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

Final Report

Table 1 Primary milestones and their accomplishment status.

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 *Metarhizium anisopliae* and sub lethal dose of imidacloprid/fipronil established	15.9.05	Accomplished
2.2	Efficacy of *M. anisopliae* for control of BVW larvae and WFT pupae in different media determined – *Metarhizium* enhancing medium identified	15.12.05	Accomplished
3.1	*Metarhizium* 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	Accomplished
6.2	Profile of microbial community based on molecular and substrate utilisation studies determined	15.10.07	Accomplished
6.3	Impact of natural microbial population on the efficacy of *M. anisopliae* assessed	15.11.07	Accomplished
7.1.	Shelf life studies completed and data analysed	1.01.08	Accomplished
8.1	Grower trials of *M. anisopliae* assessed (linked with workshops)	15.11.07	Accomplished
	End Project	31.03.08	

Table 2 Secondary milestones and their accomplishment status.

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 sub lethal 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 sub lethal doses of insecticide and *M. anisopliae* submitted	15.5.06	Accomplished (4 papers published)
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) with *M. anisopliae* 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	Accomplished (1 paper published in BioControl)
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	Accomplished
7.1	**Manuscript** on shelf life (viability and virulence) of *M. anisopliae* in different media submitted	30.03.08	In preparation*
7.2	**Protocols for** testing impact of physical-chemical and microbial components of horticultural **growing media on fungal BCAs**	30.03.08	In preparation*
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	In preparation*

*** Being updated in response to feedback from growers and project consortium**

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 HDC Project Code HNS 133
Government sponsor(s)	DEFRA, HortiLINK
Project leader	Dr Tariq M. Butt Reader Department of Environment and Molecular Biosciences, Swansea University,SA2 8PP
Report:	Final report, June, 2008
Previous reports:	Fifth six monthly report, September, 2007
Location	Swansea University, Wales
Project coordinator:	Dr. Paul Sopp Fargro Ltd.Toddington Lane Littlehampton, West Sussex, BN17 7PP
Date commenced:	1st April 2005
Key words	*Metarhizium*, black vine weevil, western flower thrips, sub lethal doses, reduced chemical insecticides, fipronil, imidacloprid, growth media, peat, coir, bark, composted green waste

GROWER SUMMARY

Headline

- The insect-pathogenic fungus *Metarhizium anisopliae* V275 has been very effective at controlling black vine weevil (BVW) larvae and western flower thrips (WFT) pupae in a range of different plant growing media (peat, bark, coir, peat blends with 10% and 20% composted green waste).

Background and expected deliverables

Black vine weevil (BVW) is considered the most important pest of hardy nursery stock (HNS) causing annual losses of ca. £30 million in the UK. Western flower thrips (WFT) is a pest 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 these subterranean stages would contribute significantly to the overall integrated pest management programme (IPM). This project complements earlier studies funded by the HDC and the EU, aiming 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 plant growing media. It offers a benign alternative to chemical pesticides that are currently under threat of being phased out (e.g. chlorpyriphos) or where pests have developed resistance (WFT is resistant to many pesticides e.g. chlorpyriphos) or likely to develop resistance soon because of extensive use (e.g. imidacloprid).

Summary of the project and main conclusions

The main findings of this project are listed below:

- Plant growth media studies showed that composted green waste (CGW) cannot be used on its own as a plant growth medium but can be used if blended with peat i.e. CGW : peat (1:4).
- *M. anisopliae* was very effective against BVW in the different plant growth media tested (peat, coir, bark and peat blended with 10% or 20% CGW).

- *M. anisopliae* used with sub lethal doses (i.e. 1 and 10% of recommended dose) of imidacloprid, chlorpyriphos or fipronil provided excellent control (up to 100%) of BVW larvae**.**
- *M. anisopliae* is very effective in controlling WFT pupae. It reduced WFT adult emergence by 80- 90% as compared to 40% reduction provided by chemical insecticides.
- *M. anisopliae* was compatible with the entomopathogenic nematodes *Heterorhabditis bacteriophora, Steinernema feltiae* (Entonem) and *S. kraussei* (Nemasys® L) and provided significantly higher control (100%) of BVW larvae when used in combination with these nematodes.
- Percolation of *M. anisopliae* inoculum through plant growth media depends upon its application method. Very little inoculum was lost if conidia had been premixed into the growing medium before potting, however, drench application could cause up to 90% loss of inoculum. It was observed that majority of inoculum percolated at the time of drench application; therefore, volume of drench application should be optimised to avoid any run off from pot during application.
- Plant growth media had no adverse effect on *M. anisopliae* efficacy.
- Trials conducted at several growers nurseries demonstrated that *M. anisopliae* provides excellent control of BVW. *M. anisopliae* efficacy was independent of plant species and plant growth media. These trials provided reassurance to growers that *M. anisopliae* was easy to use i.e. it could be easily integrated into their production systems.

Financial benefits

1. Our findings clearly showed that there is scope for reducing the cost of insect pest control. Once *M. anisopliae* is registered in the UK, growers will be able to reduce the cost of BVW control by:
    - Using *M. anisopliae* alone and getting a premium for “organically”produced plants.
    - Combining *M. anisopliae* with low dose chemicals or entomopathogenic nematodes, thus allowing each component to be used at a lower application rate (at least 50% and potentially 99%). This results in a corresponding decrease in cost.

2. Other direct and indirect financial benefits:

    - *M. anisopliae* is far superior to chemical pesticides for controlling thrips pupae, thus contributing significantly to IPM programmes for thrips control.
    - There are few or no concerns with disposal of spent media because *M. anisopliae* has no environmental risk (unlike chemical pesticides which may leach into groundwater).
    - *M. anisopliae* (± entomopathogenic nematodes) will benefit organic growers in many sectors of horticulture (i.e. soft fruit, indoor ornamentals, hardy nursery stock) where BVW and thrips are major pests.
    - We have shown that some chemical pesticides can be used at lower than recommended rates (e.g. Vi-nil can control BVW at 1/10th of the recommended rate), thus saving growers money and concomitantly reducing pesticide inputs.

Action points for growers

- Lobby industry and PSD to accelerate registration of *M. anisopliae* in the UK.
- Support researchers by helping with registration-related matters. For example, creating groups to generate risk assessment or efficacy data.
- Support researchers to develop protocols which will allow for the rapid uptake of *M. anisopliae,* once the product is registered. These protocols must reveal:

    1. What other pests (e.g. sciarids) could be controlled by *M. anisopliae*, providing even more value for money.
    2. How to optimise control of the different pests at reduced cost e.g. incorporation of *M. anisopliae* and efficacy enhancing agent in plant plugs.
    3. Compatibility of *M. anisopliae* with other agents e.g. beneficial predators and parasitoids, fungicides used for disease control.
    4. The efficacy of the different application methods: e.g. drip versus overhead irrigation, hydraulic versus electrostatic sprayers.
    5. The efficacy of different formulations – conidia *versus* conidia on grain (significantly cheaper product).

- Develop workshops to facilitate rapid uptake of products once they reach the market (no time lag).

- The protocols and workshops would give growers more insight into the attributes of the biocontrol agents (BCA) and ways to get more “value for money”. Rapid uptake of the new technologies will ensure growers are competitive in the international arena.

Additional Outputs

In addition to accomplishing the above milestones, the following were also achieved:

Publications:

1. Shah, F.A., Gaffney, M., Ansari, M.A., Prasad. M. & Butt, T.M. (2008). Neem seed cake enhances the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for the control of black vine weevil, *Otiorhynuchs sulcatus* (Coleoptera: Curculionidae. *Biological Control*. 44:111-115
2. Ansari, M.A., Brownbridge, M., Shah, F.A. & Butt, T.M. (2008). Efficacy of entomopathogenic fungi against soil-dwelling life stages of western flower thrips, *Frankliniella occidentalis,* in plant growing media. *Entomologia Experimentalis et Applicata*127: 80–87
3. Shah, F.A., Ansari, M.A., Prasad, M. & Butt, T.M. (2007). Evaluation of black vine weevil (*Otiorhynchus sulcatus)* control strategies using *Metarhizium anisopliae* with sub lethal doses of insecticides in disparate horticultural growing media. *Biological Control,* 40**:** 246-252
4. 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
5. Shah, F.A. Prasad, M. & Butt, T.M. (2007). A novel method for the quantitative assessment of the percolation of *Metarhizium anisopliae* conidia through horticultural growing media. *BioContro*l. 52:889-893
6. A grower article “Early Control” in Grower, pp. 17. July 2006.
7. Poster presentations at Horticulture Link events organized by Defra in 2006 and 2007.
8. An article in HDC Newsletter entitled “*Metarhizium anisopliae*- a potential bioinsecticide which works synergistically with chemical insecticides and entomopathogenic nematodes”

Papers in preparation/submitted

1. Shah, F.A., Hutwimmer, S., Greig, C., Dyson P., Strasser, H., & Butt T. M. Influence of natural microbial populations in horticultural growing media on the efficacy of *Metarhizium anisopliae*. Environmental Microbiology.
2. Ansari, M.A., Shah, F. A., & Butt, T. M. Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) control. *Entomologia Experimentalis et Applicata* **(submitted).**
3. Ansari, M.A., Shah, F.A. & Butt, T.M. Compatibility between cold tolerant entomopathogenic nematodes and *Metarhizium anisopliae* for black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) control in strawberries produced in growbags
4. Shah, F.A. Ansari, M.A., & Butt, T.M. Influence of plant growth media and storage conditions on the shelf life of *Metarhizium anisopliae*.

Oral Presentations:

The project co-ordinator Dr Tariq Butt and research officer Dr Farooq Shah presented oral presentations at following conferences and road shows.

1. Presentations at technical road shows organized by HDC at following locations.
    - Tuesday 21st August 2007 - Lancashire (Mere Brow Village Hall, Mere Brow, Tarleton, near Southport)
    - Wednesday 22nd August 2007 - West Midlands (Bank House Hotel, Bransford, Worcester)
    - Wednesday 29th August 2007 - East Anglia (Ramada Hotel, Kings Lynn, Norfolk)
    - Thursday 30th August 2007- Kent (Bradbourne House, East Malling
2. Butt T.M., Shah, F.A., and Ansari, M.A. (2007). Use of entomopathogenic fungi for vine weevil and thrips control at IOBC-Soft fruit meeting. Kent, UK. 25-27th Sep. 2007.
3. Butt T.M., Ansari, M.A. and Shah, F.A. (2007). Use of *Metarhizium anisopliae* and efficacy enhancing agents for the control of black vine weevil larvae and thrips pupae in plant growing media. AAB meeting. Worcester, UK. 11th Oct. 2007

4. 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
5. 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.

Website:

http://www.swansea.ac.uk/metarhizium

SCIENCE SECTION

Objective 1: Characterization of growing media properties

Introduction

Peat, bark, coir and composted green waste (CGW) were characterized to evaluate their performance as horticultural growth media as well as to gain a better understanding of their physical and chemical properties which might influence the efficacy of *M. anisopliae* V275.

Materials and Methods

pH and electric conductivity (EC) were determined in water using the standardized methods BS EN 13037:2000 and BS EN 13038:2000, respectively. Nutrient levels were determined in a $CaCl_2$/DTPA extract using the method according to the BS EN 13651:2001 protocol. Nitrogen retention and E4/E6 studies were done as described by Prasad (1997) and Schnitzer (1982), respectively. Biological stability of the media was determined using the SOUR method (Lasaridi & Stentiford 1996) and Oxitop method (Wageningen University & Nutrient Management Institute, 2003). Physical Properties were determined using the ISEN 13041:2000 protocol.

Results

Considerable variation in pH, EC and nutrient levels were observed in the media tested (Table 3). The pH of CGW was very high, while the pH of peat was very low, and bark and coir were intermediate. The EC values were high for CGW, very low for peat whereas coir and bark had moderate values. All media, except bark, had low levels of N and P. Coir and bark had moderate levels of K whereas CGW had extremely high levels of K. All media were stabilized as regards nitrogen retention, with coir and CGW tending to retain a small amount of nitrogen, over ten weeks of incubation (data not presented). The E4/E6 test indicated high levels of humic acid in all media; however CGW and composted bark had relatively higher humic acid levels than peat and coir. Biological stability studies confirmed above findings of nitrogen retention and E4/E6 studies (Table 4).

Studies on the physical properties showed low air space and easily available water for CGW (Table 4). Peat has a good balance of air as well as easily available water. Both coir and bark have high levels of air space but easily available water is low in bark. Bulk density is very high in CGW but low in peat and coir, and intermediate in bark.

Table 3 Chemical analysis of various growing media

Sample	pH (units)	EC (us/cm)	NH_4N (mg/L)	NO_3N (mg/L)	P (mg/L)	K (mg/L)	E4/E6
Composted green waste	8.23	3290	85	7	20	1073	8.17
Peat	3.90	108	1	2	0	0	12.8
Coir	6.70	398	1	0	7	400	10.8
Bark	5.80	145	0	0	5	82	3.94

Table 4 Biological stability and physical properties of plant growing media

Sample	Oxitop (mmol O2/kg OS/h)	SOUR (o_2/g dm/ h)	Bulk Density (g/L)	% Air (v:v) After applying 10 cm pressure IS EN 13041:2000	% Easily Available Water (v:v)	Total pore space
Composted green waste	3.11	0.70	411.77	27	16	63
Peat	0.70	2.37	252.97	34	8	87
Coir	0.90	0.40	80.96	34	20	92
Bark	6.50	0.69	148.63	25	31	93

Discussion

Although the plant growth media differed considerably in their physical and chemical properties all except CGW were suitable for horticultural use. However, good plant growth was observed in peat blends containing 10% or 20% CGW. The exact combination of peat and CGW blends would depend upon the source and degree of green waste composting. The results of this study will help determine the robustness of *M. anisopliae* (i.e. its ability to control pests in different growing media).

Objective 2: Elucidate synergistic interactions between *M. anisopliae* and low doses of insecticide

Task 2.1: Pot bioassay to determine synergistic interactions between *M. anisopliae* and low doses of insecticides for the control of BVW and WFT in different plant growth media

A. Studies against black vine weevil

Introduction

BVW is an important and widespread pest of ornamental nursery stock and soft fruit (Cross and Burgess, 1997; Masaki *et al.,* 1984, Moorehouse *et al*., 1992; Van Tol *et al*., 1998; Lola-Luz *et al*., 2005). Adult weevils feed on leaves causing mostly cosmetic damage whilst the larvae feed on root systems, which can lead to plants being stunted, or collapsing and dying. Current control is dependent on the use of chemical insecticides (e.g. imidacloprid, chlorpyrifos) but there is considerable interest to reduce the input of such pesticides because of the risks they pose to humans and the environment and increased resistance among pest populations. Growers are currently trapped between the diminishing number of available chemical insecticides and the availability of non-chemical alternatives. Entomopathogenic nematodes offer a more benign alternative for BVW control in potted plants and glasshouse crops (Kakouli-Duuarte *et al*., 1997; Fitters *et al*., 2000; Georgis *et al*., 2006) but they have limited success due to a relatively short shelf life and inconsistent results (Georgis *et al*., 2006; Koppenhofer, 2000; van Tol *et al*., 2004).

M. anisopliae shows considerable potential for the control of BVW larvae (Bruck, 2005; Moorhouse *et al.,* 1992, 1993; Shah *et al*., 2007). However, an inherent weakness of *M. anisopliae* is that it is slow acting, particularly at low temperatures. Since the grower often wants rapid protection then a strategy needs to be devised where pesticide inputs can be reduced yet benefit from the control given by *M. anisopliae*. One approach is the exploitation of synergistic interactions between *M. anisopliae* and low doses of chemical insecticides. Of the few studies to date, synergy has been observed in the control of termites, root weevils and aphids (Quintela and McCoy, 1998; Roditakis *et al*., 2000; Inglis *et al*., 2001). Low doses of insecticide usually alter insect behaviour. Depending on the species, insects may increase in mobility with a corresponding increase in the acquisition of conidia (Roditakis *et al*., 2000), or they may stop grooming (i.e. dislodging conidia from the cuticle) and feeding (Quintela and McCoy, 1998; Boucias *et al*., 1996). Insects that are debilitated due to starvation or other forms of stress may be more susceptible to fungal infection.

This study showed for the first time that *M. anisopliae* and pesticides work synergistically in the control of BVW larvae. These studies initially focussed on the insecticides fipronil (phenyl pyrazole) and imidocloprid (chloronicotinyl) but were extended to chlorpyrifos (organophosphate) and neem seed cake (botanical fertiliser), to help obtain a better understanding of this phenomenon. The synergy studies showed that these agents could significantly enhance the efficacy of *M. anisopliae* for control of BVW larvae.

Materials and Methods

Fungal strain, maintenance and mass production

M. anisopliae strainV275 was used in all studies. Details of its maintenance and production are given in Shah *et al*., (2005).

Inoculation of plants with BVW eggs

Rooted cuttings of *Euonymus fortunei* 'Emerald Gold' kindly provided by Johnsons of Whixley (York, UK) were transplanted in 0.5 l pots filled with one of the test media and inoculated 7 days later with 15 melanized BVW eggs which were gently placed around the base of the plant. The media provided by Bord Na Mona (Ireland) included: peat (seed and potting compost), bark (multipurpose peat free), coir and peat blended with 10% (v/v) or 20% (v/v) composted green waste.

Application of *M. anisopliae* and insecticides

M. anisopliae was applied as a drench or premixed such that the final concentration was 1x 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 and pre-determined sub lethal rates. Imidacloprid and fipronil sub lethal rates corresponded to 1% and 10% of the recommended rate. These were used alone or with *M. anisopliae* V275. Untreated plants constituted one of the controls. Trials were conducted between July-October, 2005 when the average day and night temperatures ranged between 15-25ºC and 10-15ºC, respectively.

Additional studies were conducted in potted strawberry plants to determine synergistic interaction between *M. anisopliae* and another chemical insecticide Cyren (46% w/w chlorpyrifos; Cheminova, Denmark). Young strawberry plants (c.v. Elsanta) kindly provided by Hargreaves Nursery, Plants Ltd, Lincolnshire, UK. *M. anisopliae* V275 was used as a drench or premixed at the rate of 1 x 10^{10} conidia/l of growth medium. *M. anisopliae* V275

was used with or without a reduced rate (1/10 dilution) of chlorpyrifos. Chlorpyrifos was also used alone at the recommended and reduced rates. 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 of both trials was replicated 20 times. 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.

Task 2.2 Pest susceptibility and spore acquisition

To determine if *M. anisopliae* spore acquisition increased in the presence of sub lethal doses of insecticides, additional plants were prepared in peat with *M. anisopliae* being applied as a drench with and without a sub lethal dose of imidacloprid or fipronil. Both insecticides were also applied alone at the sub lethal and recommended rates. All studies included untreated controls.

Each pot was infested with two 2nd instar BVW larvae. Each treatment was replicated 5 times and the whole experiment repeated twice. Larvae were recovered from two pots per treatment 2 days post-infestation. Half the larvae were fixed in 2% formaldehyde and stored at 4ºC until required. The specimens were stained with calcofluor and examined in a Nikon, Eclipse E600 microscope equipped with epifluorescence (Butt, 1997). Spore adhesion to the second larval group was determined as outlined by Moorehouse (1993). Briefly, the larvae were macerated in 0.5ml of 0.03% Aq. Tween and conidial numbers counted using an improved Neubaur haemocytometer (Weber Scientific Ltd., UK). The remaining pots from each treatment were destructively assessed 2 weeks post infestation to determine larval mortality.

Statistical analysis

Percentage efficacy data 6 weeks post-treatment were corrected for control mortality (Abbott's 1925), arcsine square root transformed, and analyzed using ANOVA and Duncan test for means separation (SPSS, 2003). Differences among means were considered significant at $P < 0.05$.

Results

Efficacy experiments

Both *M. anisopliae* and chemical insecticides provided good control of BVW larvae; however, efficacy was dependant on the application method, plant growth medium and insecticide dose (Figs 1-5). For example, *M. anisopliae* appeared to give better control if applied as a drench as opposed to premixing, providing between 85% and 100% control irrespective of the growing medium (Figs 1-5). Premixed applications gave less than 80% control and appeared to be influenced by the plant growth medium ($P < 0.05$) (Figs 1-5). In reality, *M. anisopliae* efficacy using the two application methods differed non-significantly ($P > 0.05$) for the all the media tested with the exception of peat blends with 10% and 20% CGW.

Imidacloprid when used at the recommended and sub lethal rates gave 70-85% and 30-50% control, respectively in the different media (Figs. 1-5). Its activity appeared to be influenced by compost type. Fipronil usually gave 100% control at the recommended rate but 85-100% control at the sub lethal dose in all the media tested (Figs 1-5). At the reduced rate it appeared to be least effective in peat and peat blends with 10% CGW ($P < 0.05$) (Figs. 1-5).

Application of *M. anisopliae* with sub lethal doses of insecticides provided excellent control of BVW larvae independent of the chemical used. Usually the control was higher than use of the *M. anisopliae* or chemical insecticides used alone. In combined applications, *M. anisopliae* efficacy was consistently over 75% irrespective of application method and/or plant growing medium.

Compost type significantly influenced BVW infestation with significantly ($P < 0.05$) more larvae being recovered from coir than all other media (Figs. 6-7). Non-significant ($P > 0.05$) differences in larval infestation were observed for the remaining growth media (Fig 6). As stated earlier, compost type influenced the efficacy of the different control agents (Figs.1-5). For example, drench application of *M. anisopliae* was most efficacious in peat blended with 10 and 20% CGW than all other media tested (Figs 1-5). Use of *M. anisopliae* with fipronil or imidacloprid gave good control of BVW larvae in all growing media with 95-100% control being achieved if the fungus was applied as a drench but 78-92% control achieved if it was premixed into the different media (Figs.1-5, 8).

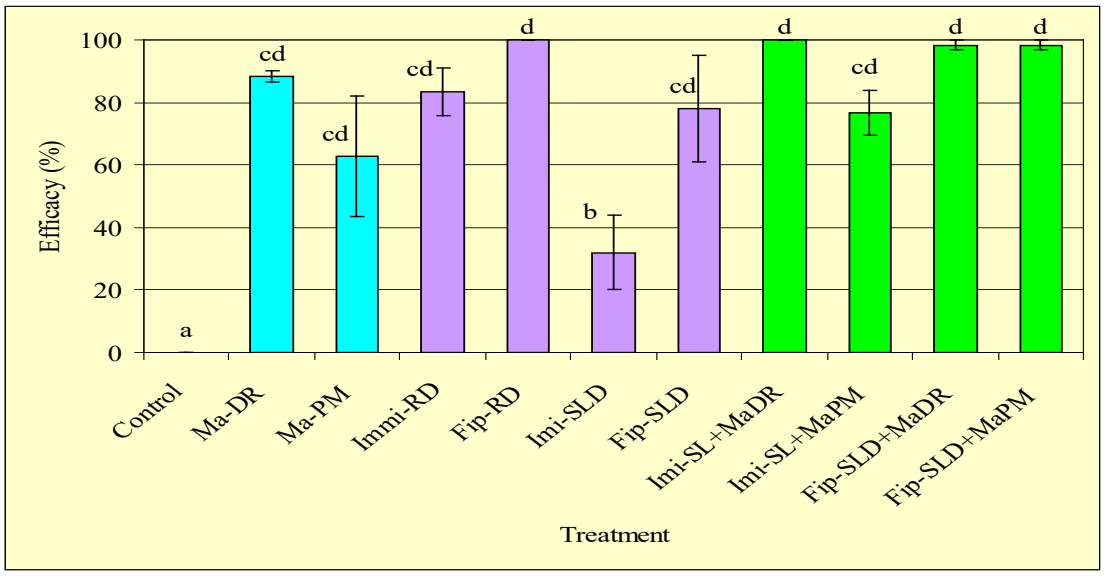

Fig. 1. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat growth media. The treatments are listed below: No treatment- (control), *M. anisopliae* applied as drench (Ma-DR); *M. anisopliae* premixed (Ma-PM), Imidacloprid applied as a drench at the recommended rate (Imi-RD); Fipronil premixed at the recommended rate (Fip-RD); Imidacloprid applied as a drench at the sub lethal rate (Imi-SLD); Fipronil premixed at the sub lethal rate (Fip-SLD); Imidacloprid used at the sub lethal rate with *M. anisopliae* applied as a drench (Imi-SL+MaDR); Imidacloprid used at the sub lethal rate with *M. anisopliae* premixed (Imi-SL+MaPM); Fipronil used at the sub lethal rate with *M. anisopliae* applied as a drench (Fip-SLD+MaDR) and Fipronil used at the sub lethal rate with *M. anisopliae* premixed (Fip-SLD+MaPM).

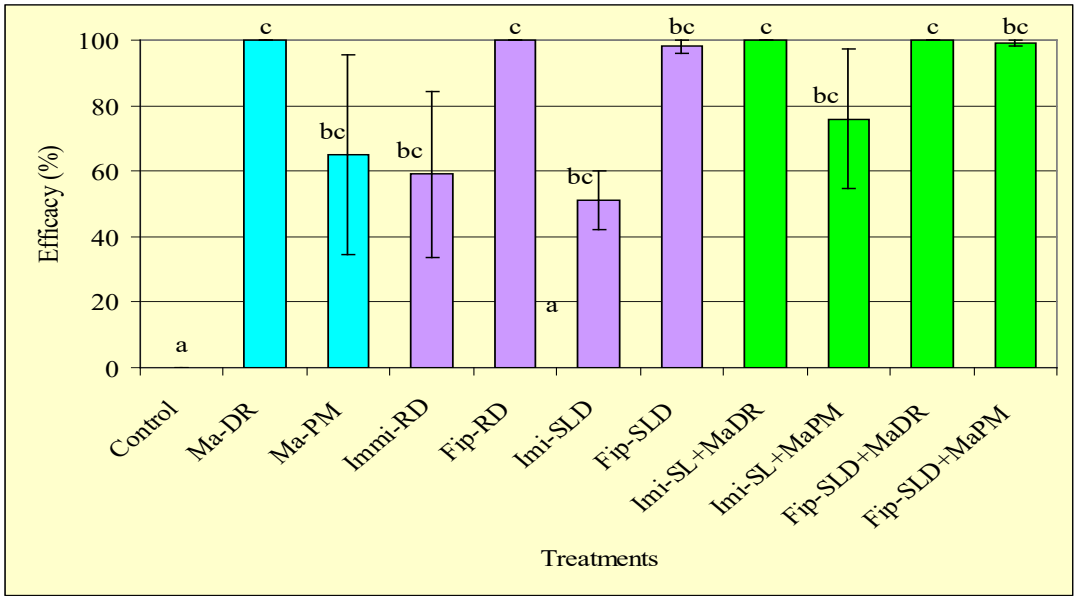

Fig. 2. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in bark growth media. Treatments are the same as those described in Fig.1.

