40: 293-297

3. A grower article "Early Control" in Grower, pp. 17. July 2006.

4.

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1. Butt, T.M., Shah, F.A. and Prasad, M. (2005). Fungal biocontrol of insect pests in peat alternatives (green waste, bark, coir) at Chartered Institution of Wastes Management, Machynlleth. 22nd April.2005.
2. Butt T.M., Shah, F.A., Ansari, M.A., Prasad, M. and Ravensberg, W. (2006). Progress in vine weevil and thrips control using the fungus *Metarhizium anisopliae* at British Berry Conference, Birmingham, 14-15 November, 2006

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Lindstrom, J.E., Barry, R.P., Braddock, J.F. (1998). Microbial community analysis: a kinetic approach to constructing potential C source utilization patterns. Soil Biology and Biochemistry 30: 231-239

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