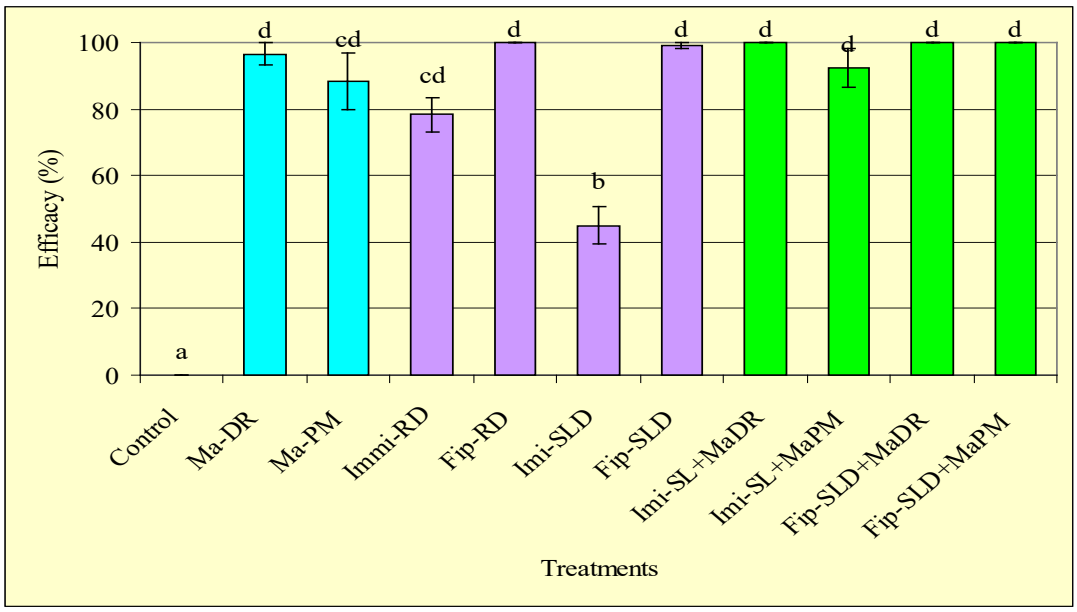

Fig. 3. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in coir growth media. Each pot was treated with either of following treatments. Treatments are the same as those described in Fig.1.

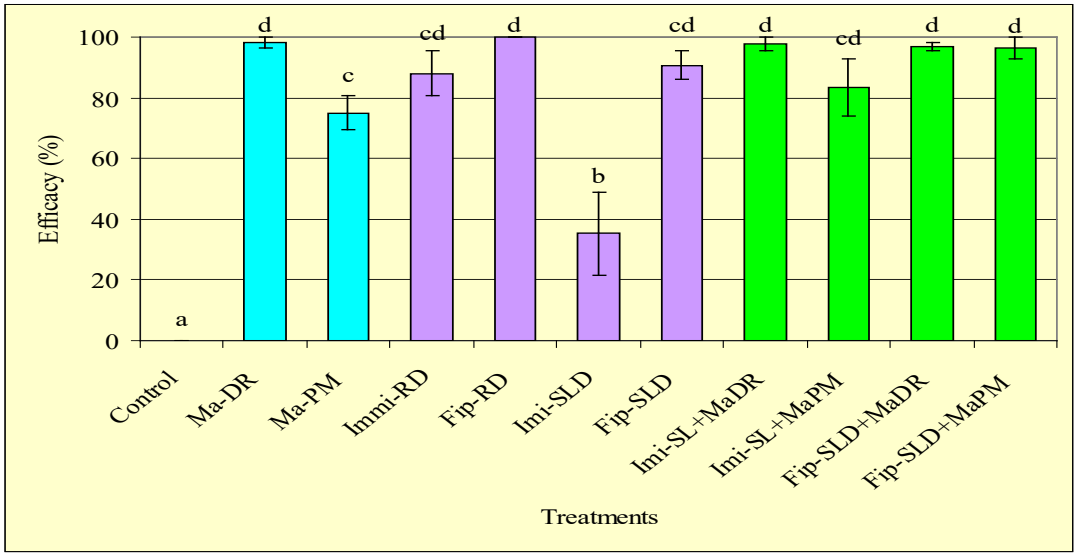

Fig. 4. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in 10% green waste blend growth media. Each pot was treated with either of following treatments. Treatments are same as described in Fig.1.

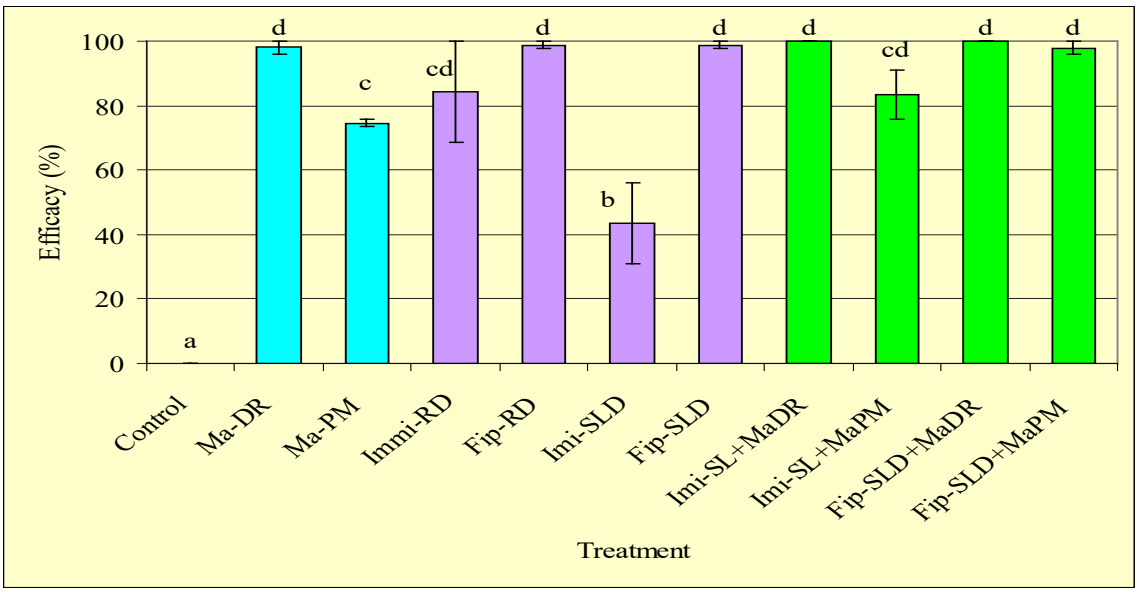

Fig. 5. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in 20% green waste blend growth media. Each pot was treated with either of following treatments. Treatments are same as described in Fig. 1.

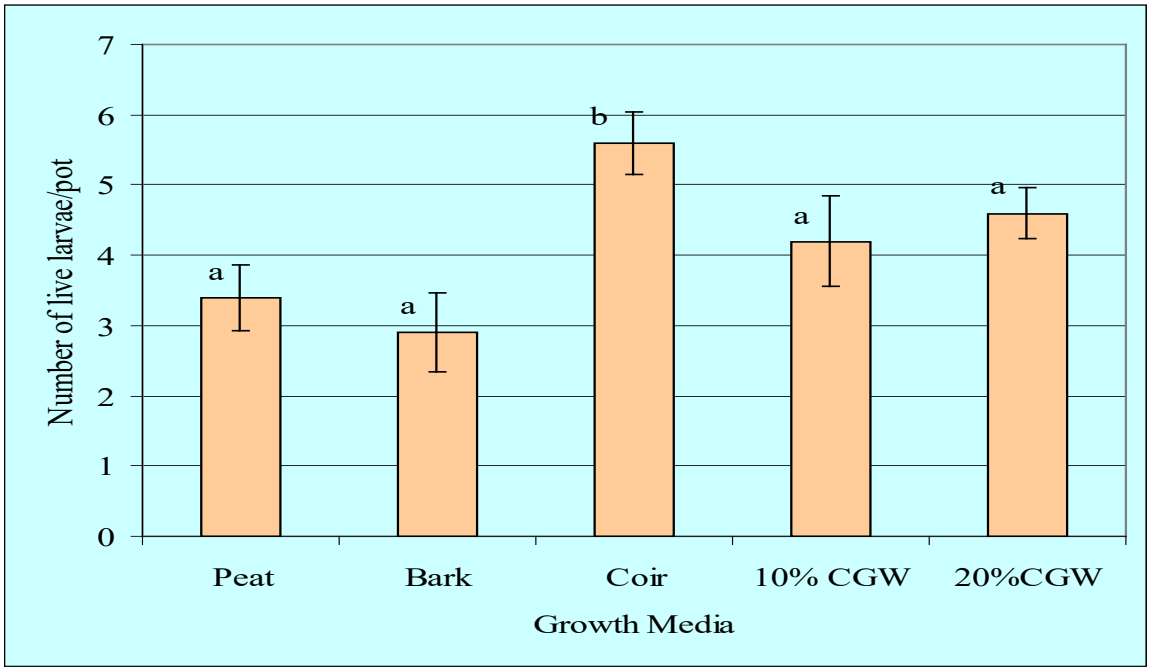

Fig. 6. Establishment of BVW larvae in different plant growth media. Each pot was infested with 15 melanized eggs and destructively assessed 6 weeks post infestation

Fig. 7. An untreated *Euonymus* plant with the root system infested with BVW larvae

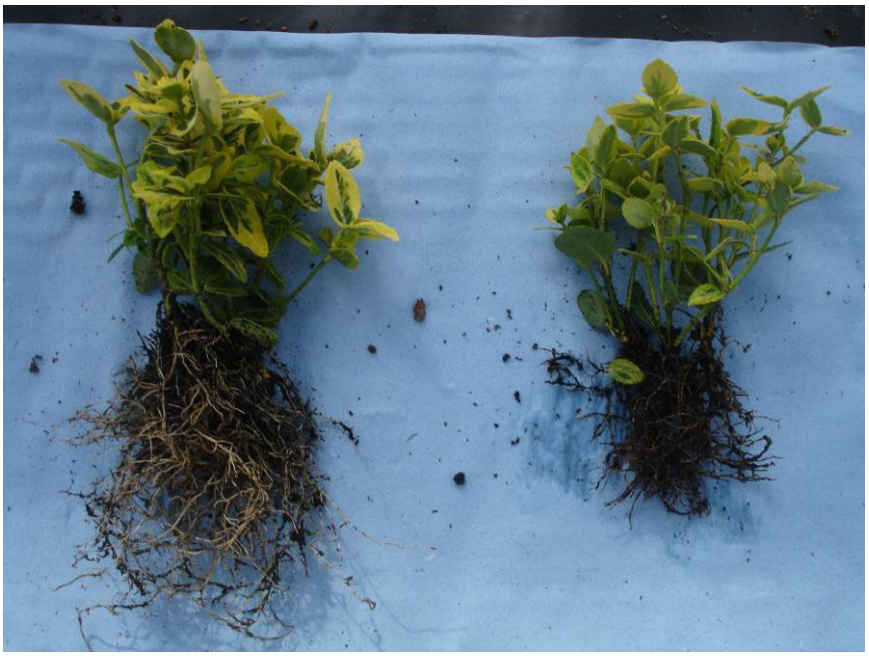

Fig. 8. Comparison between untreated (right) and plant treated with combined application of premixed application of *M. anisopliae* and sub lethal dose of imidacloprid (left). Note differences in the root system

Interactions between *M. anisopliae* and chlorpyrifos against BVW in potted strawberry plants

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. 9). 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. 9). Plants treated with *M. anisopliae* V275 or chlorpyrifos grew vigorously while untreated plants were stunted and had poorly developed root systems (Fig. 10).

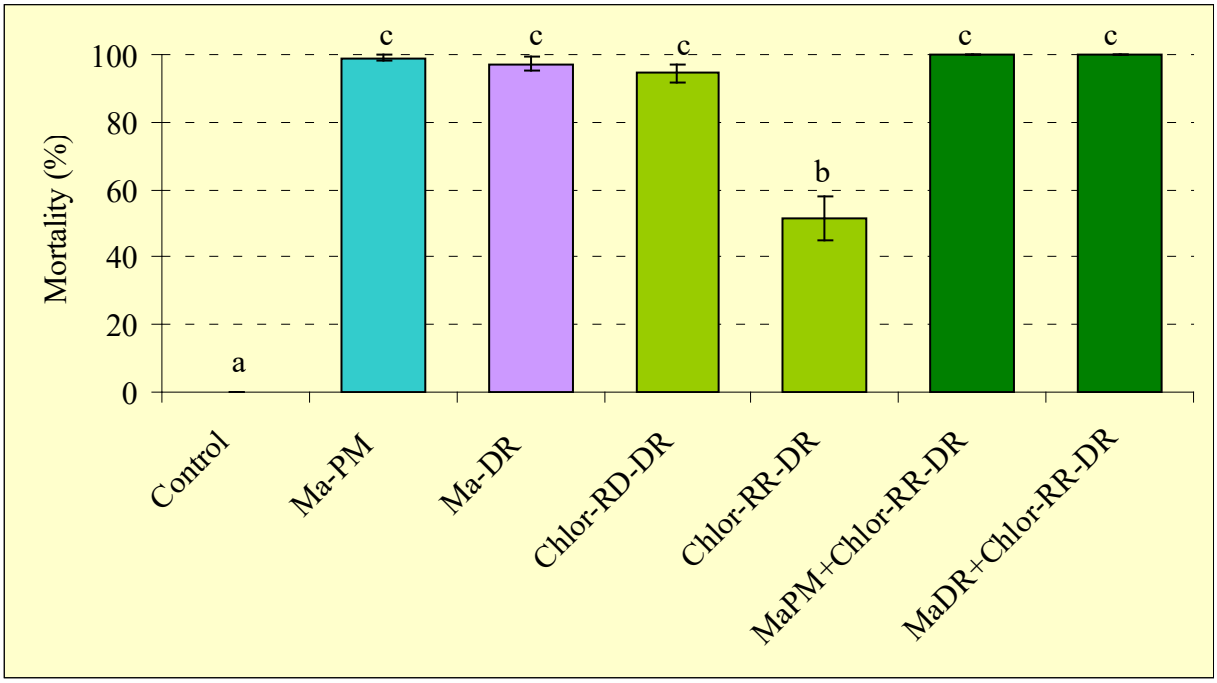

Fig. 9. Comparison of the efficacy of *M. anisopliae* V275 and chlorpyrifos for BVW control in strawberries. The treatments included: *M. anisopliae* premixed into the compost (Ma-PM) or applied as a drench (Ma-DR); Chlorpyrifos applied as a drench at the recommended dose (Chlor-RD-DR); or reduced rate (Chlor-RR-DR); *M. anisopliae* premixed into growth media and reduced rate of chlorpyrifos applied as a drench (MaPM+Chlor-RR-DR) and *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, $P < 0.05$).

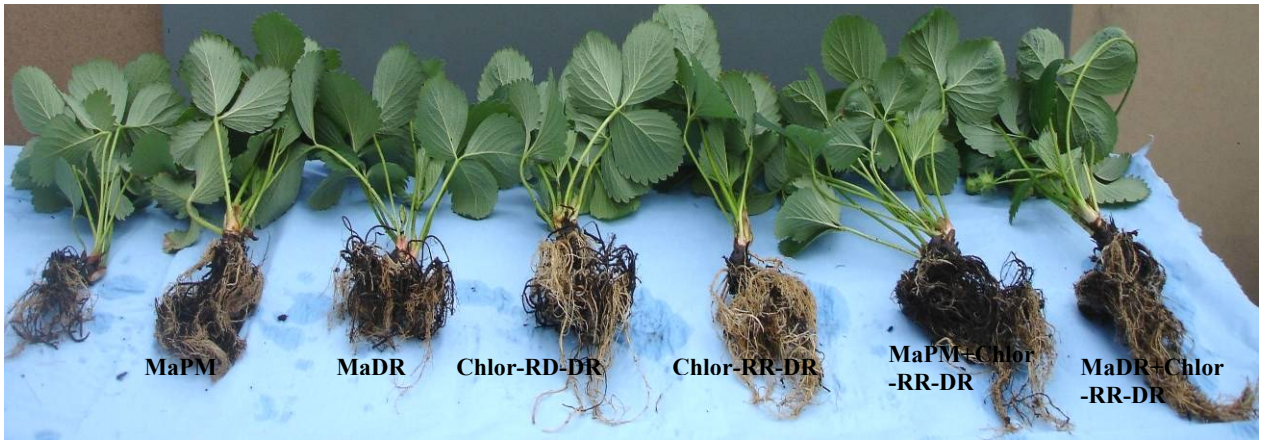

Fig. 10. Comparison of the root system of strawberry plants exposed to the following treatments: From left: untreated control; *M. anisopliae* premixed into the compost (MaPM); or applied as a drench (MaDR); Chlorpyrifos applied as a drench at the recommended dose (Chlor-RD-DR); or reduced rate (Chlor-RR-DR); *M. anisopliae* premixed into 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

Conidial adhesion

Significantly more conidia of *M. anisopliae* adhered to BVW larvae when used with sub lethal doses of fipronil than imidacloprid or if used alone (10.66 ± 0.84 versus 3.5 ± 0.34 versus 2.17 ± 0.54 conidia per 10μl of macerate). In the fipronil treatment, conidia were usually clustered and had germinated with many producing appressoria (Fig. 11). None of the conidia in the imidacloprid or chemical free controls had germinated during the same timeframe. *In vitro* studies show that neither chemical inhibited *M. anisopliae* conidia (Butt & Shah, unpublished observations). In these studies, *M. anisopliae* used with sub lethal doses of fipronil resulted in 100% control 2 weeks post inoculation whereas *M. anisopliae* and fipronil when used alone caused 25% and 75% mortality, respectively (data not shown). Use of *M. anisopliae* with sub lethal doses of imidacloprid resulted in 50% larval mortality. Imidacloprid alone caused 50 and 25% mortality at the recommended and sub lethal doses, respectively.

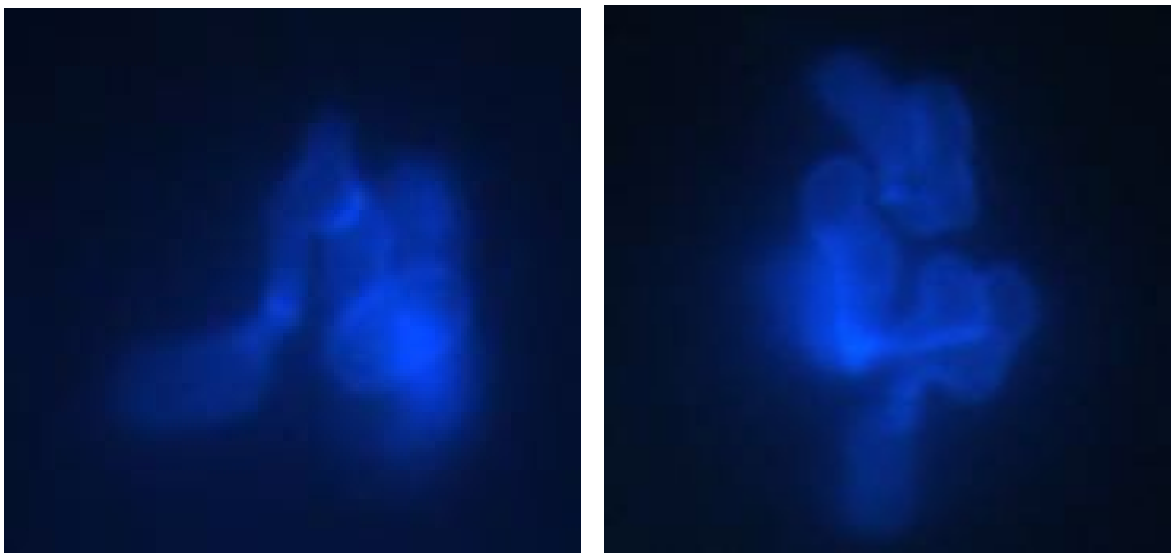

Fig 11. Spore adhesion on larvae recovered from pots treated with sub lethal dose of fipronil and *M. anisopliae*. Note spores are attached in clusters and most have germinated and differentiated appressoria (infection structures). Photographed at 60x magnification of fluorescent microscope (Nikon, Eclipse E600) using Cool Snap digital camera.

Discussion

This study shows that *M. anisopliae* V275 can be used for the prophylactic control of BVW larvae in a range of horticultural growing media. The level of control appears to be just as good as imidacloprid and chlorpyrifos but not that of fipronil. Since the objective is to reduce inputs of chemical pesticides, we show for the first time that *M. anisopliae* can give excellent control when used with 1% and 10% recommended rates of imidacloprid and fipronil or chlorpyrifos, respectively. The level of control achieved was similar to that of the recommended rates for these synthetic pesticides. The chemicals give almost immediate crop protection and concomitantly provide *M. anisopliae* more time to kill its host. Presumably the low rate insecticides stress the target or alter their behaviour in such a way as to make it more susceptible to fungal infection.

Maximum recovery of *M. anisopliae* conidia was from larvae exposed to sub lethal doses of fipronil. Compared with the latter, almost 67% and 80% fewer conidia were recovered from larvae exposed to sub lethal doses of imidacloprid and chemical free treatments, respectively. It is tempting to speculate that fipronil provided an unfavourable environment causing the larvae to move and acquire more inoculum. Indeed, chemical insecticides can induce greater movement and acquisition of inoculum as observed by Roditakis *et al* (2000). The fact that the insects are moving and not feeding can result in starvation stress which is also considered to make insects susceptible to infection (Amiri *et al*., 1999; Thomsen and Eilenberg, 2000). Since the conidia had also germinated and differentiated infection

structures suggest that either fipronil stimulated germination or induced changes in the insects which accelerated infection and may explain why more larvae were killed compared with the other treatments. Almost 38% more conidia were recovered from larvae exposed to sub lethal doses of imidacloprid and mortality was higher compared to the insecticide free treatment suggesting that this insecticide is working in an additive or synergistic way with *M. anisopliae*. Sub lethal doses of imidacloprid are considered to prevent grooming and subsequent dislodging of inoculum from insect cuticle and since mortality is dose-related it may improve pest control (Quintela and McCoy, 1998).

The physical-chemical properties of the different media may account for some of the variation observed in the control of BVW larvae. The media may exert some inhibitory effect and/or enhance pest establishment. Our studies showed that more BVW larvae survived in untreated coir than bark or peat and peat blends with CGW. These observations corroborate the findings of Buxton (2003) who reported better BVW establishment in coir and fine bark than peat or coarse bark media.

The efficacy of *M. anisopliae* was also influenced by a complex interaction between the method of application and type of growing media. With the exception of coir, the level of control was usually between 15% and 25% better using drench application than premixing into media. Chandler and Davidson (2005) observed a high concentration of *M. anisopliae* inoculum in the top 10 cm of compost following drench application. This would expose newly hatched BVW larvae to a relatively high dose and since mortality is dose related, it would result in better targeted control of the pest. Inoculum would be diluted when premixed into composts but in spite of this it was still a highly efficacious application method. It should be noted that whatever the application method or media used, all the live larvae recovered from *M. anisopliae*-treated pots ultimately died of this pathogen (Fig. 12).This observation suggests that, at the time of destructive assessment, these larvae were at the early stages of infection.

In conclusion, our study shows that *M. anisopliae* V275 can reduce inputs of synthetic insecticides for BVW larval control either when used alone or in combination with low doses of chlorpyrifos, fipronil or imidacloprid. The latter approach not only gives more immediate protection but at a level correspondingly similar to the recommended rate of the chemical pesticide, independent of the application method and/or growing medium used.

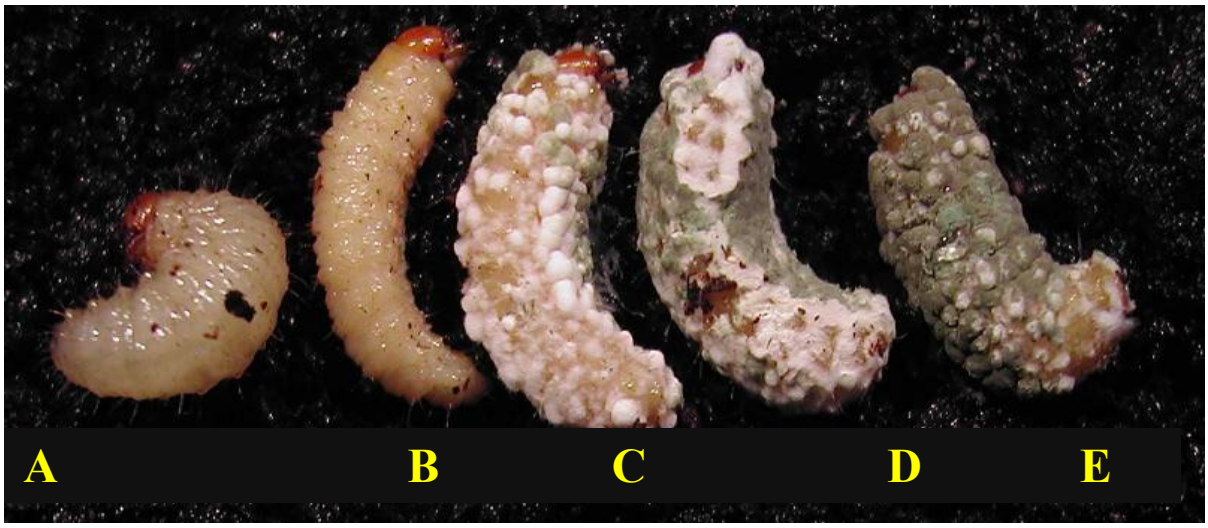

Fig. 12. BVW larvae at different stages of *M. anisopliae* infection. A. Healthy larvae, B. 3-5 days post inoculation, C-E, 2-3 post mortem.

Objective 2: Interactions between neem seed cake and *Metarhizium anisopliae* for the control of BVW. (Additional study which contributes significantly to our understanding of why some agents enhance the efficacy of *M. anisopliae*)

Introduction

Having established that sub lethal doses of synthetic insecticides enhance *M. anisopliae* efficacy against BVW, we extended our studies to determine whether such interactions exist with plant based product such as neem seed cake. These studies are important as many chemical insecticides are being withdrawn from the EU by 2008. Furthermore, it will provide an opportunity to develop chemical pesticide free strategy for the control of media-borne pests.

Materials and Methods

Fungal strain, BVW eggs, plants and neem seed cake source

Details of the source, maintenance and production of *M. anisopliae* strain V275, plants and BVW eggs were same as described above. Neem seed cake powder (GreeNeem) was obtained from GreeNeem-K. Sivaram Bros, Virudhunagar, India.

Application of *M. anisopliae* and neem seed cake

Both *M. anisopliae* V275 and neem seed cake were premixed into the peat substrate either alone or together. The former was premixed at the recommended and hundred fold lower rates of 1 x 10^{10} and 1 x 10^{8} and conidia/l of substrate, respectively. Neem seed cake was premixed at the rate of 0.5, 2.5 or 5g/l of compost alone or in combination with the lower dose (1 x 10^{8} conidia/l) of *M. anisopliae*. Untreated plants constituted one of the controls.

Trials were conducted outdoors between July-October 2006 when the average day and night temperatures ranged between 15-25ºC and 10-15ºC respectively. Plants were destructively assessed six weeks post egg infestation to determine the efficacy of the above treatments. Each treatment was replicated ten times and the whole experiment was replicated twice. Treatments were arranged in randomized complete block design with each pot adequately spaced to avoid cross contamination among treatments.

Larval susceptibility and spore acquisition

To determine if *M. anisopliae* spore acquisition increased in the presence of neem seed cake, additional plants were prepared in peat with *M. anisopliae* alone and in combination with 0.5 and 5g/l of neem seed cake. *M. anisopliae* was applied at 1 x 10^{10} conidia/l of peat. Neem seed cake was also applied alone at 0.5 and 5g/l of peat. Both *M. anisopliae* and neem seed cake were premixed in the peat before transplantation of *Euonymus* cuttings. Each treatment was replicated 10 times. All other procedures were same as described in task 2.2.

Influence of neem seed cake and *M. anisopliae* on larval development

Influence of neem seed cake or *M. anisopliae* on larval development was determined by examining the body size and weight of larvae recovered from pots treated with different treatments. Larvae were divided in groups of five and weighed. Each treatment was replicated five times. Larval size particularly head capsule width was used to determine larval instars. The whole experiment was repeated twice with each treatment replicated ten times.

Influence of neem seed cake or *M. anisopliae* on BVW oviposition

In addition to the bioassays above we tried to determine if the neem seed cake acted as an oviposition deterrent. Oviposition assays were conducted in plastic cups (125 ml). Each cup was filled with 100 ml of peat medium and a single *Euonymus* plug provided as a food source for adult BVW. Treatments included neem seed cake premixed at either 2.5 or 5g/l of peat, *M. anisopliae* premixed at 1 x 10^{10} conidia/l and untreated control. Each treatment was randomly replicated 4 times in a secured plastic box (2 x 1.5 x 1.5 feet) and infested with 10 adult BVW. The box set up was replicated 3 times, thus each treatment was replicated 12 times. The whole experiment was repeated with a fresh cohort of adult BVW and *Euonymus* plants. Adults were allowed to oviposit for 72 hours at 20-22° C with 12:12 hrs light and dark photoperiod. Each pot was then destructively assessed under magnifying glass to recover eggs from each pot. The impact of the different treatments on adult feeding was assessed by counting the number of notches in *Euonymus* leaves. Statistical analyses of data were done as described above.

Results

Efficacy of *M. anisopliae* and neem seed cake against BVW

Both *M. anisopliae* and neem seed cake were efficacious against BVW establishment but varied significantly ($P < 0.05$) among treatments (Fig. 13). On average, more than 10 larvae were recovered from each untreated pot, whereas, no larvae were recovered from pots treated with *M. anisopliae* at the low dose with either 2.5 or 5g/l of neem seed cake (Fig.13).

The number of larvae recovered from pots treated with *M. anisopliae* alone at the high and low doses were 5 and 12 larvae/pot, respectively. Larval recovery from media treated with neem seed cake was dose dependant and ranged from 0.5 to 8 larvae/pot. There was no significant difference ($P < 0.05$) in neem seed cake efficacy used at the lower concentration (0.5g/l) with or without *M. anisopliae* (Fig. 13). All the larvae recovered from *M. anisopliae* treatments died of this fungus within one week upon incubation at 25°C.

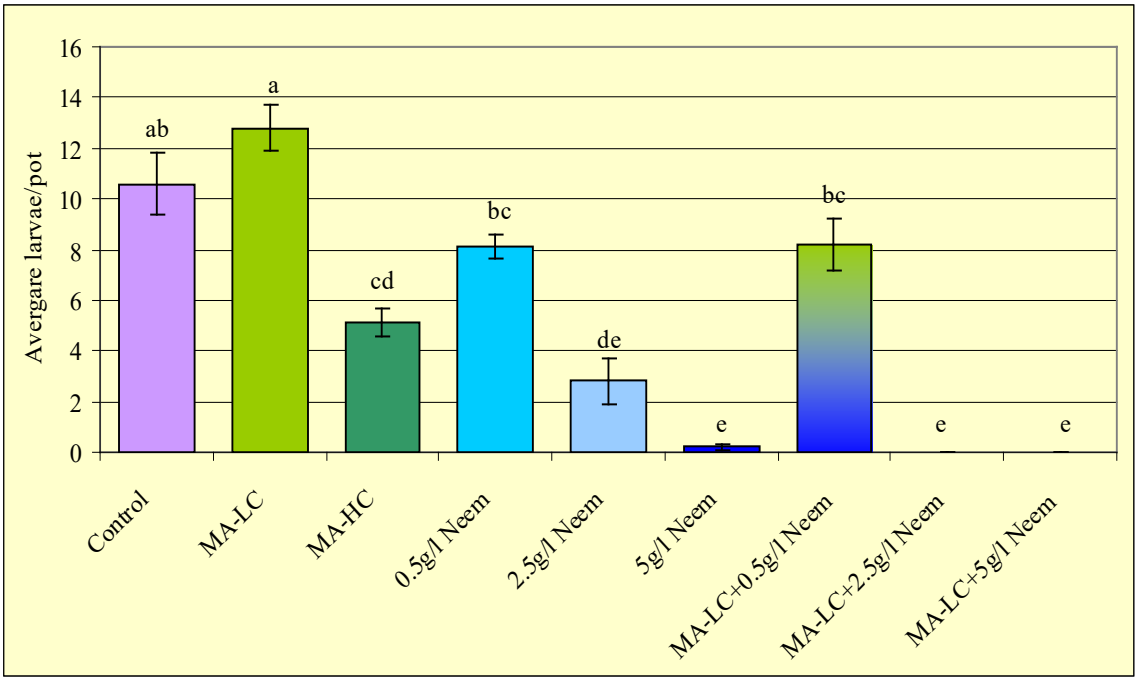

Fig. 13. Effect of *M. anisopliae* and neem seed cake against BVW establishment in *Euonymus* potted in peat growth media. Each pot was infested with 20 BVW eggs and destructively assessed at 6 weeks post infestation to recover live larvae from each pot. The treatments included: No treatment- (untreated control); *M. anisopliae* applied as premixed premixed at lower inoculum dose of 1 x 10^8 conidia/l of compost (MA-LC); *M. anisopliae* applied as premixed at higher inoculum dose of 1 x 10^{10} conidia/l of compost (MA-HC); neem seed cake premixed at the rate of 0.5g/l of compost (0.5g/l Neem); neem seed cake premixed at the rate of 2.5g/l of compost (2.5g/l Neem); neem seed cake premixed at the rate of 5g/l of compost (5g/l Neem); *M. anisopliae* premixed at lower inoculum dose in combination with neem seed cake premixed at the rate of 0.5g/l of compost (MA-LC + 0.5g/l Neem); *M. anisopliae* premixed at lower inoculum dose in combination with neem seed cake premixed at the rate of 2.5g/l of compost (MA-LC + 2.5g/l Neem) and *M. anisopliae* premixed at lower inoculum dose in combination with neem seed cake premixed at the rate of 5g/l of compost (MA-LC + 5g/l Neem). Treatments were replicated 10 times and the whole experiment was repeated twice.

Larval susceptibility and spore acquisition

Use of *M. anisopliae* with neem seed cake was more effective than use of either alone. *M. anisopliae* on its own provided 60% control whereas neem seed cake gave 30 and 40% protection at 0.5 and 5g/l of media, respectively (Fig. 14). Combined applications of *M. anisopliae* with 0.5g or 5g/l of neem seed cake provided 75 and 95% protection against BVW larvae respectively (Fig. 14). Conidial attachment was significantly higher in larvae recovered from pots treated with higher dose of neem seed cake dose (3-4 times) than *M. anisopliae* alone. The lower dose of neem seed cake had no significant effect on conidial attachment (Fig. 15).

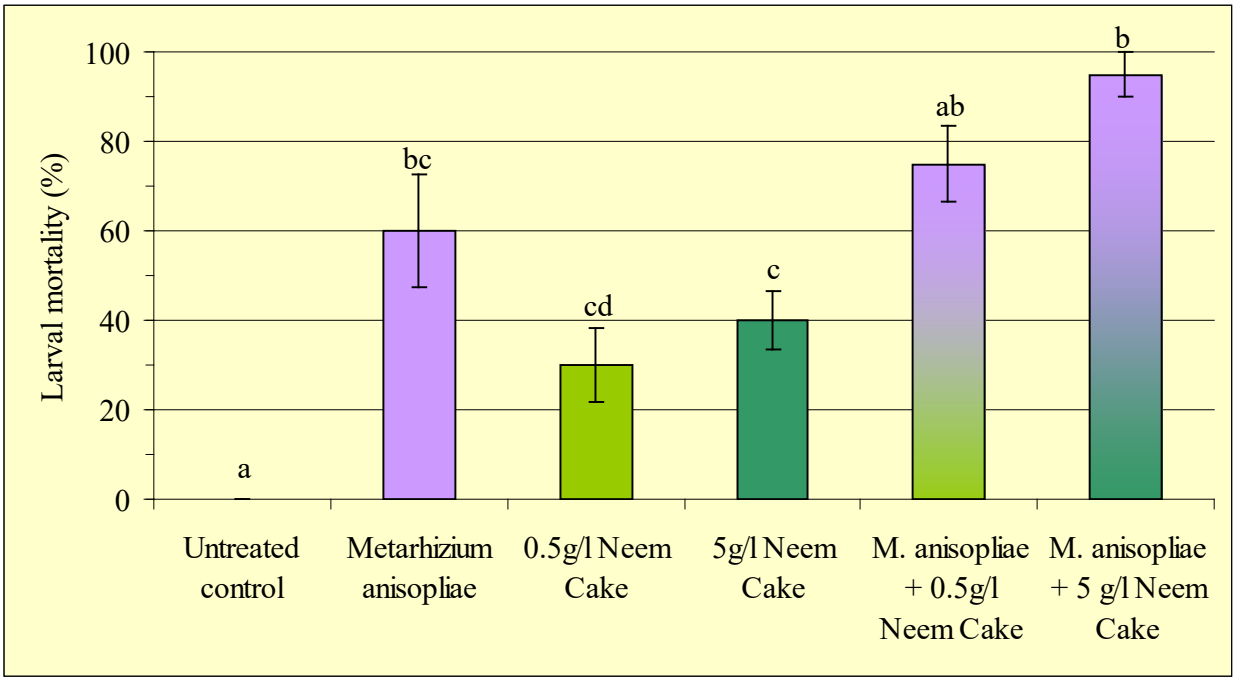

Fig. 14. Mortality (%) of BVW larvae in potted *Euonymus* plants. Each pot was infested with two 2nd instar larvae and mortality was recorded two weeks post inoculation. Treatments included: No treatment (untreated control); *M. anisopliae* applied as premixed at higher inoculum dose of 1 x 10^{10} conidia/l of compost (*Metarhizium anisopliae*); neem seed cake premixed at the rate of 0.5g/l of compost (0.5g/l Neem); neem seed cake premixed at the rate of 5g/l of compost (5g/l Neem); *M. anisopliae* premixed at higher inoculum dose in combination with neem seed cake premixed at the rate of 0.5g/l of compost (*M. anisopliae* + 0.5g/l Neem) and *M. anisopliae* premixed at higher inoculum dose in combination with neem seed cake premixed at the rate of 5g/l of compost (*M. anisopliae* + 5g/l Neem). Treatments were replicated 5 times and the whole experiment was repeated twice.

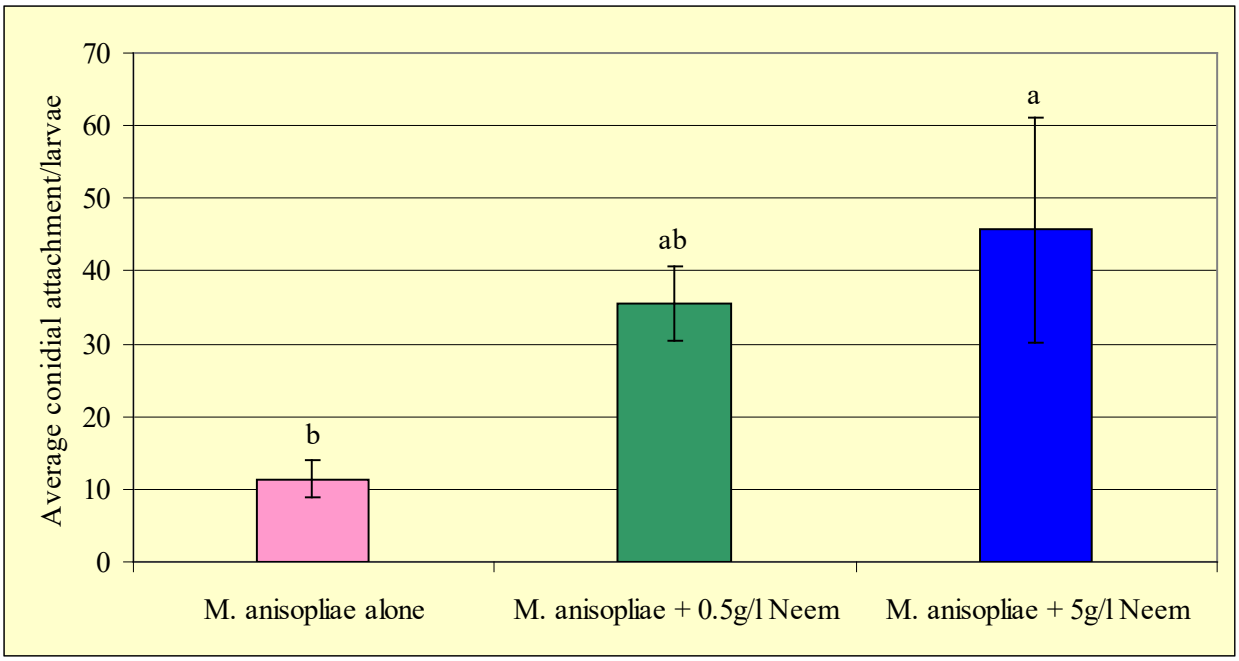

Fig.15. Spore adhesion on larvae recovered from pots treated *M. anisopliae* alone or in combination with 0.5 and 5g/l of neem seed cake

Influence of neem seed cake and *M. anisopliae* on larval development

Larvae recovered from neem seed cake treated media were significantly ($P < 0.05$) smaller than all other treatments (Fig. 16). Few larvae (on average 0.5/pot) survived in media containing high levels of neem seed cake (5g/l peat) and those recovered from intermediate (2.5g/l) and lower concentration (0.5g/l) of neem seed cake had lower body weights than those from the untreated control (Fig. 16). Larval weight of *M. anisopliae* treated insects varied non-significantly with those of untreated ($P < 0.05$). Furthermore, larval development was slower in neem treatments with most remaining at the 2nd instar stage while those from the untreated control or *M. anisopliae* treated pots were found to be 3rd instar larvae.

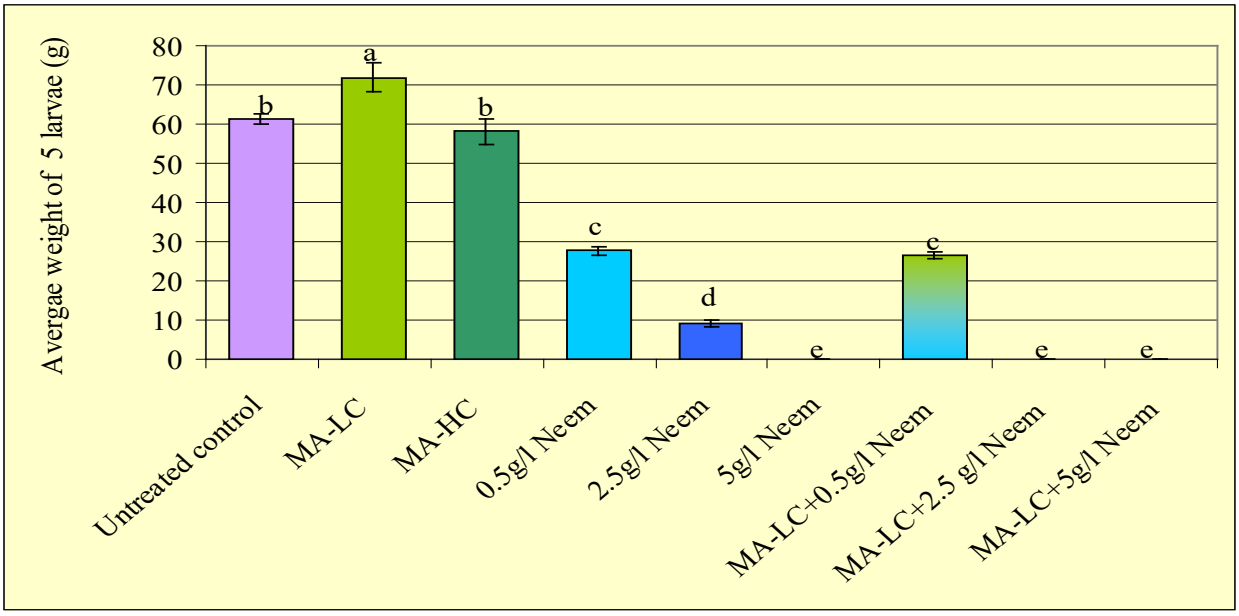

Fig.16. Average larval weight (group of five larvae) recovered from different treatments. Treatments and all other conditions are same as described in Fig.13.

Influence of neem seed cake or *M. anisopliae* on BVW oviposition

Neither neem seed cake nor *M. anisopliae* influenced adult BVW oviposition or feeding on *Euonymus* leaves as no significant difference between the treatments was observed compared with untreated control (Fig. 17).

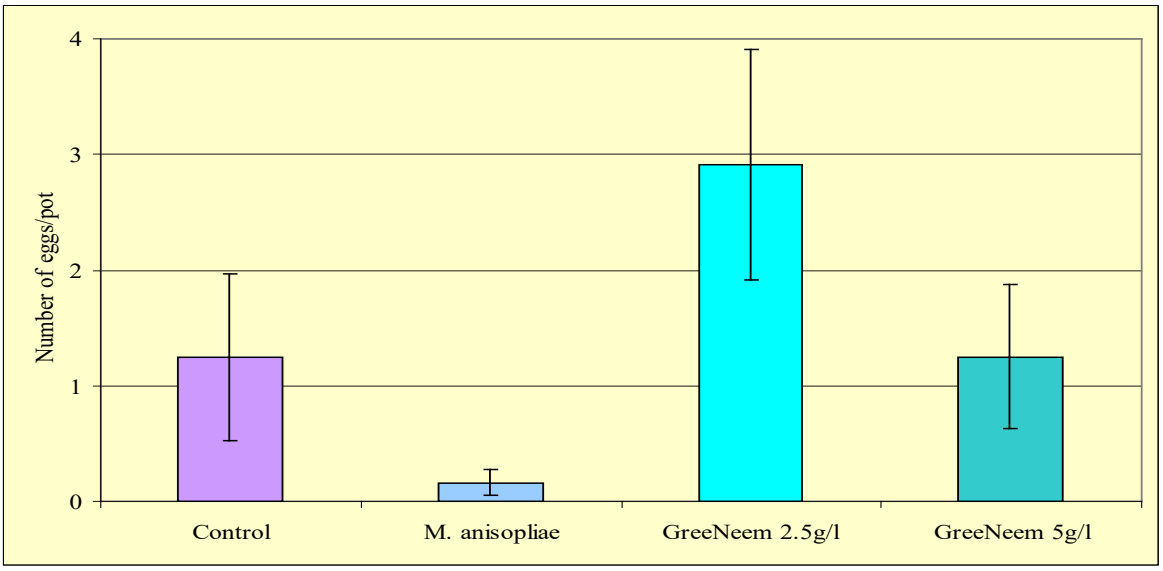

Fig.17. Average number of eggs recovered from each pot treated either with 2.5, 5g/l of neem seed cake or *M. anisopliae* (1 x 10^{10} conidia/l) of compost. Each treatment was replicated 12 times.

Discussion

This study clearly shows that neem seed cake enhances the efficacy of *M. anisopliae* for control of BVW larvae, probably through repellent or antifeedent properties. This is supported by the fact that larvae exposed to neem treatments had more spores adhering to their surface which could only be due to increased movement. Indeed, search for better feeding sites in response to neem application has been reported in other insects. For example, larvae of *Cosmopolites sordidus* (Germar) took longer to locate feeding sites, initiate feeding and bore into pseudostem discs treated with extract of powdered neem seed or kernel (Musabyimana *et al*., 2001). It is important to note that insect starvation and higher inoculum doses improve *M. anisopliae* efficacy (Amiri *et al.,* 1999; Butt, 2002), therefore, the increased movement and starvation associated with the neem treatment may have stressed the insects making them more susceptible to infection. Increasing the susceptibility of insects by exposing them to sublethal doses of synthetic insecticides is well documented (Shah *et al*., 2007; Roditakis *et al*., 2000; Quintela and McCoy *et al*., 1998), however, this is the first report of neem seed cake enhancing the efficacy of an insect pathogenic fungus.

It is possible that the lower body weight recorded for the BVW larvae was due to the antifeedant properties of neem which forced the larvae to seek better feeding sites. Neem products are known to reduce food intake (Ascher, 1993; Schmutter, 1990) and/or influenced physiological changes (Mordue and Blackwell, 1993). Starvation stress may increase their susceptibility to *M. anisopliae*. The fact that most BVW larvae remained in the 2nd instar may be due to the growth regulatory properties of neem (Mordue and Blackwell, 1993; Ascher, 1993). If so, it suggests that sufficient residues are present in the neem seed cake to influence larval development. Since early instar larvae are generally more susceptible to fungal infections this would explain why neem seed cake increased the efficacy of *M. anisopliae*.

Cowles (2004) noted that azadirachtin influenced the BVW fecundity and egg viability. Our studies show that neither neem seed cake nor *M. anisopliae* influenced adult BVW feeding and oviposition. Presumably, the azadirachtin residues in neem seed cake were insufficient to deter adults. Since neem products and by-products are considered safer than synthetic insecticides, they could be used to enhance the efficacy of entomopathogenic fungi as part of a chemical pesticide free pest control strategy (it should be noted that at present neem seed cake is not commercially available in UK, therefore, consultation with PSD would be required to determine its use as efficacy enhancing agent in UK). Furthermore, our studies clearly showed that when used in combination with neem seed cake, even 100 fold lower dose of *M.*

anisopliae could provide protection against BVW which would benefit the growers in reducing their costs for controlling media-borne pests like BVW larvae.

Task 2.1 cont'd: Pot bioassay to determine synergistic interactions between *M. anisopliae* and sub lethal doses of insecticides for the control of BVW and WFT in different plant growth media

B. Studies against Western Flower Thrips

Introduction

Western flower thrips is one of the world's major pests causing damage to a wide range of economically important crops directly through feeding and indirectly through the transmission of harmful plant virus diseases (van Lenteren *et al*., 1992; Kirk & Terry, 2003). Thrips are difficult to control because of their high reproductive rate, cryptic habit (larvae hide in closed buds and pupate in soil) and resistance to many insecticides (Jensen, 2000; Herron and James, 2005). In addition to chemical insecticides, a range of biological agents are available for thrips control including arthropod predators and parasitoids, and insect pathogenic nematodes and fungi (Jacobson *et al.,* 2001; Blaeser *et al*., 2004; Georgis *et al*., 2006; Xu *et al.,* 2006). Whereas most attention has focused on the control of adults and larvae in the crop canopy little effort has been made to interrupt the life cycle by controlling the pupae.

Earlier studies by Helyer *et al* (1995) showed that fungal BCAs applied to peat-based composts was effective in killing WFT pupae and helped reduce thrips populations. In light of the studies conducted against BVW, we initiated these studies with following objectives: i) to determine the efficacy of *M. anisopliae* against WFT pupae in disparate horticultural growth media, ii) to explore synergistic interactions between sub lethal doses of chemical insecticides (fipronil and imidacloprid) and *M. anisopliae* against WFT puape, iii) to compare *M. anisopliae* application method on its efficacy against WFT pupae.

Materials and Methods

Plant growth media, fungal inoculum and chemical insecticides were applied as described in studies against BVW. Details of WFT rearing and bioassay protocols are described below.

Source and maintenance of WFT

WFT were reared in ventilated plastic containers (29 cm ×29 cm ×16 cm) kept at 24 ± 2°C, 50-60% relative humidity (RH), and 16 L: 8D h photoperiod. Between 40-50 adult WFT were introduced into the containers and provided 3-4 pieces of green bean (*Phaseolus vulgaris* L.) and 2-3 yellow chrysanthemum flowers. After three days, the egg infested beans were transferred to fresh ventilated plastic containers (28 cm × 20 cm × 10 cm). First instar larvae usually started to hatch 2 days later. Three days post-eclosion second instar larvae (L2) were collected for experimental use.

Bioassays against WFT

Assays were done using 250 ml white opaque plastic pots (8 cm dia) obtained from Tesco, UK. Ventilation holes were made in the lids and subsequently sealed with thrips-proof nylon gauze. A 5cm × 4cm yellow/blue sticky trap (AgriSense, UK) was attached to the inner part of the lid to trap any emergent adult WFT. Approximately 125 ml of medium at field capacity was added to each pot. Each pot was inoculated with twenty L2 WFT and a small piece of bean provided as a source of food. The pots were sealed and kept at 24 ± 2°C, 50-60% RH, and 16:8 light and dark photoperiod. Four days after the introduction of the L2, adult WFT started to emerge. These either adhered to the sticky traps or were found on top of the compost. Adults were counted daily for 7 days until no more WFT were observed. Trapped adults on sticky card were incubated in Petri dishes lined with moist filter paper and examined in a binocular microscope to see if they were infected with *M. anisopliae*. Both the number of mycosed cadavers and any surviving emergent adults were recorded. Each treatment consisted of five replicates and the whole experiment repeated twice. Percentage efficacy data 11 days post-treatment were corrected for control mortality (Abbott's 1925), arcsine square root transformed, and analyzed using ANOVA and Duncan test for means separation (SPSS, 2003).

Results and Discussion

M. anisopliae V275 was far more efficacious in controlling WFT pupae than either fipronil or imidacloprid (Figs. 18-22). Pest mortality, whether using fungi or chemicals, is dose-related which suggests that thrips larvae acquired sufficient conidia of V275 to cause infection. Pupal mortality ranged between 72% and 91% with marginally more pupae being killed if the pathogen was premixed into the compost than if applied as a drench. Presumably, more conidia adhere to the larvae as they burrow in the compost to pupate. Our results corroborate the findings of other workers. For example, Heyler *et al* (1995) reported 75% control of WFT pupae in peat-based media treated with *M. anisopliae.* Brownbridge (1995) found that applying *M. anisopliae* as a soil drench reduced the glasshouse population of

WFT by approximately 72%. These observations show the importance of targetting the pupal as well as thrips larval and adult stages.

We show that of the few adults (ca. 10%) that emerged over 40% were infected and ultimately killed by V275, and subsequently covered with conidiophores and conidia (Fig. 23). The latter provide a source of fresh inoculum to infect larvae and adults in the crop canopy. In contrast, few pupae (20-51 %) were killed when exposed to fipronil and imidacloprid suggesting that these had low to moderate contact activity (Fig. 18-22).

There was no statistical difference between use of V275 alone or with sub lethal doses of insecticide suggesting that there was no additive or synergistic activity between these agents against WFT pupae (Fig. 18-22). Significant differences were observed between the overall treatments ($P < 0.001$) including the different potting media ($P < 0.001$); however, interactions between the treatments and potting media were not significant ($P > 0.26$). This suggests that *M. anisopliae* V275 is robust and can be used in both the conventional peat and new generation peat alternative and peat blend growing media. The studies also show that the WFT is a robust pest since adult emergence was high in all the untreated media ($P < 0.181$). Marginally more adult WFT emerged from bark (90.5 ± 3.9 %) than peat (84.0 ± 4.3%) and coir (83.0 ± 6.6%). Thrips emergence was lowest in peat media blended with either 10% (76.0 ± 6.6 %) or 20% (73.5 ± 4.5%) CGW.

Overall, our results show that premixing *M. anisopliae* into composts can be used as a part of an integrated pest management programme to control thrips populations in glasshouses. Premixing the pathogen into growing media is not only ergonomic for the growers but offers immediate control of thrips pupae.

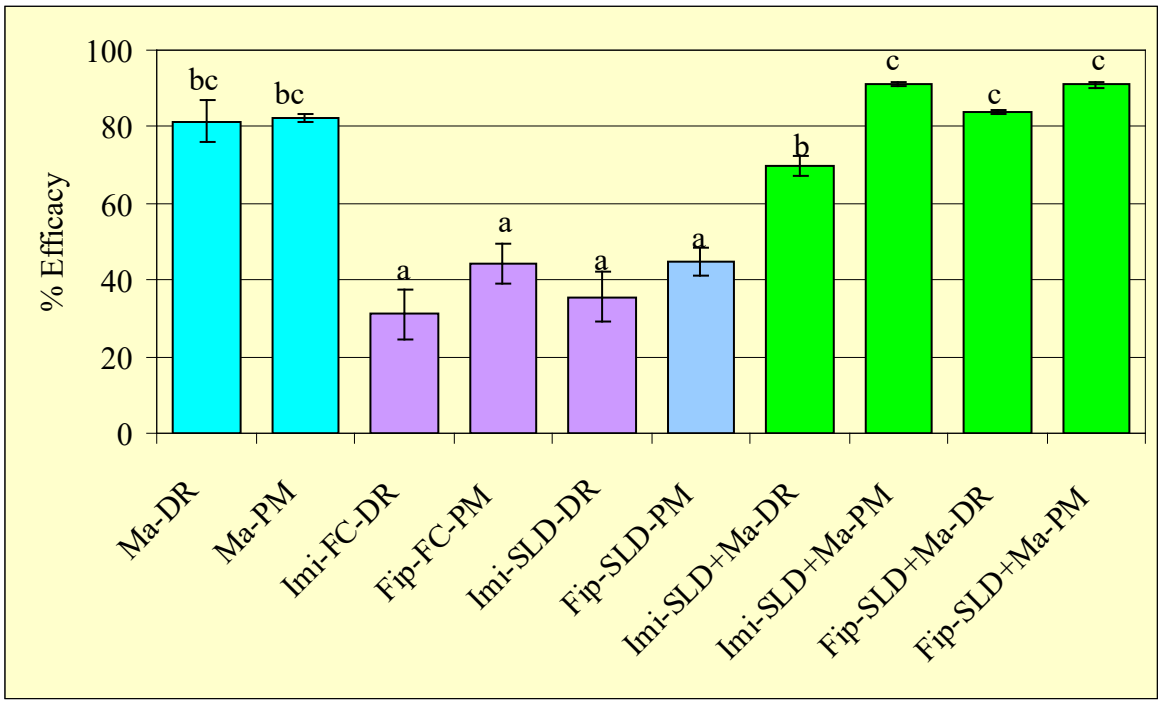

Fig. 18. Mortality (% ± SE) of WFT with *M. anisopliae* (1×10^{10} conidia/l compost) alone, sub lethal dose or recommended dose of imidacloprid or fipronil alone, or the combination of *M. anisopliae* and sub lethal dose of insecticides in 250-ml cups with different potting media. Ma-DR: *M. anisopliae* applied as drench, Ma-PM: *M. anisopliae* premixed, Imi-FC-DR: Imidacloprid applied as a drench at the recommended dose, Fip-FC-PM: Fiprinol premixed at the recommended dose, Imi-SLD-DR: Imidacloprid applied as a drench at the sub lethal dose, Fip-SLD-PM: Fiprinol premixed at the sub lethal dose, Imi-SLD+Ma-DR: Imidacloprid used at the sub lethal dose with *M. anisopliae* applied as a drench, Imi-SLD+Ma-PM: Imidacloprid used at the sub lethal dose with *M. anisopliae* premixed, Fip-SLD+Ma-DR: Fiprinol used at the sub lethal dose with *M. anisopliae* applied as a drench, Fip-SLD+Ma-PM: Fiprinol used at the sub lethal dose with *M. anisopliae* premixed. Means (± SE) with same letter (11 days after treatment) are not significantly different by Duncan test ($P < 0.05$).

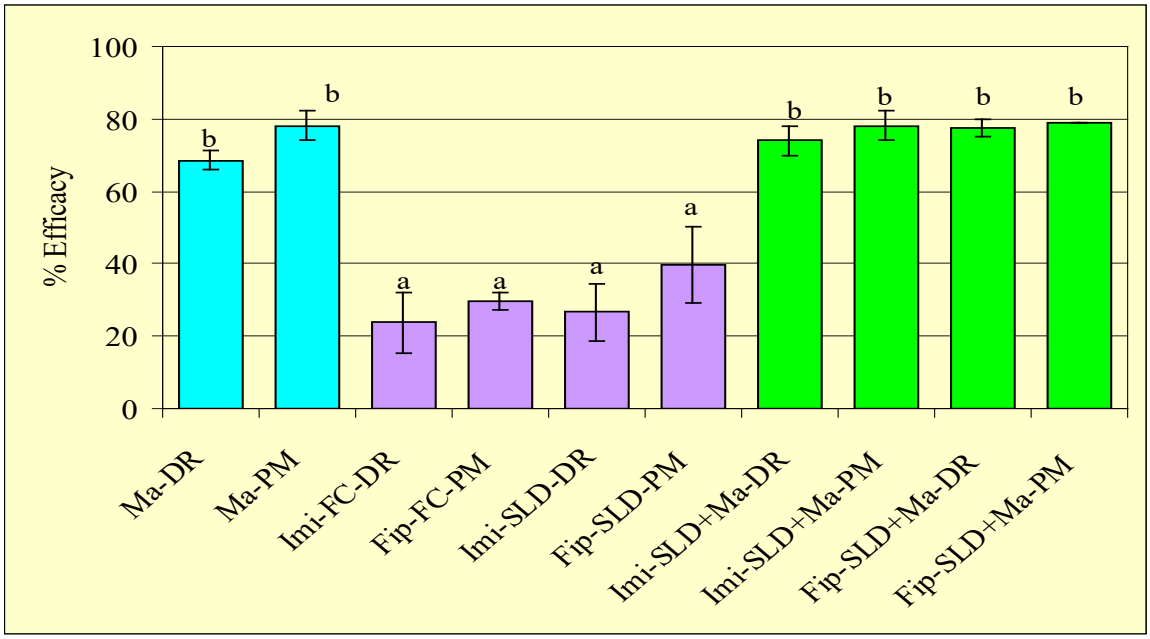

Fig. 19. Efficacy (%) of *M. anisopliae* and insecticides against WFT in bark based media
Treatments are same as described in Fig.18.

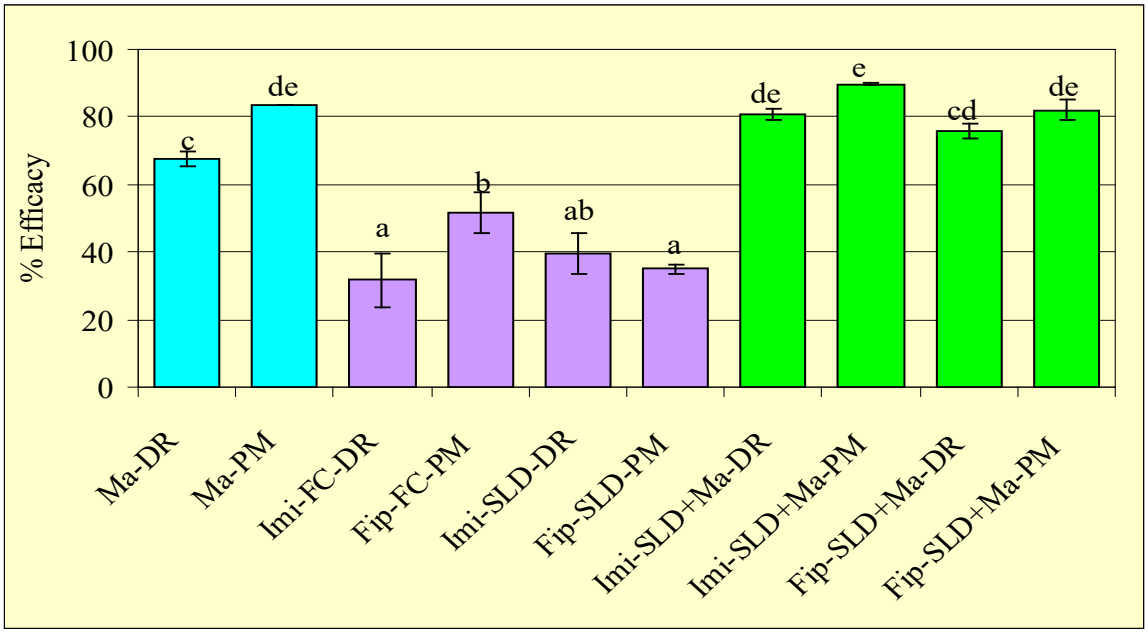

Fig. 20. Efficacy (%) of *M. anisopliae* and insecticides against WFT in coir based media
Treatments are same as described in Fig.18.

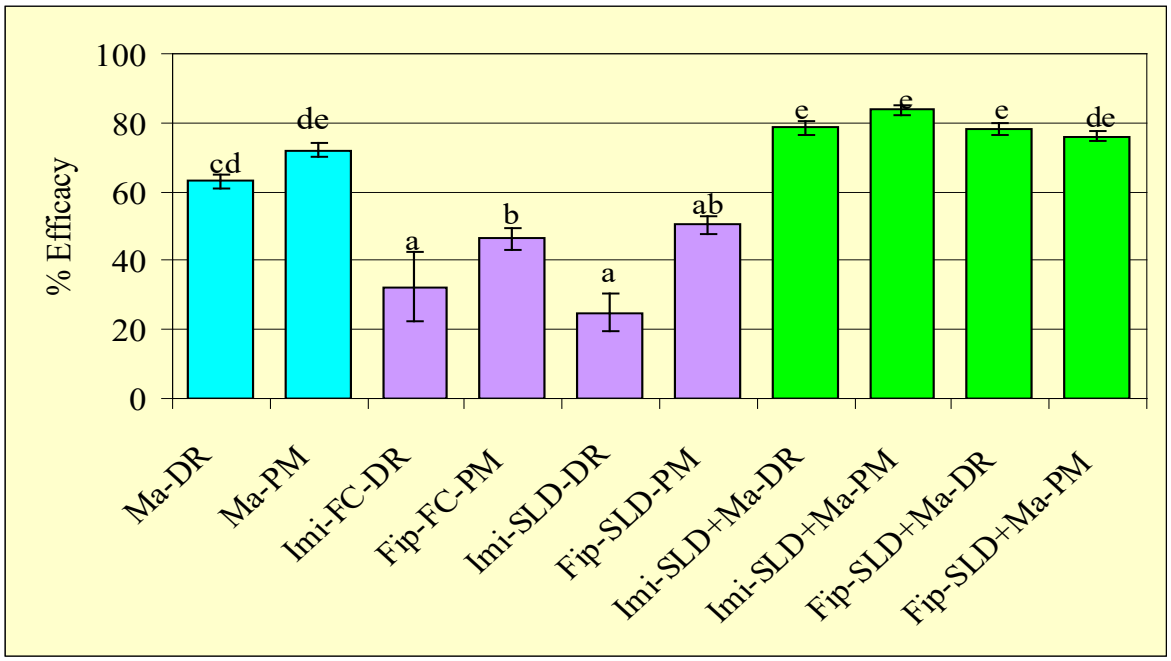

Fig 21. Efficacy (%) of *M. anisopliae* and insecticides against WFT in peat blended with 10% CGW. Treatments are same as described in Fig.18.

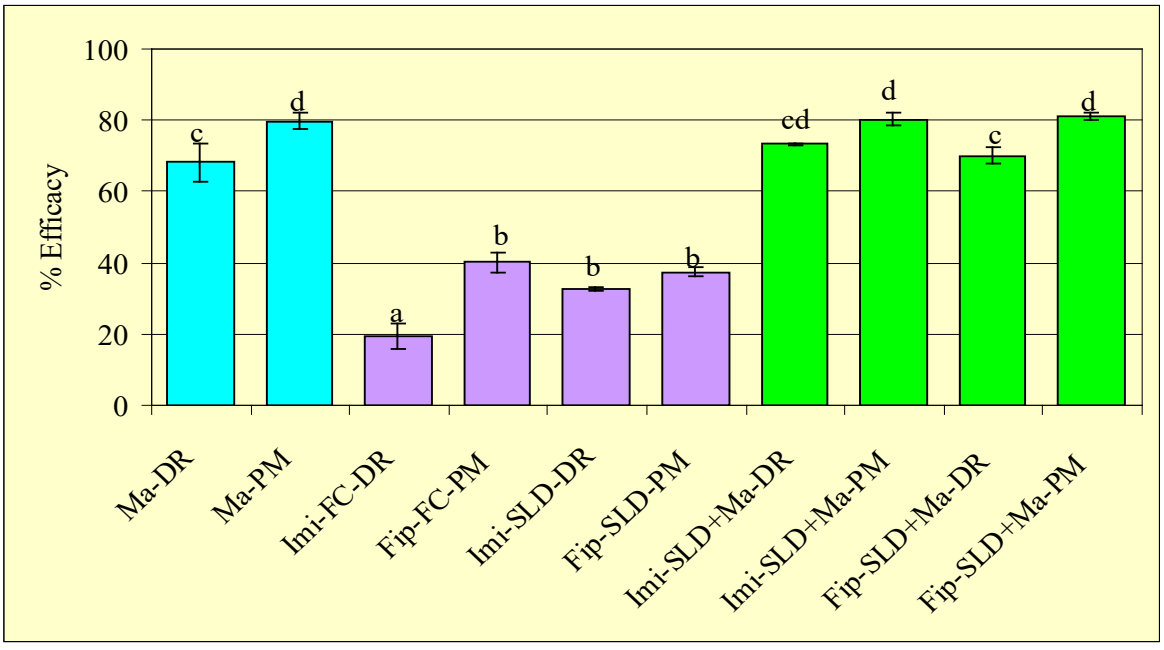

Fig 22. Efficacy (%) of *M. anisopliae* and insecticides against WFT in peat blended with 20% CGW. Treatments are same as described in Fig.18.

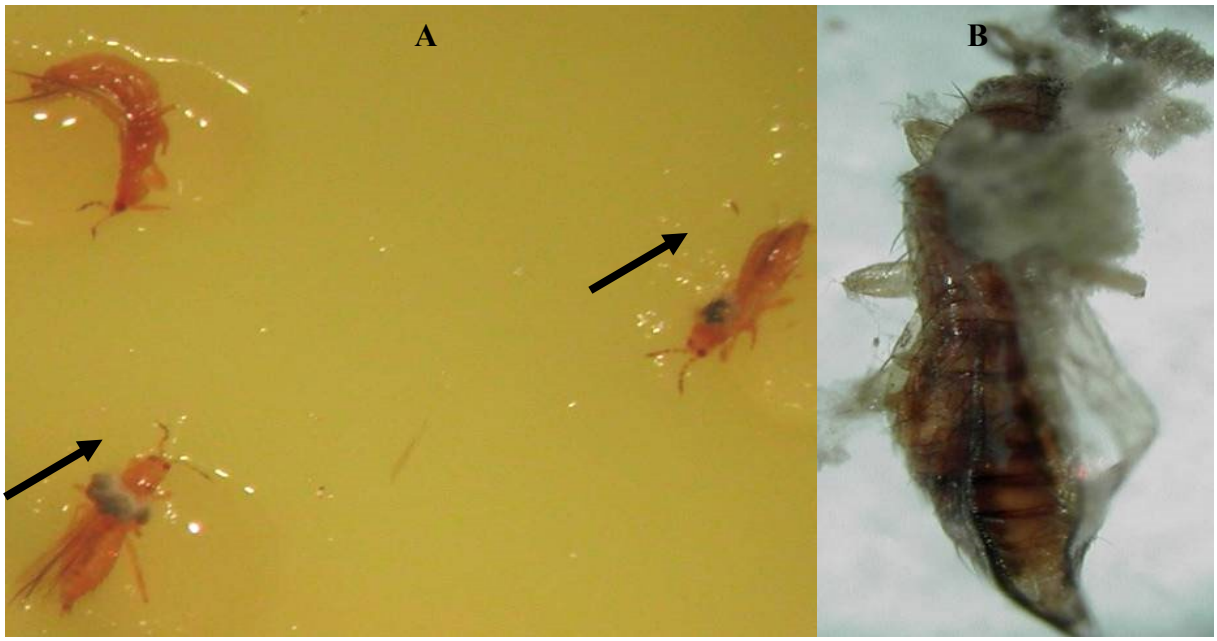

Fig. 23. (A) Adult WFT stuck to yellow sticky card including mycosed individuals (arrow). (B) Adult WFT infected with *M. anisopliae anisopliae*.

Objective 3: Determine compatibility of *M. anisopliae* with cold tolerant entomopathogenic nematodes

Cold tolerant entomopathogenic nematodes (CTEN) offer an alternative to low dose insecticides for use with *M. anisopliae* to control BVW larvae during the winter when the fungus is less active. Studies on the compatibility of *M. anisopliae* with CTEN would benefit growers in different ways. It gives growers choice – they can choose between the CTEN or the low dose insecticides to use with *M. anisopliae.* For soft fruit (e.g. raspberry, strawberry) growers this strategy would allow for continuous cropping since the absence of chemicals means that there would no harvest interval. Organic growers would have a fully integrated pest control strategy. Here we report for the first times that CTEN are not only compatible with *M. anisopliae* but also increase its efficacy against BVW.

Material and Methods

Nematodes and Fungal cultures

Two commercial products Entonem (*Steinernema. feltiae,* Koppert Biological Systems, The Netherlands) and Nemasys® L (*S. kraussei,* Becker Underwood, UK) and a UWS strain of *Heterorhabditis bacteriophora* was included in this study. All nematodes species were maintained in last instar *Galleria mellonella* according to Kaya and Stock (1997). *M. anisopliae* V 275 was used in all studies.

Insect, plants and growth media

Third-instars BVW were obtained from a colony maintained at the Swansea University. Rooted cuttings of *Euonymus fortune* “Emerald Gold” were kindly provided by Johnsons of Whixley, York, UK for greenhouse experiments whereas plant growth media was provided by Bord Na Mona, Ireland.

Laboratory experiments

General experimental details

Laboratory and greenhouse experiments were based on methods described by Ansari *et al*., (2004). Briefly, 3rd -instar BVW larvae individually transferred to 30-ml plastic cups (surface area: 15.9 cm²) containing 25 ml peat-based medium and a carrot slice. Conidia of *M. anisopliae* were premixed into dry compost to the appropriate concentration. The compost moisture was adjusted to ca. 45%. Each treatment was replicated three times with 10 individual BVW larvae per replicate. Experiments were conducted at 23 ± 1°C and the cups

were kept in trays lined with moist filter paper to reduce moisture loss. New carrot slice were added to cups with surviving BVW larvae as needed.

Determination of LC_{50} for nematodes

The LC_{50} of the nematodes against 3rd-instar BVW was determined in 30 ml cup as described above in which 0, 10, 30, 60 and 120 infective juveniles (IJs) of *H. bacteriophora*, *S. feltiae* or *S. kraussei* suspended in 500 µl distilled water were applied using a pipette onto the compost surface and larval mortality assessed 3, 6 and 9 days post application.

Determination of LC_{50} for *M. anisopliae*

The LC_{50} of *M. anisopliae* V275 against 3rd-instar BVW was determined by exposing the larvae to compost premixed with 1×10^7,10^8, 10^9, 10^{10} and 10^{11}conidia/l of compost. Larval mortality was assessed at weekly intervals for 3 weeks.

Determination of interaction between fungus and nematodes

In an earlier study with white grubs, synergism between *M. anisopliae* and nematodes had been observed if grubs had been exposed for at least 3 or 4 weeks to the *M. anisopliae* (Ansari *et al*., 2004). In this study we expected a similar interaction between *M. anisopliae* and *H. bacteriophora*, *S. feltiae* or *S. kraussei*. Therefore, in these experiments, BVW larvae were exposed to nematodes or fungus concentrations causing 50% mortality i.e. BVW larvae were exposed to *M. anisopliae* at 1.1×10^9 conidia/l compost, 12 IJs of *H. bacteriophora*, 37 IJs of *S. feltiae* or 22 IJs of *S. kraussei*/cup either applied simultaneously or nematodes applied 1 and 2 weeks after *M. anisopliae* application. Mortality was assessed weekly after fungus or nematode application for 3 weeks.

Reproduction of nematodes in BVW

To determine nematode yield, dead larvae from different treatments were placed on White traps (Kaya and Stock, 1997). The concentration of IJs was then determined by taking 1 ml sample (three times) and counting IJs under the microscope. Mortality due to *M. anisopliae* was confirmed by fungal emergence from cadavers.

Greenhouse experiment

Rooted cutting of *Euonymus* were transplanted in 0.5 l plastic pot filled with peat-based medium. Four 3rd instars BVW larvae/pot were placed around the base of the plant 4 h before start of an experiment. Larvae that had not entered into the compost within 2 h were replaced. The air temperature in the greenhouse averaged 18.5°C (range: 8.6- 28.4°C) and compost temperature in the pots at 5 cm depth averaged 16.5°C (range: 15-25°C).

Treatments consisted of two concentrations (1×10^8 conidia/l and 1×10^9 conidia/l compost) of *M. anisopliae*, two concentrations of *H. bacteriophora* (1 IJs/cm^2 = 1.0×10^8 IJs/ha and 2 IJs/cm^2 = 2.0×10^8 IJs/ha) and *S. feltiae* (3 IJs/cm^2 = 3.0×10^8 IJs and 6 IJs/cm^2 = 6.0×10^8 IJs/ha) and all combinations of both nematode species with *M. anisopliae* concentrations. Experiments started with the premixing of fungal conidia into dry compost to the appropriate concentration. The nematode suspensions were applied to the appropriate pots 2 weeks later. In all experiments, treatments were applied in 50 ml of tap water per nematode or fungal agent. In treatments with only one agent, an additional 50 ml of water was added; in the control 100 ml water was added. After treatment, pots were arranged in a randomized block design. There were 5 pots per treatment; pots were destructively assessed 1 week after nematode application and the number of surviving BVW was recorded.

Statistics analysis

LT_{50} data for BVW were analyzed using POLO-PC (LeOra Software, 1987). Before analysis all mortality data were corrected for control mortality (Abbott, 1925). Interaction between *M. anisopliae* and EPN (synergistic, additively, or antagonistic), if any, between nematodes and *M. anisopliae* using a procedure originally described by Finney (1964) and modified by McVay *et al*. (1977). Larval mortality was calculated by subtracting the number of surviving larvae from the number of larvae released for each replicate. The expected additive proportional mortality M_E for the nematode- *M. anisopliae* combinations was calculated by $M_E = M_N + M_M (1- M_N)$, where M_N and M_M are the observed proportional mortalities caused by nematodes and *M. anisopliae* alone, respectively. Results from a χ^2 test, $\chi^2 = (M_{NM}- M_E)^2/M_E$, where M_{NM} is the observed mortality for the nematode- *M. anisopliae* combination, were compared to the χ^2 table value for 1 degree of freedom. If the calculated χ^2 values exceeded the table value, there would be reason to suspect a non-additive effect, i.e. synergistic/antagonistic, between the two agents (Finney, 1964). If the differences M_{MN}-M_E = *D* had a positive value, a significant interaction was then considered synergistic, and if *D* had a negative value, a significant interaction was considered antagonistic.

In the greenhouse experiment, the effect of the treatments on BVW was analyzed using ANOVA followed by Tukey's test (SPSS, 2003). The type of interaction between nematodes and *M. anisopliae* was determined using the procedure described above. Differences between means in all experiments were considered significant at $P < 0.05$.

Results

Laboratory experiments

LC_{50} for nematodes

BVW larval susceptibility towards different nematodes species was significantly different. (P < 0. 003). The LC_{50} values calculated after 9 days of *H. bacteriophora* strain UWS1 was significantly lower compared to *S. feltiae* or *S. kraussei* (Table 5).

Table 5. Lethal concentration (LC_{50}) values of 3rd instar black vine weevil at different concentration of *H. bacteriophora*, *S. feltiae* and *S. kraussei* 9 days post exposure at 23 ± 1°C.

Treatment	LC_{50} (95% CL)*	Slope ± SE	χ^2	df
H. bacteriophora	12 (2.2-20.6) [a]	1.73 ± 0.33	10.9	6
S. feltiae	37 (21-61.76) [c]	1.55 ± 0.29	8.05	6
S. kraussei	22 (7.5-39.1) [b]	1.75 ± 0.30	13.75	6

* Concentrations are expressed in IJs/larva

LC_{50} for fungus

The LC_{50} (95% confidential limits) values of BVW for *M. anisopliae* V275 was 1.1×10^9 (6.3×10^8 to 6.5×10^9) conidia/l of compost after 3 week of exposure.

Determination of the interaction between *M. anisopliae* and nematodes

The interactions between the EPN and *M. anisopliae* were mostly synergistic but in a few cases they were additive (Table 6). The synergy between the EPN *H. bacteriophora* and *S. feltiae* with *M. anisopliae* was independent of application time but the degree of synergy increased significantly when nematodes were applied 2 weeks after the fungus. Interaction between *S. kraussei* and *M. anisopliae* differed significantly on application time. Synergistic effect was observed when *S. kraussei* and *M. anisopliae* were applied simultaneously (χ^2 = 14.9; df = 1; P < 0.004), but additivity was observed when nematodes were applied 1 week (χ^2 = 3.3; df = 1; P > 0.069) or 2 weeks (χ^2 = 2.9; df = 1; P > 0.088) after fungal application (Table 6).

Table 6. Interactions between EPN and *M. anisopliae*[a] when used against 3rd -instar BVW in 30 ml cup with peat based medium.

Nematode[b] species	Application rate IJs/cup	Intervals week[c]	Observed mortality[d]	Expected mortality[e]	χ^2 Interactions[e]
Hb	12	0	86.7 ± 8.8[ab]	66.0	7.0 Synergistic
Hb	12	1	93.3 ± 3.3[ab]	60.0	19.7 Synergistic
Hb	12	2	100 ± 0[b]	53.0	54.3 Synergistic
Sf	37	0	80.0 ± 15.3[ab]	66.0	8.2 Synergistic
Sf	37	1	93.3 ± 3.3[ab]	57.0	24.0 Synergistic
Sf	37	2	100 ± 0[b]	58.0	29.0 Synergistic
Sk	25	0	100 ± 0[b]	68.3	14.9 Synergistic
Sk	25	1	63.3 ± 8.8[a]	59.3	3.3 Additive
Sk	25	2	66.2 ± 8.8[ab]	61.3	2.9 Additive

[a] *M. anisopliae* was premixed at 1.1×10^9 conidia/l peat medium.

[b] Hb, *H. bacteriophora*; Sf, .*S. feltiae*; Sk, *S. kraussei*

[c] Interval between application of *M. anisopliae* and entomopathogenic nematodes.

[d] Observed mortality (%) in average of three replicates of 10 BVW larvae (n= 30). Pots were destructively sampled 1 or 2 week after application of nematodes. Mortality means (± SE) within column followed by the same letters are not significantly different (Tukey test).

[e] Expected mortality $M_E = M_N + M_M (1 - M_N)$, where M_N and M_M are the observed proportional mortalities caused by nematodes and *M. anisopliae* alone

f Interaction was based on χ^2 ratio of expected: observed mortality

Reproduction of nematodes in BVW

The production of *H. bacteriophora* IJs differed significantly among treatments ($P < 0.001$) (Table 7). No significant difference in IJs production was observed whether *H. bacteriophora* was applied simultaneously with *M. anisopliae* or 1 week post fungal application. Production of IJs declined significantly upon *H. bacteriophora* application 2 weeks after *M. anisopliae* (Table 7). Larvae killed by *S. feltiae* or *S. kraussei- M. anisopliae* combination produced nematodes ranged between (20,000-30,000 IJs/larva). Production of IJs was not significantly different compared with nematodes alone but significantly reduced when the nematodes were applied 2 weeks after fungus (data not shown).

Table 7. Mean number of nematodes produced when exposed to *H. bacteriophora* alone and *H. bacteriophora* plus *M. anisopliae* V275.

Treatmen	Mean no. nematodeproduced per larva[a]
Control	0.0± 0.0[a]
Hb (12IJs/cup) applied alone	29642 ± 4643[c]
Hb + *Ma* (12IJs + 1.1 × 10^6) applied simultaneously	26167 ± 3193[bc]
Hb + *Ma* (12IJs + 1.1 × 10^6) *Hb* applied 1 weeks after *Ma* application	30640 ± 1477[c]
Hb + *Ma* (12IJs + 1.1 × 10^6) *Hb* applied 2 weeks after *Ma* application	16833 ± 600[b]

[a]Nematode production data are means of 3 replications of 10 BVW larvae (n = 30). Experiment was done in 30-ml cup with peat-based medium and both agents applied alone or in combinations. Mean ± SEM followed by the same letter within a column are not significantly different ($P < 0.05$ Tukey test).

Greenhouse experiments

Individual application of either *M. anisopliae* or nematodes provided limited protection against BVW (Table 8). BVW mortality was significantly ($P < 0.001$) higher when nematodes were applied 2 weeks after *M. anisopliae* application. Irrespective of dose, *M. anisopliae* and *H. bacteriophora* interacted synergistically ($P < 0.001$, *D*: 32 to 36%) against 3rd instar BVW. Similarly, interaction between *S. feltiae* and *M. anisopliae* was also synergistic at all combinations of nematode and fungus ($P < 0.001$, *D*: 9 to 17.5%) but the degree of interaction was low as compared to *H. bacteriophora* (Table 8).

Table 8 Mortality (± SE) of 3rd -instar BVW after treatment with two doses of *M. anisopliae* (1×10^8 and 10^9/l compost) and two doses of *Heterorhabditis bacteriophora* (1 and 2 IJs/cm^2) or *Steinernema feltiae* (3 and 6 IJs/cm^2) alone or the combination of both agents in peat-based growing media.

Treatments	Observed[b] mortality	Expected[c] mortality	χ^2	Interactions[d]
Untreated control	12.5 ± 5.6[a]	-	-	-
Ma (1×10^8/l peat)	40.0 ± 4.1[abc]	-	-	-
Ma (1×10^9l peat)	50.0 ± 10.5[bcd]	-	-	-
Hb (1 IJs/cm^2)	40.0 ± 8.5[abc]	-	-	-
Hb (2 IJs/cm^2)	37.5 ± 6.7[abc]	-	-	-
Ma (1×10^8/l peat) + Hb (1 IJs/cm^2)[a]	100 ± 0[f]	63.8 ± 6.8	53.6	Synergistic
Ma (1×10^8/l peat) + Hb (2 IJs/cm^2)[a]	97.5 ± 2.5[f]	63.1 ± 4.3	28.3	Synergistic
Ma (1×10^9/l peat) + Hb (1 IJs/cm^2)[a]	100 ± 0.0[f]	69.4 ± 7.9	40.4	Synergistic
Ma (1×10^9/l peat) + Hb (2 IJs/cm^2)[a]	100 ± 0.0[f]	68.1 ± 7.2	38.9	Synergistic
Sf (3 IJs/cm^2)	30.0 ± 6.2[ab]	-	-	-
Sf (6 IJs/cm^2)	35.0 ± 6.7[abc]	-	-	-
Ma (1×10^8/l peat) + Sf (3 IJs/cm^2)[a]	62.5 ± 9.3[cde]	57.5 ±3.6	12.0	Synergistic
Ma (1×10^8/l peat) + Sf (6 IJs/cm^2)[a]	72.5 ± 8.7[def]	58.8 ± 4.5	14.9	Synergistic
Ma (1×10^9/l peat) + Sf (3 IJs/cm^2)[a]	85.0 ± 5.5[ef]	67.5 ± 11.6	71.9	Synergistic
Ma (1×10^9/l peat) + Sf (6 IJs/cm^2)[a]	77.5 ± 4.5[def]	68.8 ± 10.7	31.5	Synergistic

Ma= *Metarhizium anisopliae*; Hb = *Heterorhabditis bacteriophora* Sf = *Steinernema feltiae*

[a] Nematodes were applied in combination treatments 2 weeks after *M. anisopliae* application.

[b] Observed mortality (%) in average of five pots of 4 BVW larvae (n= 20). Pots were destructively sampled 1 week after application of nematodes. Mortality means (± SE) within column followed by the same letters are not significantly different (Tukey test).

[c] Expected mortality $M_E = M_N + M_M (1 - M_N)$, where M_N and M_M are the observed proportional mortalities caused by nematodes and *M. anisopliae* alone.

[d] Interaction was based on χ^2 ratio of expected: observed mortality.

Discussion

This study clearly shows that EPN and *M. anisopliae* work synergistically in controlling BVW but the degree of synergy varies with nematode species and/or timing of nematode application. These results corroborate earlier studies which showed additive or synergistic interactions between *M. anisopliae* and EPN for control of white grub larvae (Ansari *et al*., 2004, 2006).

Prior exposure of BVW to *M. anisopliae* for 2 weeks retarded nematode reproduction whereas simultaneous or 1 week exposure had no effect. Ansari *et al*. (2004) made similar observations when targeting white grubs which suggests that *M. anisopliae* alters the internal environment of its host making it less conducive for EPN reproduction. Since both *M. anisopliae* and nematodes interacted synergistically even at lower inoculum doses, provides an opportunity to reduce the cost of BVW control. Our study shows that BVW can be effectively controlled by using a combination of 4 - 10 times lower fungus (1×10^{13} conidia/ha) and 25 times lower nematode rates (1×10^{8}IJs/ha).

Objective 4: Elucidate the impact of physical properties of media on conidial leaching

Introduction

Insect-mortality is dose-related; therefore, inoculum rapidly leached from growing media will result in poor pest control. Conversely, media that retain inoculum will give better pest control. At present the interactions between conidia of *M. anisopliae* and the substrate are poorly understood. Furthermore, extrapolation from field soil studies may not apply to horticultural growing media. By elucidating which physical aspects of growing media affect leaching it may be possible to devise better formulations of the fungus and design media that improve pest control.

Materials and Methods

Percolation of inoculum through disparate growth media was evaluated using leaching columns. Each leaching column was filled with one litre of growth medium, and conidia of *M. anisopliae* applied as a drench or premixed into the compost. The growth medium was subsequently flushed with one litre of water at 0 hr, 1 hr, 24 hrs and then daily up to 7 days post inoculation. The columns were flushed weekly for another 4 weeks. After each flushing, leachates from media were collected in a beaker and samples were taken to determine the number of conidia using a haemocytometer. Each sample was fixed with 2% formaldehyde and stored at 4ºC until evaluated. Each treatment was replicated and the whole experiment repeated twice.

Results and Discussion

Both the application method and growth medium influenced conidial leaching. Drench applications resulted in greater (95%) leaching from all media (Fig. 24). Relatively more conidia were leached from bark and coir media as compared to peat and peat blend media. Leaching of conidia if premixed was less than 15% irrespective of growth medium but was more pronounced in peat than the other media (Fig. 24). Over 90% of the conidia, following drench application had been leached from the media at 0 and 1 hr after flushing (Fig. 25). Premixed inoculum leaching varied non significantly till 2 days post application and accounted for up to 80-90% of total loss (Fig. 26). These observations suggest that immediately after potting that channels exist for rapid loss of conidia applied as a drench. Subsequent watering of medium results in compaction of the medium and less conidia being lost from the medium. In contrast, premixing appears to facilitate better entrapment or adhesion of spores to the substrate since very little is lost irrespective of the media type.

None of the conidia collected had germinated suggesting that plant growth media do not stimulate germination of *M. anisopliae* conidia or conversely the media contain factors that inhibit germination.

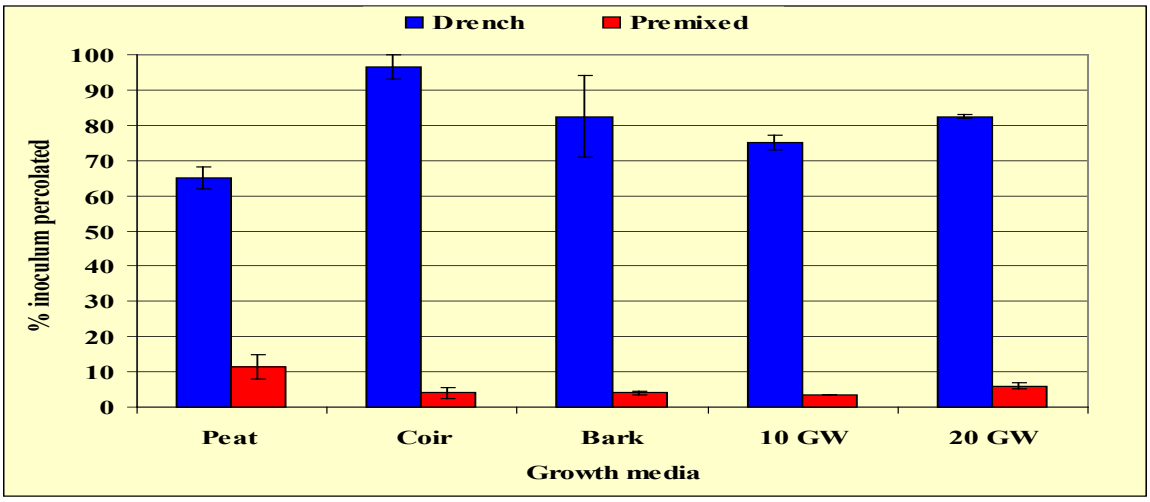

Fig. 24. Influence of application method on the percolation (total) of *M. anisopliae* inoculum from different plant growth media

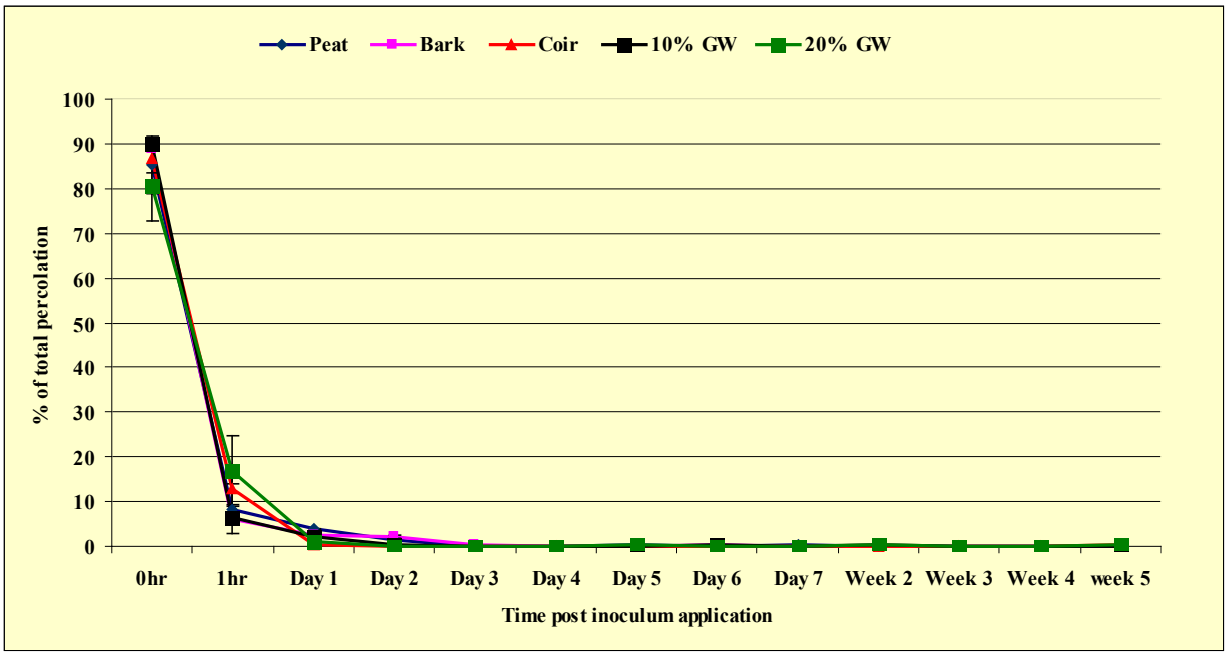

Fig. 25. Time course analysis of the inoculum percolation applied as drench. Note more than 90% of the total inoculum loss occurs at the time of application.

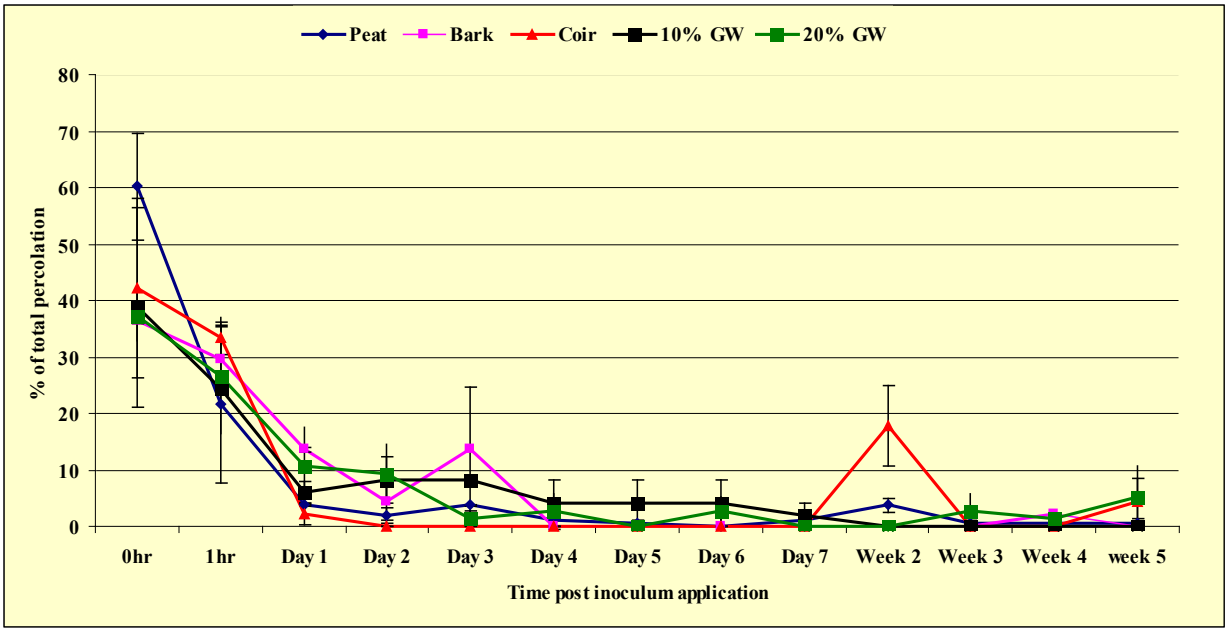

Fig. 26. Time course analysis of the inoculum percolation when applied as premixed. Note up to 90% of the total inoculum loss occurs during the first 2-3 days.

Objective 5: Elucidate the impact of chemical properties of media on the infectivity of *M. anisopliae* conidia

Introduction

Humic-fulvic substances are ubiquitous, heterogeneous macromolecules derived from biological, chemical and physical degradation of organic matter. They play an important role in plant nutrition and detoxification and will influence the microbial composition of growing media (Hoitink and Boehm, 1999; Ashley, 2002). The quantity of fulvic and humic substances vary with the different media. At present, there is no information on their influence on the efficacy of insect-pathogenic fungi. Many other compounds are present in the media such as tannins (oligomeric and polymeric phenolics) and terpenoids but detailed evaluation of these substances would be outside the scope of this project. In addition to humic-fulvic substances, additional studies were conducted to see whether leachates from different plant growth media influence *M. anisopliae* germination and virulence.

Task 5.1. Effect of humic/fulvic substances on *M. anisopliae* germination

Materials and Methods

Preparation of growth media lactates

Humic-fulvic substances were provided by Bord Na Mona, Ireland. Leachates were prepared by suspending 400 ml of plant growth medium in 800 ml of distilled water for 1 hour at room temperature. The suspension was blended in a laboratory shaker for 30 sec. and filtered through a double layer of cheese cloth. The filtrate was then divided into two parts with one part being filtered through a 0.2µm Millipore filter to exclude microorganisms.

M. anisopliae incubation in leachates and humic/fulvic acids

M. anisopliae conidia were suspended in leachates from the different plant growth media or generic humic-fulvic substances at a final concentration of $1x10^7$ conidia/ml. Conidia were also suspended in 2, 4 and 20 fold dilutions of humic-fulvic substances. Conidia suspended in 0.03% aq. Tween 80 (Fisher Scientific, UK) were used as a control. Samples were incubated at 25ºC (LEEC incubator) for 24 hrs. Each treatment was replicated three times and the whole experiment was repeated twice.

Germination assays

Influence of humic-fulvic substance on *M. anisopliae* germination was assessed in sterile 24 well microtire plates (Nunc™). Each well contained 500 µl of Sabouaraud dextrose broth and was inoculated with 500 µl of the conidial suspension from the above treatments. Plates were incubated in a Gallen-Kamp orbital incubator (Sanyo) at 25ºC and 70 rpm. At 8 and 24 hpi, samples were fixed with 2% formaldehyde and germination assessed at 40x magnification of light microscope (Leitz WETZLAR, Germany). For each replicate sample, three fields of 100 conidia/field were randomly observed. Conidia exhibiting germ tube equal or greater than conidial width was regarded as germinated. Each treatment was replicated three times and the whole experiment was repeated twice.

Task 5.2 Bioassays - influence of humic-fulvic substances on virulence

The influence of humic-fulvic substances and leachates on *M. anisopliae* virulence was determined in peat based media. *M. anisopliae* was applied with the solutions of humic-fulvic substances or leachates at 1 x 10^7 conidia/ml of peat. Controls consisted of water with and without the conidia. The treated and control peat was added to 250 ml pots and five mealworm (*Tenebrio molitor*) larvae were transferred to each vial. Each treatment was replicated three times. Mortality was recorded daily over a period of seven days.

Results and Discussion

Germination varied significantly at 8 hpi in Sabouraud broth, however, at 24 hpi no significant difference was observed. At 8 hpi germination was less than 5% in conidia incubated with different dilutions of generic humic-fulvic substances (Table 9). Conidia incubated in leachates (un-filtered) from peat and bark also germinated relatively slow (35-40%). Conidial germination varied non-significantly among conidia incubated in the remaining treatments (Table 9).

M. anisopliae was significantly less virulent when applied with full, ½ and ¼ dilutions of humic-fulvic substances (Table 9). No significant difference in virulence was observed in the rest of the treatments. These findings suggest that only very high concentrations of humic-fulvic substances can influence *M. anisopliae* efficacy but such levels are unlikely to be encountered in growing media or field conditions.

Table 9. Effect of humic/fulvic substances and plant growth media leachates on *M. anisopliae* germination and virulence.

Treatments	Germination (%)	LT_{50} (Days post inoculation)
Untreated – Conidia incubated in water	65.5 ± 5^{def}	2.7^{a}
Conidia incubated in leachate (filtered through 0.2 µm filter) from peat	64.7 ± 2.5^{def}	-
Conidia incubated in leachate (filtered through 0.2 µm filter) from bark	64.0 ± 1.0^{def}	-
Conidia incubated in leachate (filtered through 0.2 µm filter) from coir	74.2 ± 3.0^{f}	-
Conidia incubated in leachate (filtered through 0.2 µm filter) from 10% green waste blend	68.0 ± 4.0^{ef}	-
Conidia incubated in leachate (filtered through 0.2 µm filter) from 20% green waste blend	73.5 ± 4.8^{f}	-
Conidia incubated in leachate from peat	41.7 ± 4.2^{bc}	2.4^{a}
Conidia incubated in leachate from bark	34.0 ± 3.3^{b}	2.6^{a}
Conidia incubated in leachate from coir	70.5 ± 6.7^{f}	2.6^{a}
Conidia incubated in leachate from 10% green waste blend	53.0 ± 3.9^{cde}	3.2^{a}
Conidia incubated in leachate from 20% green waste blend	49.7 ± 2.5^{bcd}	2.2^{a}
Generic humic/fulvic substances-full concentration	0 ± 0^{a}	18.2^{d}
Generic humic/fulvic substances-1/2 dilution of full concentration	0 ± 0^{a}	9.0^{c}
Generic humic/fulvic substances- ¼ dilution of full concentration	0 ± 0^{a}	4.5^{b}
Generic humic/fulvic substances-1/20 dilution of full concentration	4.2 ± 1.4^{a}	3.1^{a}

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 of the media as described below.

1. Plant growth media alone
2. Plant growth media + *Euonymus fortunei* 'Emerald Gold'
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, 6 and 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) studies (Anderson and Domsch 1978). 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 hrs 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 lab technologies).

Results

Determination of microbial biomass and activity in different plant growth media

Irrespective of plant growth media microbial biomass increased over time (Table 10). There was no significant difference in microbial biomass of 0 and 1 month samples; however, these varied significantly with those of 6 and 12 month samples. There was a significant increase in biomass from 1 to 6 month; however, months 7-12 had no significant increase in biomass (Table 10). In general, at any particular sampling time, neither plant growth media nor the treatments had any significant influence on the microbial biomass (Table 10). The only anomaly was that of significantly higher microbial biomass in coir samples as compare to that of all other growing media. The FDA test showed high levels of microbial activity in all the samples, however, statistical analysis showed no significant difference in samples.

Table 10. Microbial biomass in different growth media (with or without *M. anisopliae* and/or plant) at different time period

Treatments	Biomass C (µg C/g dry weight)			
	0 month	1 month	6 month	12 months
Peat	64[a]	90.75[a]	674.8[b]	864.95[bc]
Peat + MA	87[a]	131.9[a]	811.2[bc]	737.05[bc]
Peat + P		241.6[a]	897.7[bc]	982.85[bc]
Peat + MA + P		245.75[a]	682.3[b]	1083.5[c]
Bark	82[a]	121.25[ab]	740.85[c]	1027.6[de]
Bark + MA	113[ab]	172.35[ab]	661.9[c]	692.9[c]
Bark + P		363.3[b]	781.9[cd]	1154.3[e]
Bark + MA + P		325.5[ab]	1033.6[de]	1009[de]
Coir	188[a]	-*	1429.2[bc]	2254.6[d]
Coir + MA	216[a]	-	1678.1[cd]	1045.3[b]
Coir + P		-	1491.1[bc]	1766.5[cd]
Coir + MA + P		-	1080.6[b]	998.35[b]
10 % CGW	72[a]	193.3[a]	1269.1[e]	869.6[bcd]
10 % CGW + MA	106[a]	221.65[a]	617.5[b]	823.25[bc]
10 % CGW + P		275.55[a]	1085.3[cde]	1159.9[de]
10 % CGW + MA + P		283.3[a]	1223.3[e]	999.9[cde]
20 % CGW	79[a]	146.7[a]	890.8[bc]	926.75[bc]
20 % CGW + MA	92[a]	156.1[a]	747.15[b]	678.5[b]
20 % CGW + P		226.7[a]	997.55[bc]	936.65[bc]
20 % CGW + MA + P		223.25[a]	1200.7[c]	705.6[b]

* Samples exhibited high intensity background signal, therefore an accurate measurement couldn't be recorded. MA= *M. anisopliae*; P= Plant; CGW= composted green waste

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 i.e. Community level physiological profiling (CLPP) and automated ribosomal intergenic spacer analysis (ARISA)

A. Community level physiological profiling (CLPP)

Introduction

This method is based on the microbe's ability to utilise different carbon substrates. The substrate utilisation approach, using the Biolog Micro Plate 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; 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 hrs 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

Comparison of all treatments within any one plant growth medium (0, 1, 6 and 12 month's samples with or without *M. anisopliae* and/or plant) showed a stable utilisation of 31 carbon substrates in the Biolog kit. In most cases, no significant difference in clustering was observed among the treatments and time of sampling, however, comparison of 0-12 months samples showed distinct clustering at each time suggesting an overall shift in the microbial profile. All the media had similar carbon substrate utilisation profiles at 0 and 1 months (Figs. 27- 28). However, at 6 months 3 distinct groups were distinguished: group1 consisting of bark and peat blends with 10% and 20% CGW and group 2 consisting of peat and coir (Fig. 29). At 12 months sampling (Fig. 30), PCA showed three distinct groups i.e. peat (group1), bark (group 2) and peat blends and coir (group 3).

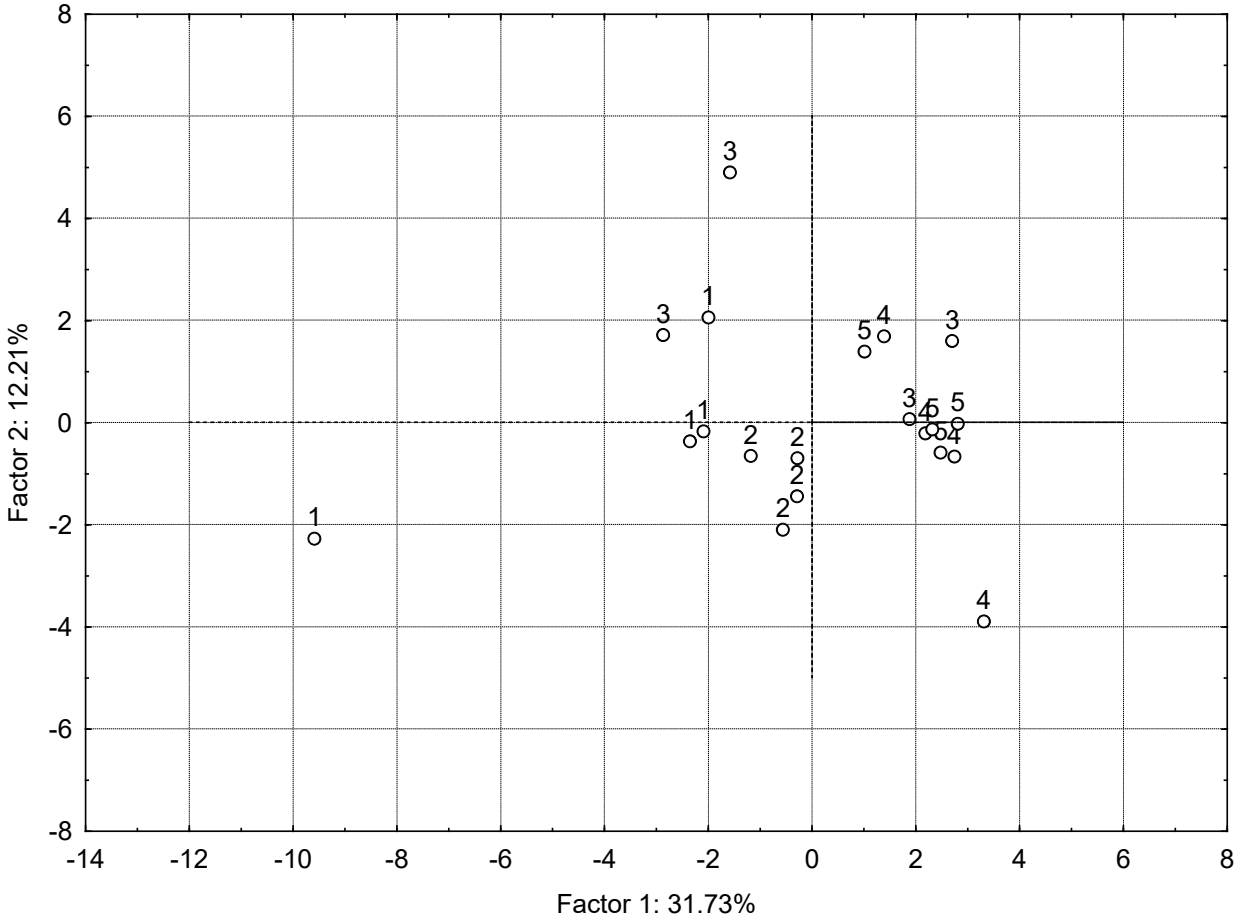

Fig. 27 PCA scaling plot of 0-month untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph correspond to following treatments:1-Peat blend with 10 % CGW, 2-Peat blend with 20 % CGW, 3- Bark,4-Peat, 5 – Coir. Note all media are clustered in one group and individual replicates of one media overlap with that of other media.

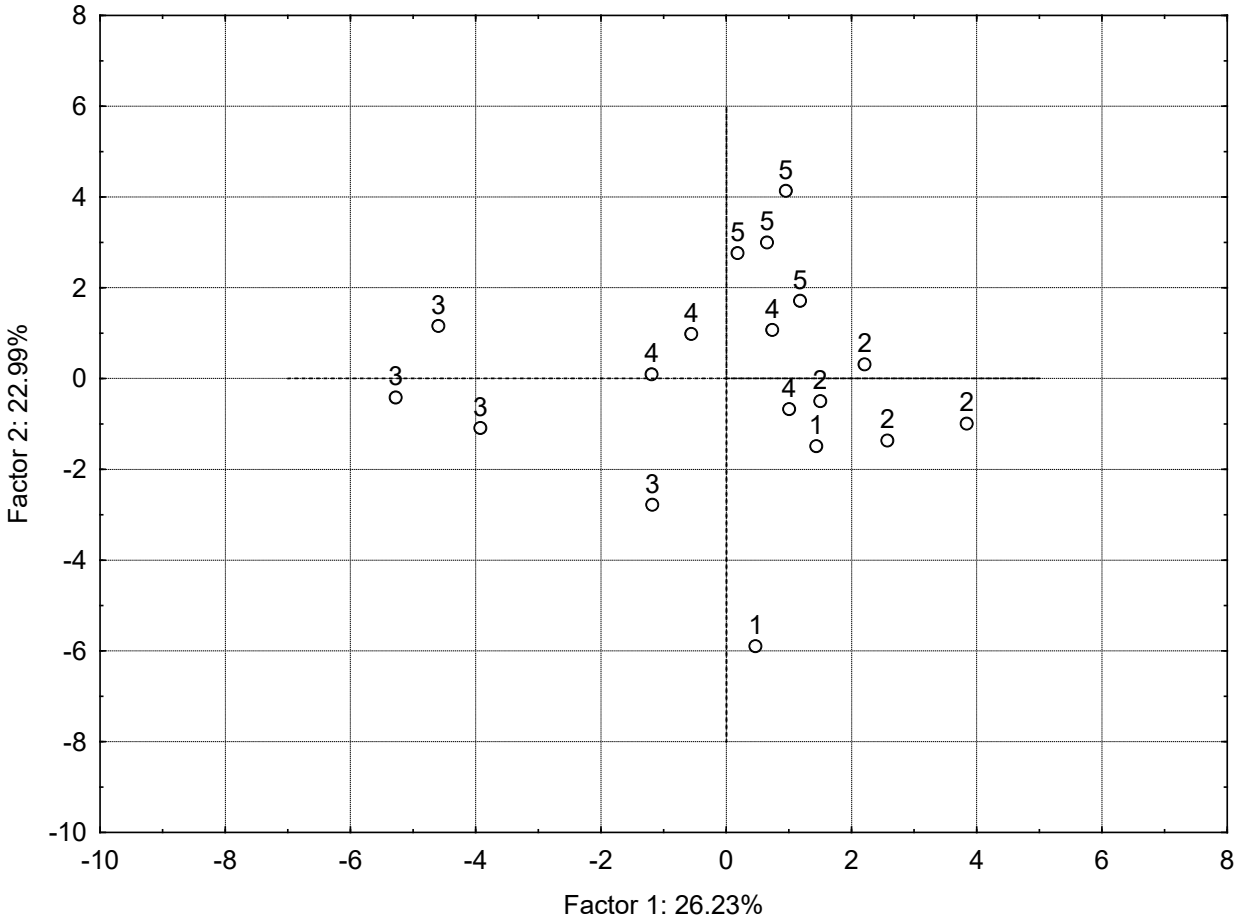

Fig. 28 PCA scaling plot of 1-month untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph correspond to following treatments: 1-Peat blend with 10 % CGW, 2-Peat blend with 20 % CGW, 3- Bark,4-Peat, 5 – Coir. Note that each media started to differentiate in distinct groups, however, each group is located very close to each other.

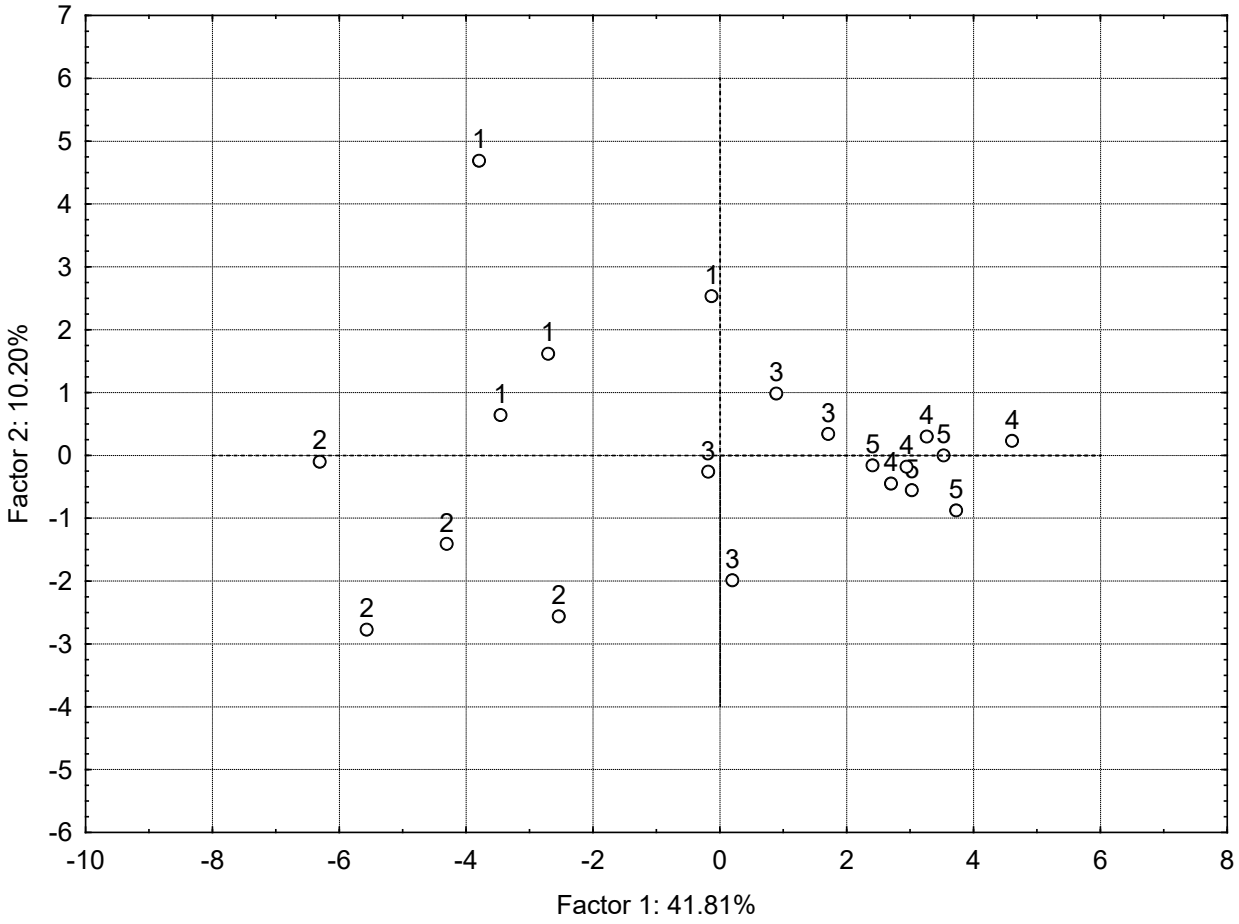

Fig 29. PCA scaling plot of 6-months untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph correspond to following treatments: 1-Peat blend with 10 % CGW, 2-Peat blend with 20 % CGW, 3- Bark,4-Peat, 5 – Coir. Note that growth media can be categorized into 3 distinct groups i.e. group 1 (1-peat blended with 20% CGW), group 2 (Peat blend with 10 % CGW), group 3 (peat, coir and bark)

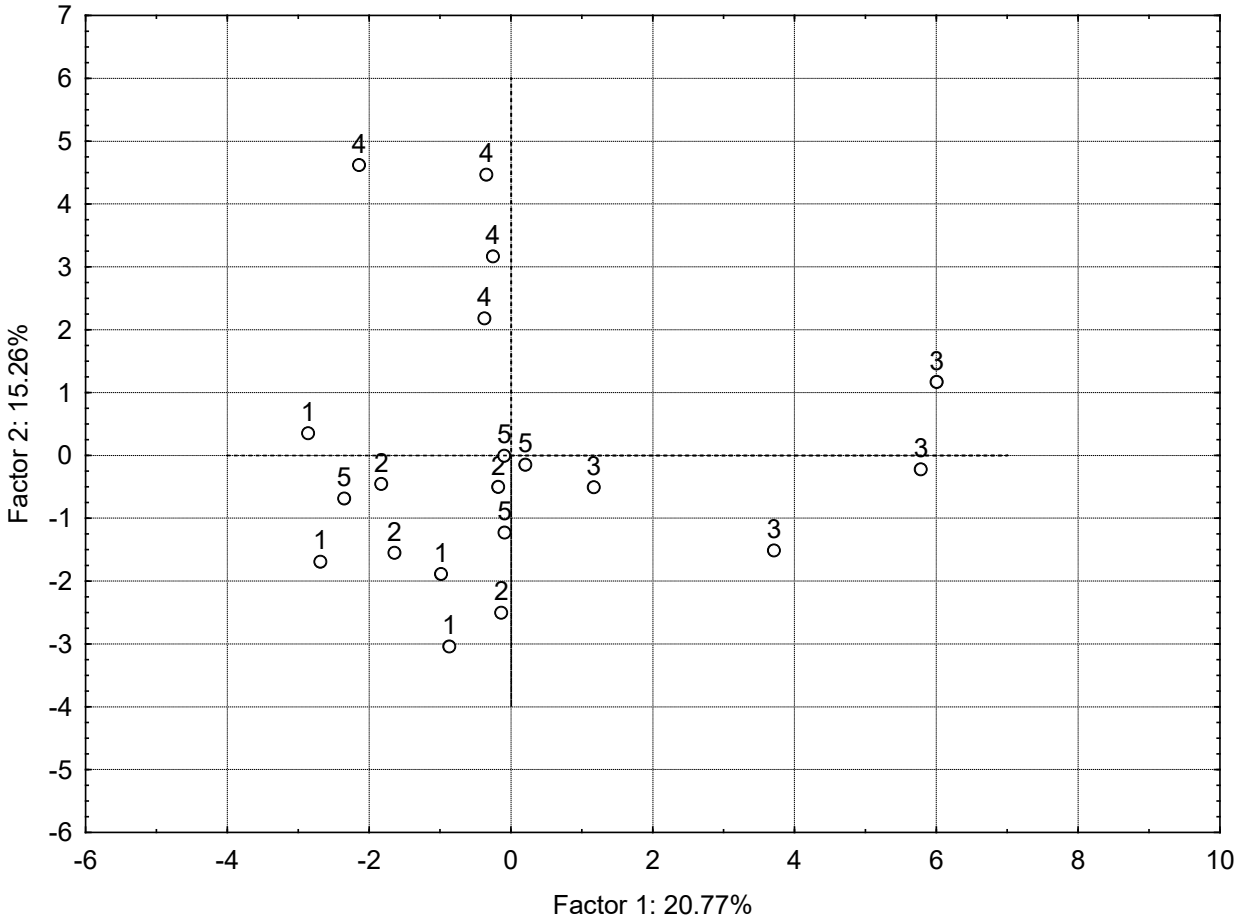

Fig 30. PCA scaling plot of 12-months untreated compost samples based on a correlation matrix of association between carbon-substrate utilisation rates. Numbers (dots) in graph correspond to following treatments: 1-Peat blend with 10 % CGW, 2-Peat blend with 20 % CGW, 3- Bark,4- Peat, 5 – Coir.

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 using 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, 0.025µ/µl Taq (GoTaq, Promega) and 0.5µg/µl 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 bio film DNA (kindly provided by Simon Gregory, UWS). 40µl PCRs were done for each sample and 5µl 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. The raw data consisted of an electrogram from each sample, which was analysed for relative peak height and area. 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.

Data analysis

Two-way indicator species analysis (TWINSPAN) was used to analyse patterns in community structure between the different growing media using the Community Analysis Package (CAP) (Harrison et al., 2004; Whittikar, 1972). TWINSPAN is a divisive clustering method using iterative reciprocal averaging to subdivide groups in a hierarchal manner, resulting in a dendrogram describing community relationships. Changes in microbial diversity were calculated as the number of peaks in the fungal and bacterial ARISA (Alpha diversity) as described by (Harrison et al., 2004; Whittikar, 1972).

Determination of *M. anisopliae* efficacy

Influence of microbes on *M. anisopliae* efficacy was determined using 4-5th instar *Tenebrio molitor* larvae. Plant growth media samples collected from different treatments were aliquoted into small plastic cups. Each treatment was replicated 3 times. Five *T. molitor* larvae were released in each cup and mortality recorded daily until all the larvae died. Controls consisted of untreated growth media or media treated with fresh *M. anisopliae*.

Results

ARISA data provided complex profile of peaks for both fungal and bacterial species. Overall 176 peaks ranging from 94 bp to 999bp were observed for fungal ARISA; where as 349 peaks ranging from 133bp to 995bp were recorded for bacterial ARISA (Fig.31). Irrespective of the treatment or growth media, there was a significant increase in fungal and bacterial diversity over time. At 0 month, only few bacterial and fungal species were observed, however, at later sampling time, an increase in fungal and bacterial species was observed. In general, number of bacterial species observed was significantly higher than fungal species at each time period. Plant growth varied in their fungal and bacterial diversity with highest diversity observed in coir and least in bark (Fig. 31). Peat and peat blends had intermediate fungal and bacterial diversity (Fig. 31, Tables 11-13). At each sampling time, number of new species observed was higher than those which were observed at earlier sampling times. Similarly, majority of species

observed in any particular growth media were specific to that media whereas few were common in all media.

Influence of either plant or *M. anisopliae* on bacterial diversity was dependant upon growth media (Table 11). For example, *M. anisopliae* treated 10 % and 20% CGW showed a decline in bacterial diversity where as bark was unaffected. In peat and coir treated with *M. anisopliae*, although bacterial diversity increased over time but rate of increased was considerably lower than untreated or with plant samples. In general, sample containing both *M. anisopliae* and plant, bacterial diversity was marginally higher than *M. anisopliae* alone (Fig. 32). In the presence of plant samples, bacterial diversity increased considerably in peat and coir but declined in peat+20%CGW and bark (Table 13).

Incorporation of *M. anisopliae* with or without plant resulted in considerable increase in fungal diversity (Table 11). The most significant rise in fungal diversity was observed in bark and coir whereas a modest rise was observed in peat and peat blends. There was only one treatment (*M. anisopliae* + plant treated 10%CGW), which showed a declined in fungal diversity over time. Irrespective of growth media, the presence of plant samples resulted in significant rise in fungal diversity over time.

The TWINSPAN analysis clustered samples from the same growth medium together, illustrating their distinctive community profiles (Figs 33-35). Bark had the most distinctive profile, whereas coir was closest to peat. Samples of peat blended with CGW clustered together. Clustering from samples taken at 1 and 6 months showed similar profile as that of 12 months, except that the peat and peat +CGW samples cluster more closely (Fig. 35).

Task 6.3 Influence of microbial populations on *M. anisopliae* efficacy

Irrespective of plant growth media and/or treatments, *M. anisopliae* efficacy against *T. molitor* larvae varied non –significantly at each sampling time.

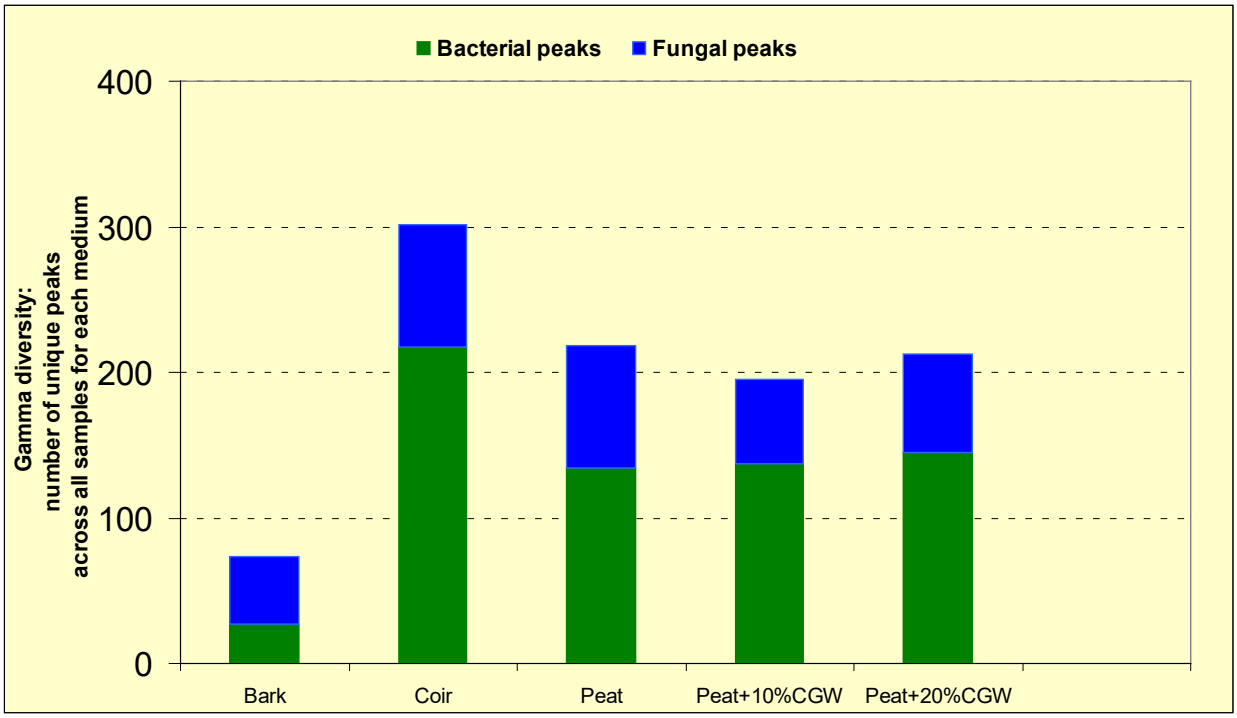

Fig.31. Number of ARISA peak observed in different plant growth media over 12 months. These peaks indicate diversity of fungal and bacterial species in different plant growth media.

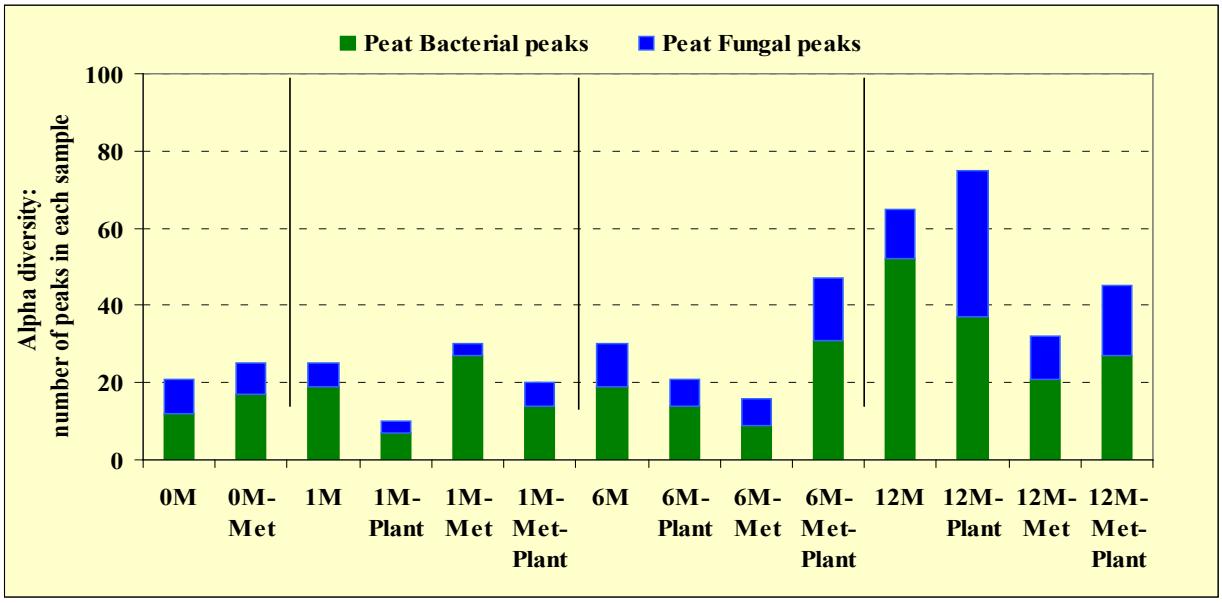

Fig. 32. Influence of different treatments on fungal and bacterial diversity in peat observed over 12 months. Similar profiles were observed for other plant growth media (data not shown).

Table 11. Alpha Diversity Indices for Bacterial (B) and Fungal (F) ARISA analyses of different growing media and percentage change in Alpha diversity from 0-12 months

Alpha Diversity Indices for Bacterial and Fungal ARISA analyses of different growing media										
	Bark		**Coir**		**Peat**		**Peat+10%CGW**		**Peat+20%CGW**	
(number of peaks)	**B**	*F*	**B**	*F*	**B**	*F*	**B**	*F*	**B**	*F*
0M	**12**	4	**16**	5	**12**	9	**13**	2	**23**	8
0M-Met	**9**	4	**35**	10	**17**	8	**21**	9	**29**	9
12M	**7**	9	**51**	7	**52**	13	**48**	7	**25**	11
12M-Plant	**10**	19	**56**	5	**37**	38	**15**	4	**22**	10
12M-Met	**9**	12	**43**	22	**21**	11	**14**	11	**25**	10
12M-Met-Plant	**8**	6	**64**	27	**27**	18	**47**	2	**24**	12
Percentage change in Alpha diversity from 0-12 months										
	Bark		**Coir**		**Peat**		**Peat+10%CGW**		**Peat+20%CGW**	
	B	F	**B**	F	**B**	F	**B**	F	**B**	F
No treatment	**-41.7**	125.0	**218.8**	40.0	**333.3**	44.4	**269.2**	250.0	**8.7**	37.5
+ Plant	**-16.7**	375.0	**250.0**	0.0	**208.3**	322.2	**15.4**	100.0	**-4.3**	25.0
+ Met	**0.0**	200.0	**22.9**	120.0	**23.5**	37.5	**-33.3**	22.2	**-13.8**	11.1
+ Met+ Plant	**-11.1**	50.0	**82.9**	170.0	**58.8**	125.0	**123.8**	-77.8	**-17.2**	33.3

Table12 Fungal diversity index (species observed in common or different) in different treatment applied to different growth media.

Treatments	Sampling time	Plant growth media									
		Bark		Coir		Peat		Peat blended with 10% CGW		Peat blended with 10% CGW	
		Common	Different	Common	Different	Common	Different	Common	Different	Common	Different
Untreated media	0 M/1M*	2	4	0	23	1	13	0	10	0	12
	0M/6M	0	6	0	13	1	18	0	18	1	23
	0M/12M	1	11	0	12	0	22	0	9	1	17
	1M/6M	0	6	3	20	1	15	4	16	3	15
	1M/12M	1	11	1	23	4	11	1	13	1	13
	6M/12M	1	9	2	11	3	18	5	13	3	22
Plant growth media + *M. anisopliae*	0 M/1M	2	4	3	25	0	11	0	20	0	15
	0M/6M	2	3	2	27	0	15	0	18	0	16
	0M/12M	1	14	2	28	2	15	1	18	0	19
	1M/6M	2	3	6	30	1	8	3	14	3	7
	1M/12M	3	10	5	33	1	12	1	20	3	10
	6M/12M	2	11	5	33	1	16	2	16	5	7
Plant growth media + plant	0 M/1M	2	3	0	13	0	12	0	11	1	18
	0M/6M	2	9	0	6	1	14	2	17	0	17
	0M/12M	2	19	0	10	1	45	0	6	1	16
	1M/6M	1	10	0	9	1	8	7	14	1	19
	1M/12M	2	18	0	13	2	37	1	11	3	16
	6M/12M	4	0	0	0	2	0	2	0	1	0
Plant growth media + *M. anisopliae* and plant	0 M/1M	2	3	3	20	0	14	0	13	0	14
	0M/6M	3	9	4	16	0	24	0	22	0	14
	0M/12M	0	10	4	29	0	26	0	11	1	19
	1M/6M	3	8	4	22	2	18	4	9	3	4
	1M/12M	0	9	5	33	3	18	1	4	0	17
	6M/12M	0	17	7	27	2	30	1	13	0	17

Table13. Bacterial diversity index (species observed in common or different) in different treatment applied to different growth media.

Treatments	Sampling time	Plant growth media									
		Bark		Coir		Peat		Peat blended with 10% CGW		Peat blended with 10% CGW	
		Common	Different	Common	Different	Common	Different	Common	Different	Common	Different
Untreated media	0 M/1M*	8	6	5	39	4	23	8	15	9	36
	0M/6M	3	11	2	76	1	29	5	37	5	60
	0M/12M	2	15	1	65	3	58	4	53	5	38
	1M/6M	3	9	14	69	3	32	5	42	10	58
	1M/12M	3	13	4	76	6	59	5	56	6	44
	6M/12M	2	8	8	99	7	57	10	62	10	52
Plant growth media + *M. anisopliae*	0 M/1M	4	9	6	39	6	32	3	20	13	34
	0M/6M	6	7	10	58	1	24	5	36	4	32
	0M/12M	5	8	6	66	2	34	2	31	3	48
	1M/6M	7	4	13	33	3	30	4	22	5	32
	1M/12M	3	11	8	43	5	38	2	15	5	46
	6M/12M	3	13	14	58	7	16	8	23	4	28
Plant growth media + plant	0 M/1M	6	9	3	64	2	15	7	29	5	23
	0M/6M	6	7	3	69	2	22	4	35	9	41
	0M/12M	6	10	4	64	2	45	1	26	4	37
	1M/6M	6	4	16	81	2	17	13	34	9	28
	1M/12M	6	7	13	84	3	38	2	41	3	26
	6M/12M	5	0	20	0	4	0	4	0	6	0
Plant growth media + *M. anisopliae* and plant	0 M/1M	5	7	5	35	3	25	9	24	9	30
	0M/6M	4	7	7	57	2	44	4	26	9	34
	0M/12M	5	7	12	75	2	40	5	58	1	51
	1M/6M	6	2	8	30	8	29	6	22	14	14
	1M/12M	3	10	5	64	5	31	5	58	3	37
	6M/12M	2	10	14	72	10	38	5	50	3	41

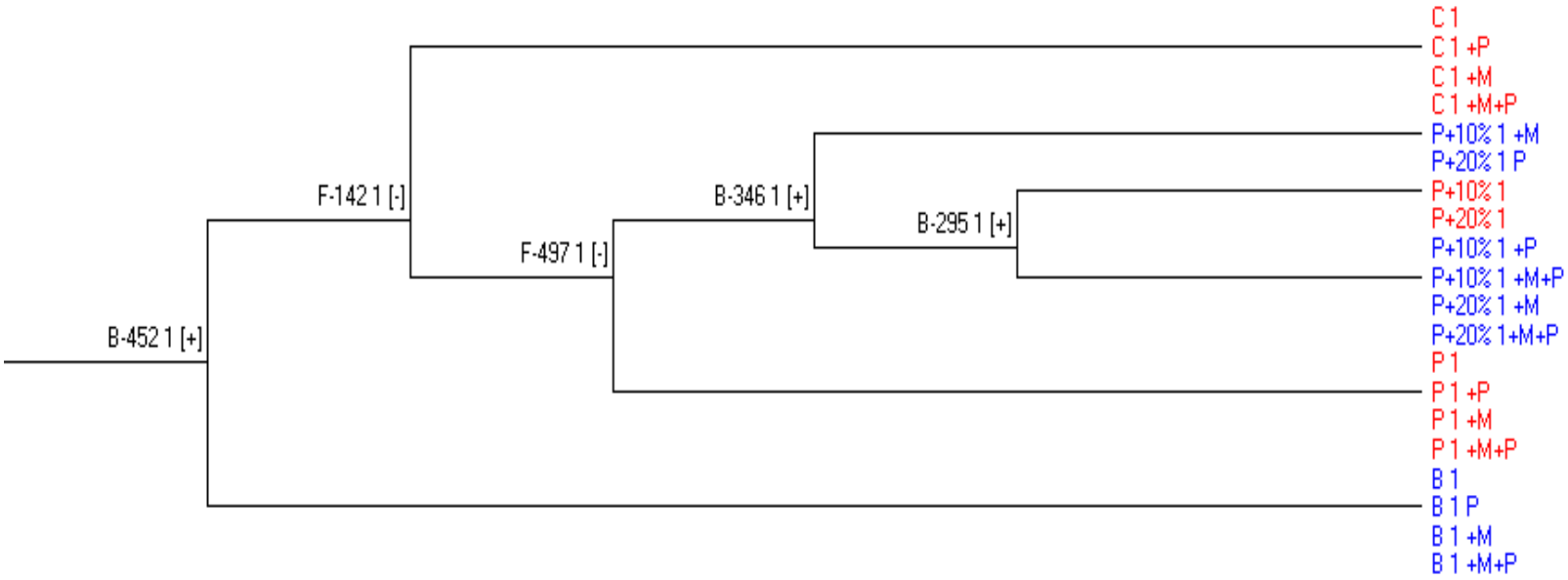

Fig. 33. Dendrogram showing TWINSPAN analysis of fungal and bacterial ARISA presence/absence data for 12 month samples. B (bark), C (coir), P (peat), P + 10% CGW (peat + 10% composted garden waste), P + 20% (peat+20% composted garden waste), + M= with *M. anisopliae,* + P= with plant.

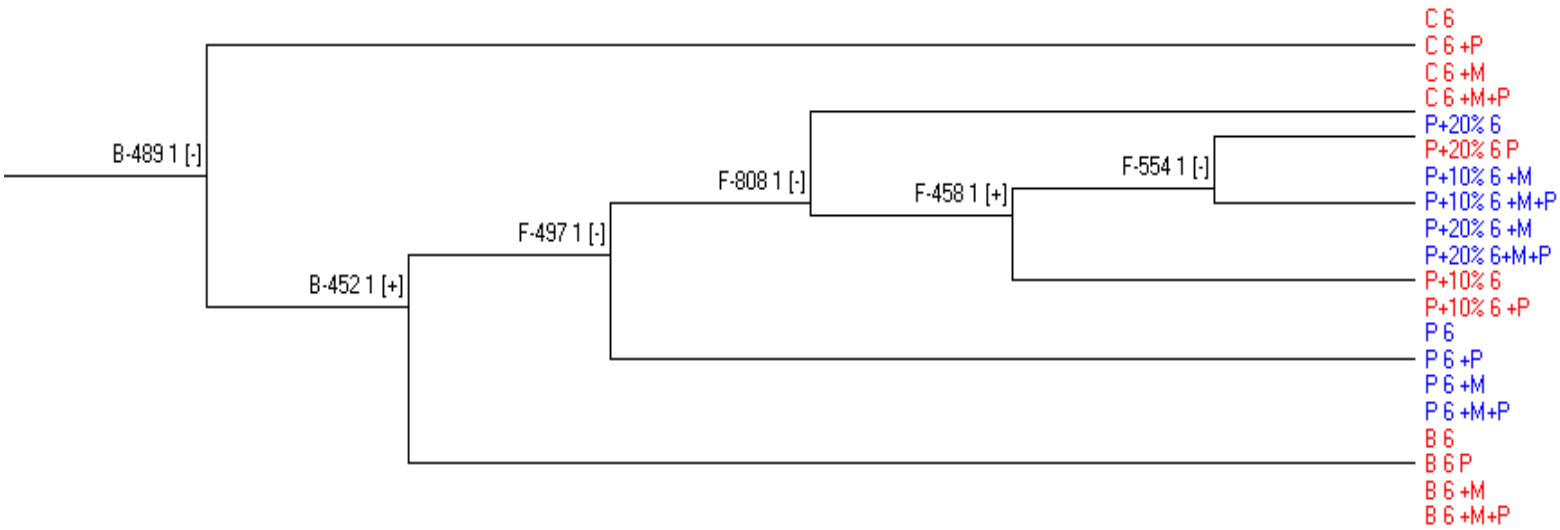

Fig. 34 Dendrogram showing TWINSPAN analysis of fungal and bacterial ARISA presence/absence data for 12 month samples. B (bark), C (coir), P (peat), P + 10% CGW (peat + 10% composted garden waste), P + 20% (peat + 20% composted garden waste), + M= with *M. anisopliae,* + P= with plant.

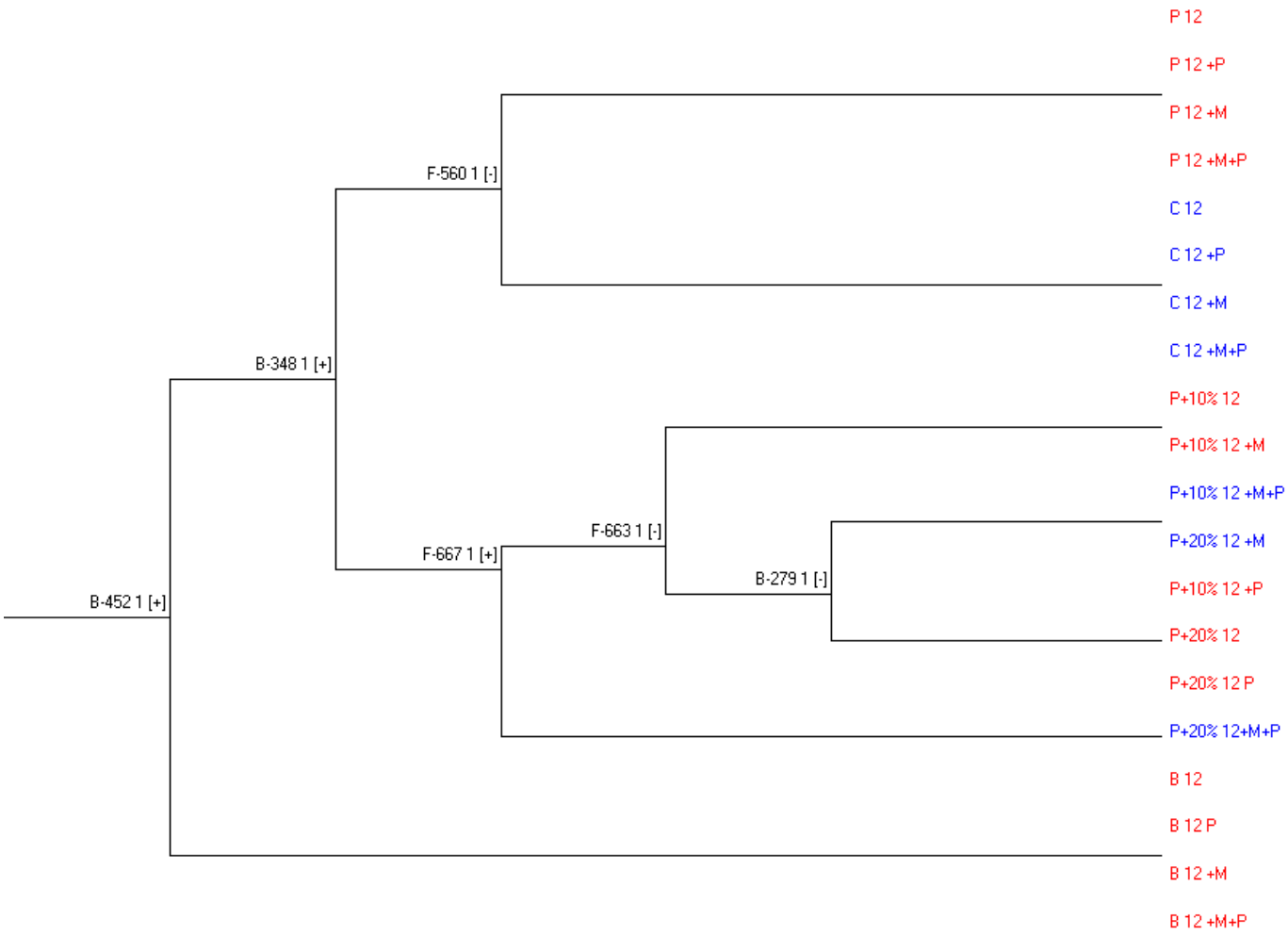

Fig. 35. Dendrogram showing TWINSPAN analysis of fungal and bacterial ARISA presence/absence data for 12 month samples. B (bark), C (coir), P (peat), P + 10% CGW (peat + 10% composted garden waste), P + 20% (peat + 20% composted garden waste), +M= with *M. anisopliae,* + P= with plant.

Discussion

This is the first study that investigates in detail the diversity and population dynamics of microbial communities in disparate plant growth media in the presence and absence of *Euonymus*. It is also the first study to determine the impact of the microbial communities on *M. anisopliae.* Results show that irrespective of media, the initial microbial activity and diversity was extremely low but increased significantly over 6-12 months. Changes in the microbial community composition may be due to changes in substrate availability, temperature, or pH (Hoitink & Boehm, 1999). Changes in microbial populations may also have been affected by more specific microbe-microbe interactions. For example, Fukui *et al*., (1999) reported a decline in *Xanthomonas axonopodis diffenbachiae* numbers in the presence of certain bacterial species. However, the decline was reduced when one of the antagonistic bacteria was removed.

Incorporation of *M. anisopliae* and/or the presence of *Euonymus* had no significant effect on either the microbial activity or CLPP. In contrast, ARISA analysis showed that incorporation of *M. anisopliae* and/or presence of *Euonymus* influenced the overall microbial diversity favouring some species and not others. Our studies showed that the majority of microrganisms were common in the presence or absence of *M. anisopliae* and few were specifically associated or inhibited under these conditions. Interestingly, *M. anisopliae* treated samples had relatively lower bacterial diversity suggesting this fungal biocontrol agent may be producing an antibiotic.

CLPP data clearly indicated that *M. anisopliae* had no significant influence on the indigenous microbe's ability to utilize nutrients in the media. Since the CLPP and ARISA profiles of unamended media were quite distinct at 6 and 12 months suggests that microbial establishment is media specific. Although diverse microbial communities were observed in each media they did not appear to affect the efficacy of *M. anisopliae* validating its robustness in different plant growth media.

Objective 7: Determine shelf life of *M. anisopliae* in different media

Success of any biocontrol agent is dependant on a good shelf life in diverse environments. Earlier studies indicate that persistence and efficacy of *M. anisopliae* is influenced by several biotic and abiotic factors. Plant growth media vary in their physiochemical properties and microbial composition, which could influence persistence of *M. anisopliae*. Our preliminary studies showed that *M. anisopliae* can persist for over 18 months in peat, however, very little is known about its persistence in disparate plant growth media. These studies were conducted to determine persistence and efficacy of *M. anisopliae* in disparate growing media under different conditions.

Materials and Methods

M. anisopliae was premixed in different media at the final inoculum concentration of (1 x 10^{10} conidia/l) of growth media. Each growth media was then potted in 2 litres pots and divided into 3 groups as follow. Each group was replicated five times.

1. Group 1: kept in potting shed to simulate storage conditions (no plants)
2. Group 2: kept out door (no plants)
3. Group 3: kept out but potted up with *Euonymus* plants

In addition to these premixed conidia, *M. anisopliae* was stored as dry conidia at 4ºC and 25ºC to determine the conidial shelf life under these conditions. Samples were collected from each treatment at 0, 6, 12, and 20 months to determine *M. anisopliae* efficacy.

Determination of inoculum concentration and virulence of conidia recovered from different media

A composite sample was collected from all replicates of each treatment. Inoculum recovery was determined by suspending a 10g sample in 90 ml of 0.03% aq. Tween 80 (Fisher Scientific, UK) in a small plastic bottle. The suspension was vigorously shaken for 2 min by hand. Inoculum concentration was determined using an improved Neubauer haemocytometer (Weber Scientific Ltd. U.K) and its efficacy assessed as described in task 6.3.

Results and Discussion

There was no significant difference in inoculum recovery from different media at different sampling times (data not shown). At 6 and 12 months, virulence of conidia premixed in media varied non-

significantly among growth media and storage conditions (Table 14). Conidia from all treatments, except for conidia stored at 25ºC, had a similar efficacy as freshly prepared inoculum. At 20 months, conidia from media stored in the potting shed were significantly less aggressive than that kept outdoors. The latter was just as efficacious as freshly prepared inoculum. Conidia kept indoors or at 4ºC and 25ºC were least virulent. These observations suggest that the shelf life of unformulated conidia of *M. anisopliae* is significantly less than conidia mixed into plant growth media. The latter clearly provides a favourable environment for the survival of *M. anisopliae.*

Table 14. Virulence of the inoculum stored under different conditions.

Treatments (Plant growth media - storage conditions)	**Virulence (LT_{50} –days post inoculation) of *M. anisopliae* stored under different conditions at different sampling time**			
	0 months	6 months	12 months	20 months
Outdoor-no plant				
Peat	4.75^{ab}	6.02^{ab}	5.35^{bc}	4.91^{d}
Bark	5.25^{b}	5.60^{ab}	5.22^{bc}	5.65^{d}
Coir -outdoor	4.45^{a}	4.86^{ab}	5.24^{bc}	4.87^{d}
Peat +10%CGW blend	4.70^{a}	5.31^{ab}	5.17^{bc}	5.74^{d}
Peat +20%CGW blend	4.96^{ab}	5.06^{ab}	5.17^{bc}	5.68^{d}
Outdoor +Plant				
Peat		4.92^{ab}	5.39^{bc}	5.78^{d}
Bark		4.99^{ab}	5.08^{bc}	5.60^{d}
Coir -outdoor		5.10^{ab}	4.84^{c}	4.76^{d}
Peat +10%CGW blend		5.24^{ab}	5.28^{bc}	5.37^{d}
Peat +20%CGW blend		4.63^{a}	5.24^{bc}	5.06^{d}
Potting shed-no plant				
Peat		6.52^{b}	6.61^{ab}	9.32^{a}
Bark		5.95^{ab}	6.36^{abc}	7.32^{b}
Coir -outdoor		5.27^{ab}	5.51^{bc}	5.91^{cd}
Peat +10%CGW blend		6.14^{ab}	6.27^{abc}	7.28^{b}
Peat +20%CGW blend		6.15^{ab}	7.42^{a}	7.16^{bc}
Positive control		5.00^{ab}	5.22^{bc}	4.97^{d}
Conidia stored at 25 ºC		5.62^{ab}	*	*
Conidia stored at 4 ºC		5.42^{ab}	7.28^{a}	*

Means within a column followed by same letter are not significantly different ($P < 0.05$ Tukey test)

* Less than 50% mortality.

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

Studies conducted at Swansea University showed that *M. anisopliae* V275 was effective in controlling BVW and WFT. However, to demonstrate that V275 was efficacious and robust trials were conducted at different locations. These trials were designed to demonstrate that the efficacy of *M. anisopliae* was independent of plant species, environmental or cultural conditions and was compatible with other biocontrol agents e.g. entomopathogenic nematodes. Details of these trials are given below:

Trials conducted at Swansea

1. Determining the efficacy of *M. anisopliae* for BVW control in different plant species.

Earlier studies showed that *M. anisopliae* strain V275 was highly efficacious in controlling BVW larvae in outdoor potted *Euonymus* (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

All experimental conditions except the plant species were the same as described in section A of task 2.1. These studies were conducted in summer 2006 and 2007 and results representing *M. anisopliae* efficacy in different plant species is presented below.

Plant species

Rooted cuttings of *Euonymus fortunei* 'Emerald Gold" and *Fuchsia* 'in cultivars' 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. *Euonymus* trials were conducted in disparate growth media (peat, bark, coir and peat blended with 10% (v/v) or 20% (v/v) composted green waste (CGW), whereas, peat and/or bark was used for rest of the plant species. In 2007, the efficacy of *M. anisopliae* was compared with thiacloprid (Exemptor) and not the related compound imidacloprid because the latter was not commercially available.

Results

BVW control in *E. fortunei*

M. anisopliae provided 73-88% (drench) and 71-95% (premixed) control and varied significantly ($P < 0.05$) with drench application only in peat, however such differences were not observed in trials conducted in 2007 (Fig. 36). In all other growth media, no significant differences in *M. anisopliae* efficacy were observed whether it was applied as a drench or premixed (Figs. 37-40). 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. 36-40). Thiacloprid provided slightly better control (over 90%) than imidacloprid against BVW.

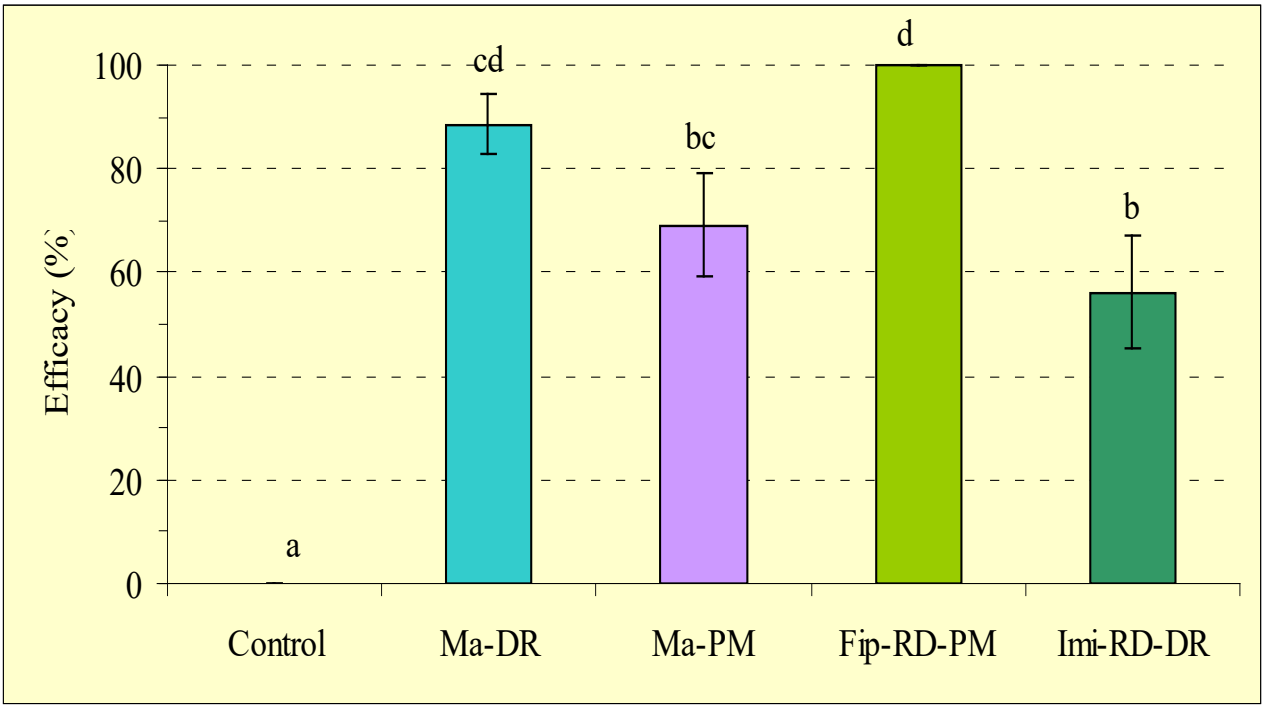

Fig. 36. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat. The treatments included: No treatment (control), *M. anisopliae* applied as drench (Ma-DR), *M. anisopliae* premixed (Ma-PM), Fipronil premixed at the recommended dose (Fip-RD-PM) and Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR). Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$).

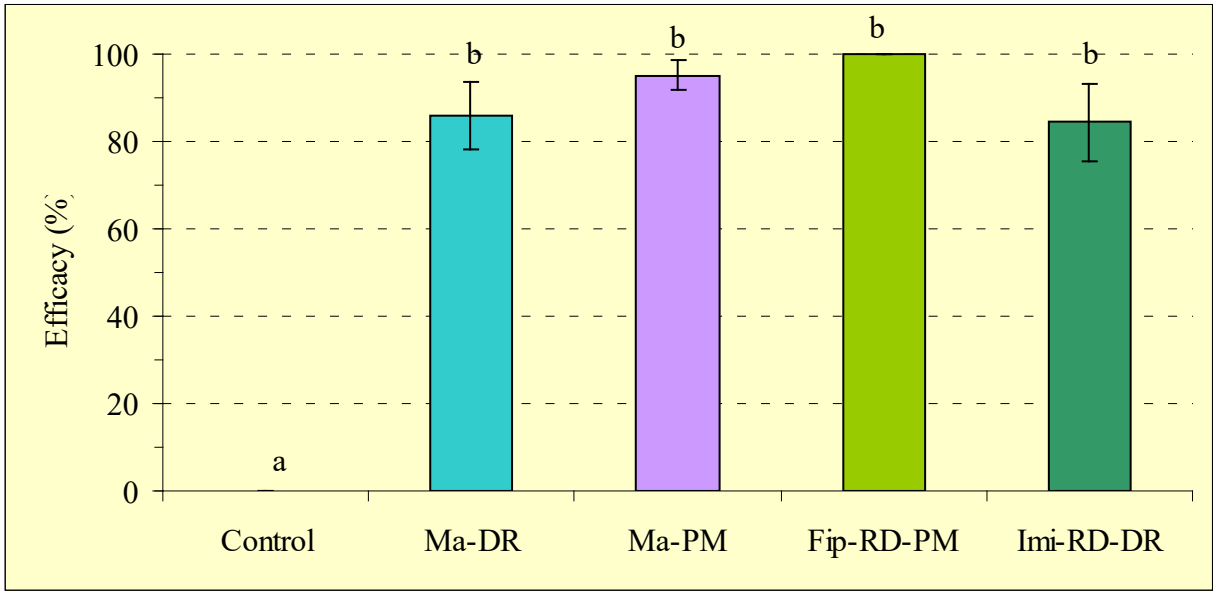

Fig. 37. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in bark. Treatments are the same as those described in Fig 36.

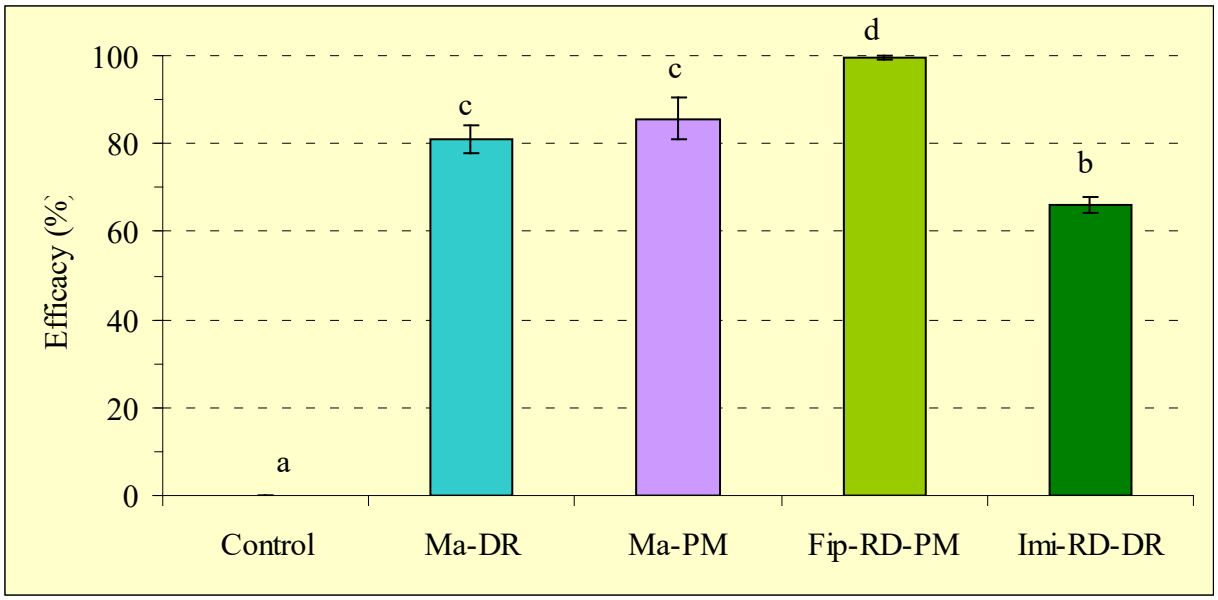

Fig. 38. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in coir. Treatments are the same as those described in Fig 36.

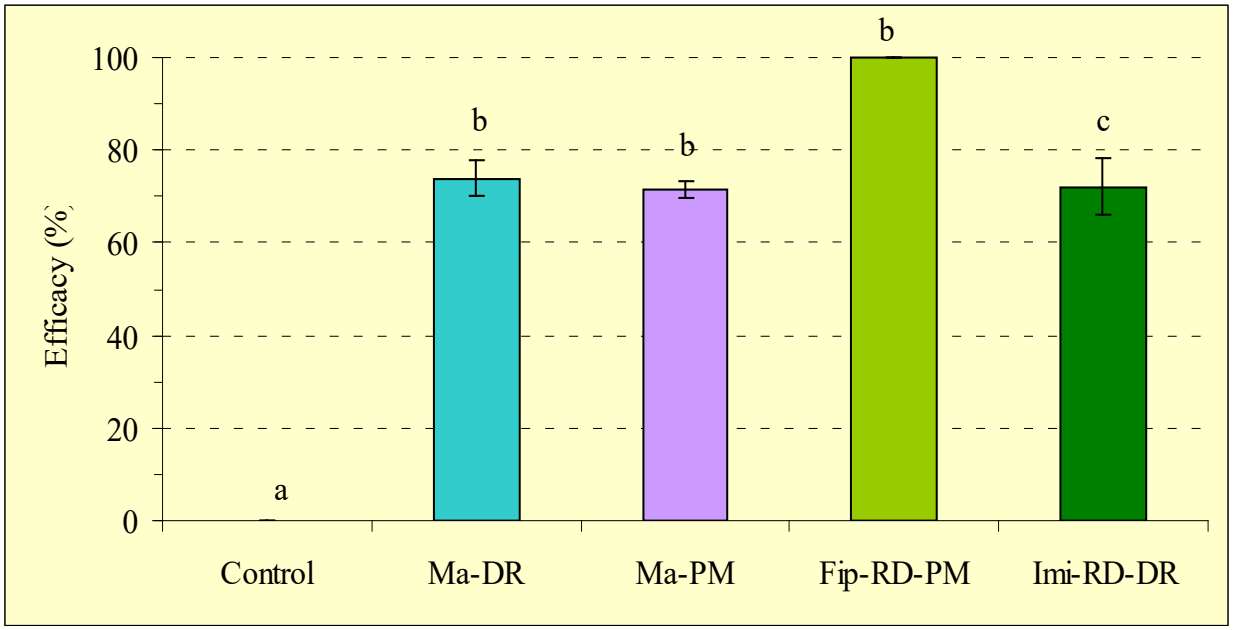

Fig. 39. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat blended with 10% CGW. Treatments are the same as those described in Fig 36.

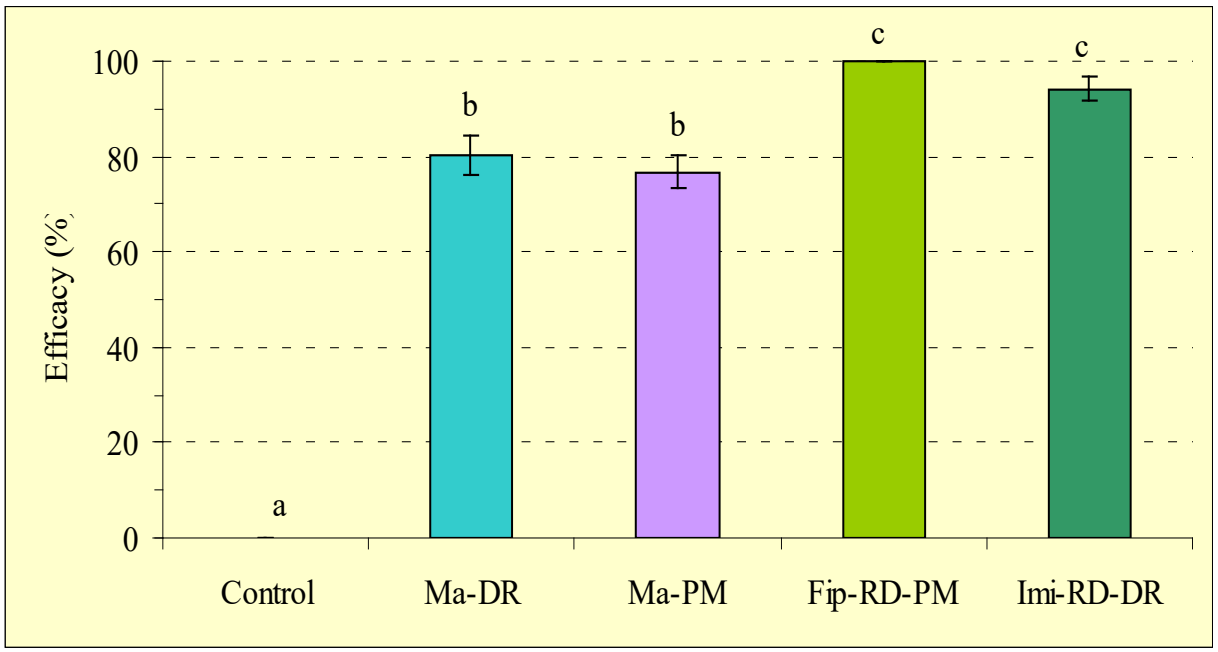

Fig. 40. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Euonymus* potted in peat blended with 20% CGW. Treatments are the same as those described in Fig 36.

BVW control in *Tellima grandiflora*

There was no significant difference in *M. anisopliae* efficacy whether it was applied as drench or premixed (Fig.41). Drench applications provided over 95% protection which varied non-significantly with fipronil and imidacloprid. Both insecticides provided 100% control of BVW (Fig. 41). Plants treated with *M. anisopliae* or insecticides appeared healthy and had extensive root systems while untreated plants were stunted and had poorly developed root systems (Figs. 42-43).

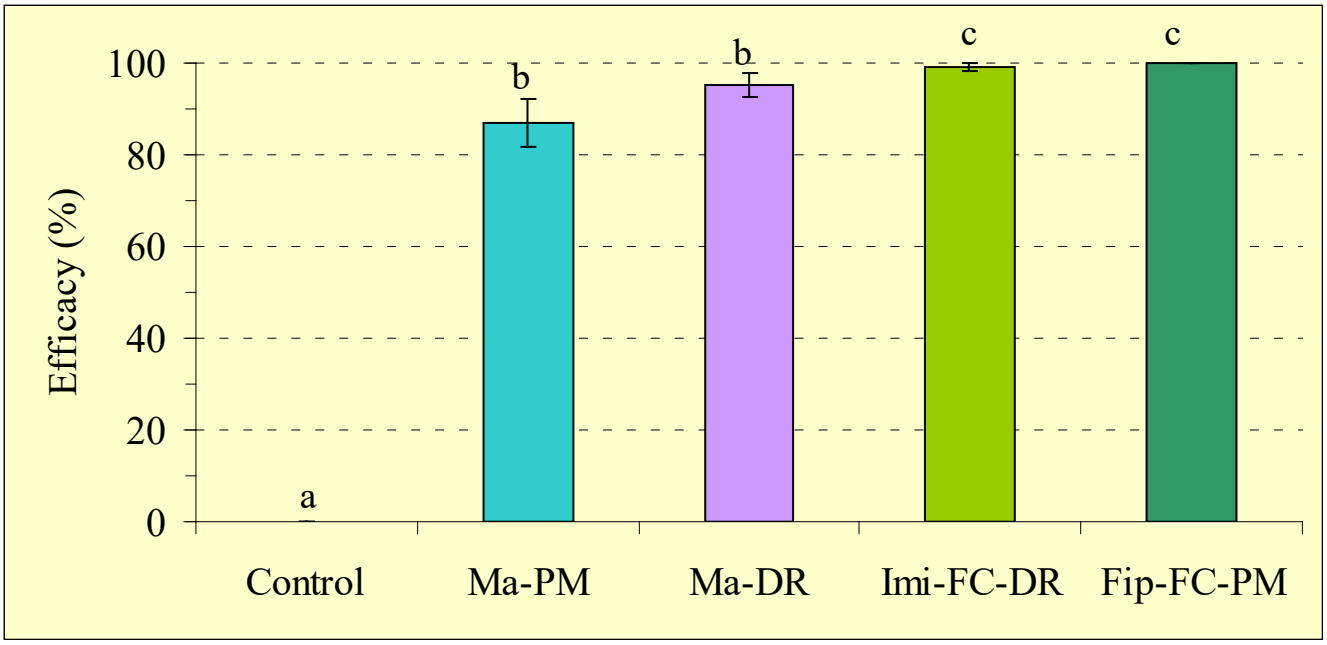

Fig. 41. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *T. grandiflora* potted in peat-based compost. The treatments are listed below. No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-FC-DR) and Fipronil premixed at the recommended dose (Fip-FC-PM). Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$).

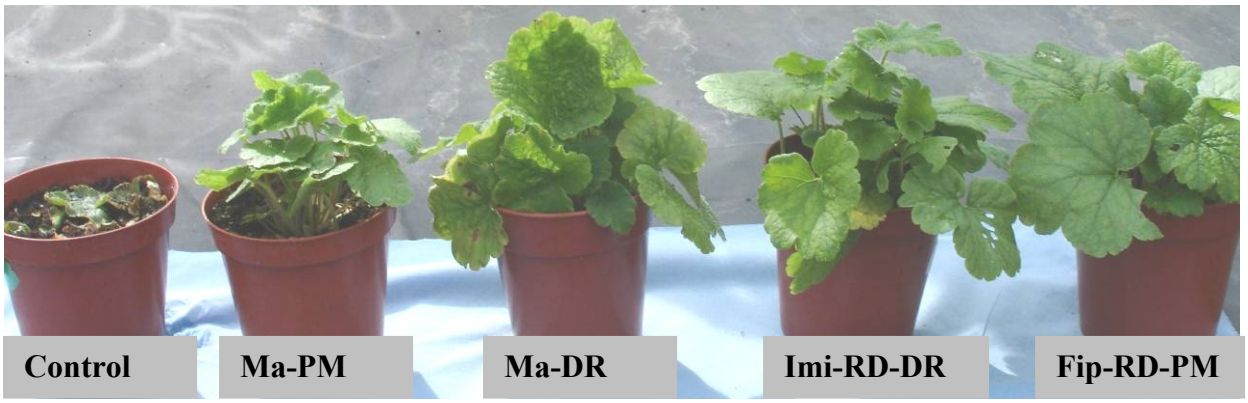

Fig. 42. Comparison of the plant growth of *T. grandiflora* exposed to the following treatments: No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed into the peat at the recommended dose (Fip-RD-PM). Each pot was infested with 20 BVW eggs and destructively assessed 6 weeks post infestation.

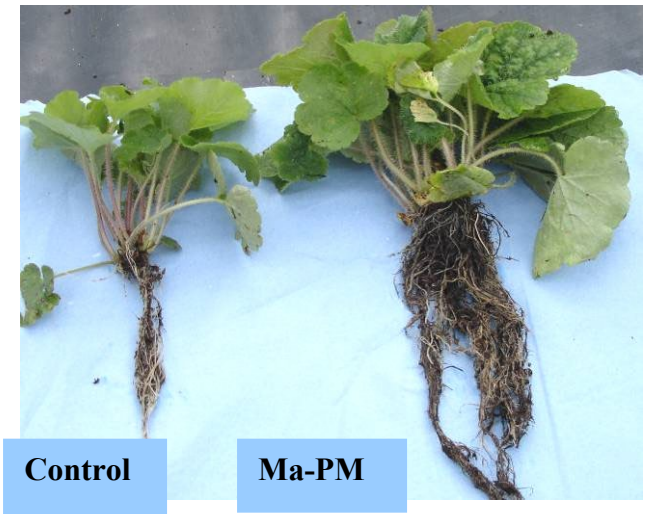

Fig. 43. Comparison of the root system of *T. grandiflora* untreated (control) *M. anisopliae* premixed (Ma-PM) in peat.

BVW control in *Sedum spurium* 'Ruby Mantle'

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. 44). Plants treated with *M. anisopliae* or insecticides appeared healthy while untreated plants appeared stunted (Fig. 45).

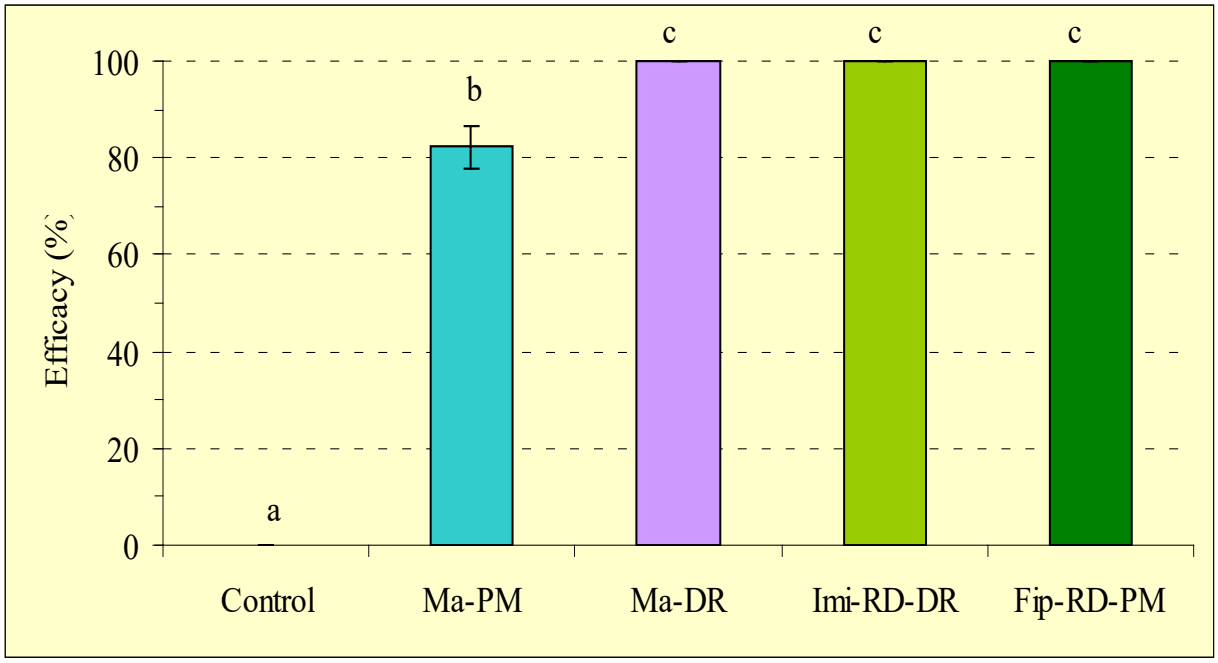

Fig. 44. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *S. spurium* 'Ruby Mantle' potted in peat. The treatments are listed below. No treatment (control), *M. anisopliae* premixed (Ma-PM), *M. anisopliae* applied as drench (Ma-DR), Imidacloprid applied as a drench at the recommended dose (Imi-RD-DR) and Fipronil premixed at the recommended dose (Fip-RD-PM). Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.05$).

Fig. 45. 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.

BVW Control in *Fuchsia*

All treatments were highly efficacious against BVW in potted *Fuchsia* plants. Irrespective of media or application method, *M. anisopliae* and exemptor provided 80-90% protection (Fig. 46). Fipronil (=Vi-nil) was most effective and provided 100% protection in both media.

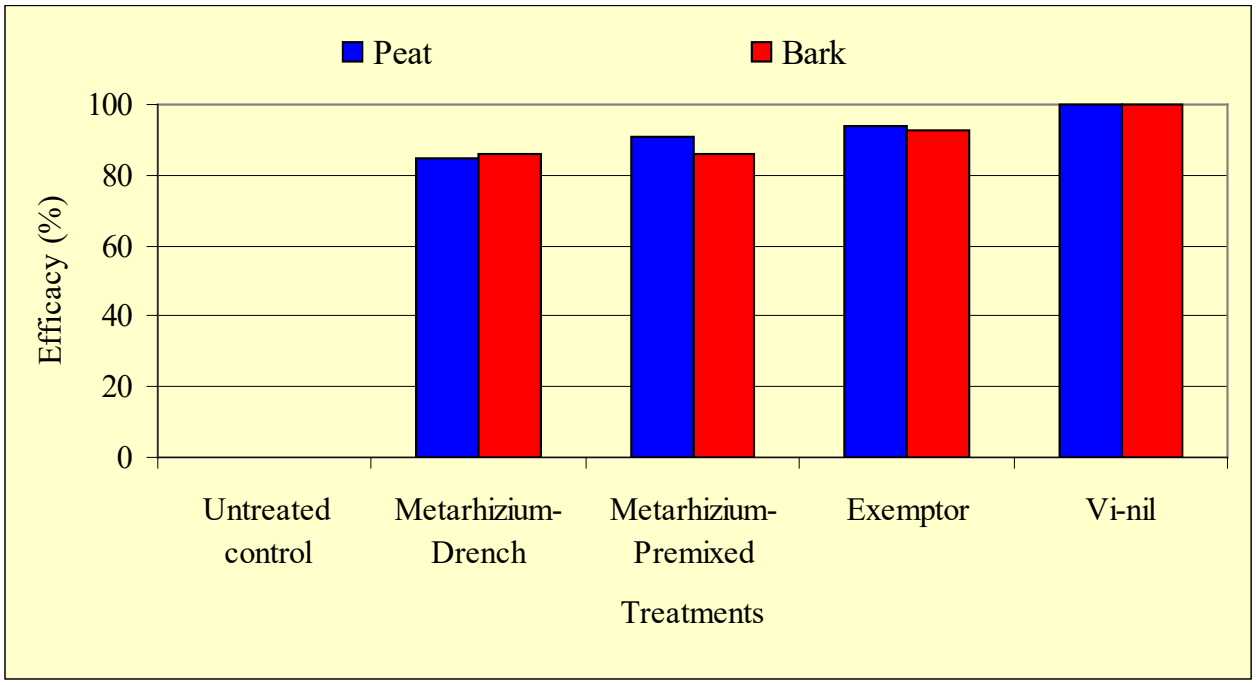

Fig. 46. Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Fuchsia* potted in peat or bark. The treatments are listed below. No treatment (control), *M. anisopliae* applied as drench or premixed in media, Thiacloprid (Exemptor) and Fipronil (Vi-Nil) premixed at the recommended dose.

2. Demonstrating *M. anisopliae* efficacy against WFT under glasshouse conditions

Earlier laboratory studies showed that *M. anisopliae* V275 was effective in controlling WFT pupae in different plant growing media (Ansari *et al*., 2007). Studies were extended (i) to compare V275 with other entomopathogenic fungi against thrips pupae (ii) to determine the efficacy and robustness of the most virulent strain against thrips pupae in a wide range of growing media under glasshouse conditions.

Materials and Methods

Rearing of thrips, bioassay protocol and statistical analysis was done as described in section B of task 2.1 except that glasshouse experiment was maintained in unheated glasshouse where the ambient night temperature ranged from 15° to 22°C (11 h). The highest temperatures were recorded around 12: 00 h and ranged from 28° to 37°C (for 2-4 h); for the rest of the day temperatures ranged between 20° and 25°C (9 to 11 h). Details of strains used in screening bioassay are summarized in table 15.

Table 15. Origin of fungal isolates screened against thrips

Fungal isolates	Host or source of origin	Geographic origin
M. anisopliae anisopliae		
V275	*Cydia pomonella* (Lepidoptera: Tortricidae),	Austria
ARSEF 3297	*Boophilus* sp. (Acari: Ixoididae)	Mexico
ARSEF 4556	*Boophilus* sp. (Acari: Ixodidae)	USA
ERL 700 (= ARSEF 1080)	*Heliothis zea* (Lepidoptera: Noctuidae);	FL, USA
CA1	Soil, *Galleria* baiting; avocado orchard;	CA, USA
F10	*Wiseana* sp. (Lepidoptera: Hepialidae)	New Zealand
M. flavoviridae		
F62	*Austracris guttulosa* (Orthoptera: Acrididae)	Australia
Beauveria bassiana		
CLO61	Soil, *Galleria* baiting; turf;	Eeklo, Belgium
CA603	Soil, *Galleria* baiting; avocado orchard;	CA, USA
Paecilomyces fumosoroseus		
CLO55	Soil, *Galleria* baiting; turf;	Lovendegem, Belgium
P34	Soil; *Galleria* baiting	UK

ARSEF, U.S. Department of Agriculture, Agricultural Research Service, Collection of Entomopathogenic Fungus Cultures, Ithaca, NY; CLO. Institute for Agricultural and Fisheries Research, Merelbeke, Belgium.

Results

Screening bioassay

All fungal strains caused significantly higher thrips mortality than fipronil (Fig 47). Overall, efficacy of fungal strains varied significantly among different strains and growing media. However, interactions between various fungal strains and growing media were not significantly different.

Irrespective of the growing medium tested, *M. anisopliae* V275 and ERL700 were consistently the most effective, causing 78-87% and 88-96% mortality, respectively (Figs.47-51). *M. anisopliae* strains ARSEF 4556, ARSEF 3297, CA1 and F10 caused 68-86%, 65-79%, 63-81% and 49-81% thrips mortality, respectively. *B. bassiana* and *P. fumosoroseus* strains caused 53-83% and 63-73% thrips mortality, respectively. Fipronil was least effective, causing <30% mortality in the different media.

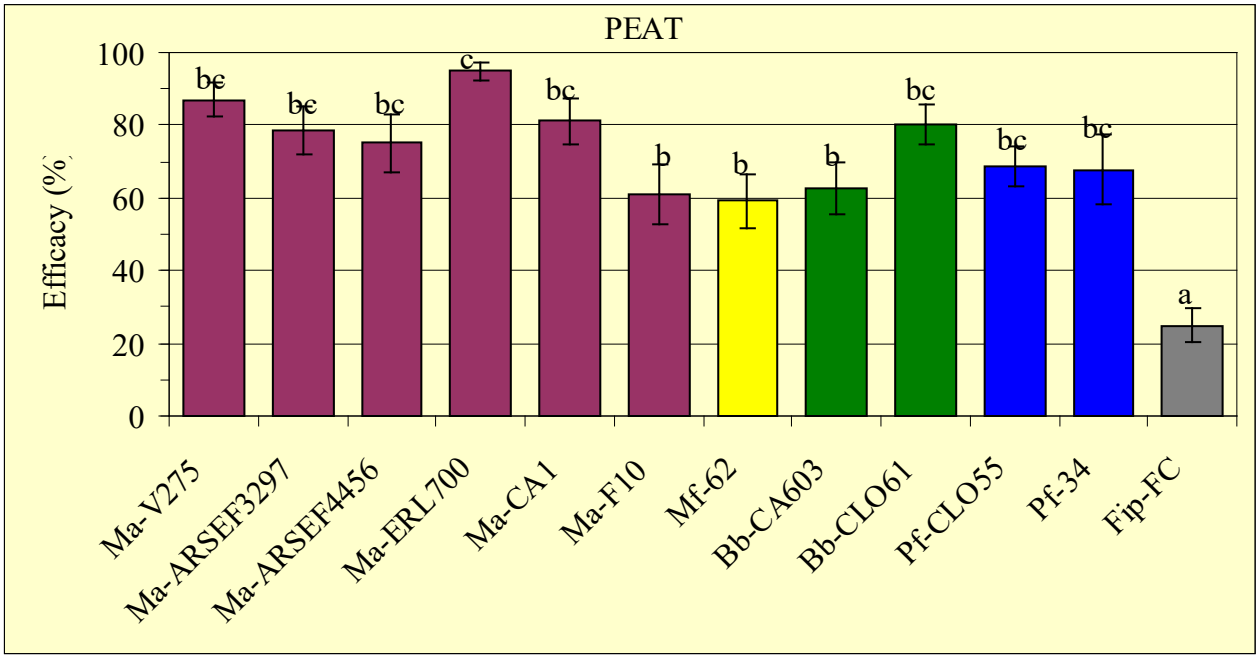

Fig.47. Thrips pupae mortality (% ± SE) in peat growth media treated with entomopathogenic fungi (1×10^{10} conidia/l medium) or the recommended dose of fipronil (1 g/l medium) in 250-ml cups, 11-d post inoculation. Means with the same letter are not significantly different by Tukey's test ($P < 0.05$). Ma = *M. anisopliae*, Mf = *M. flavoviridae*, Bb = *Beauveria bassiana*, Pf = *Paecilomyces fumosoroseus*, FIP = fipronil, FC = Recommended dose.

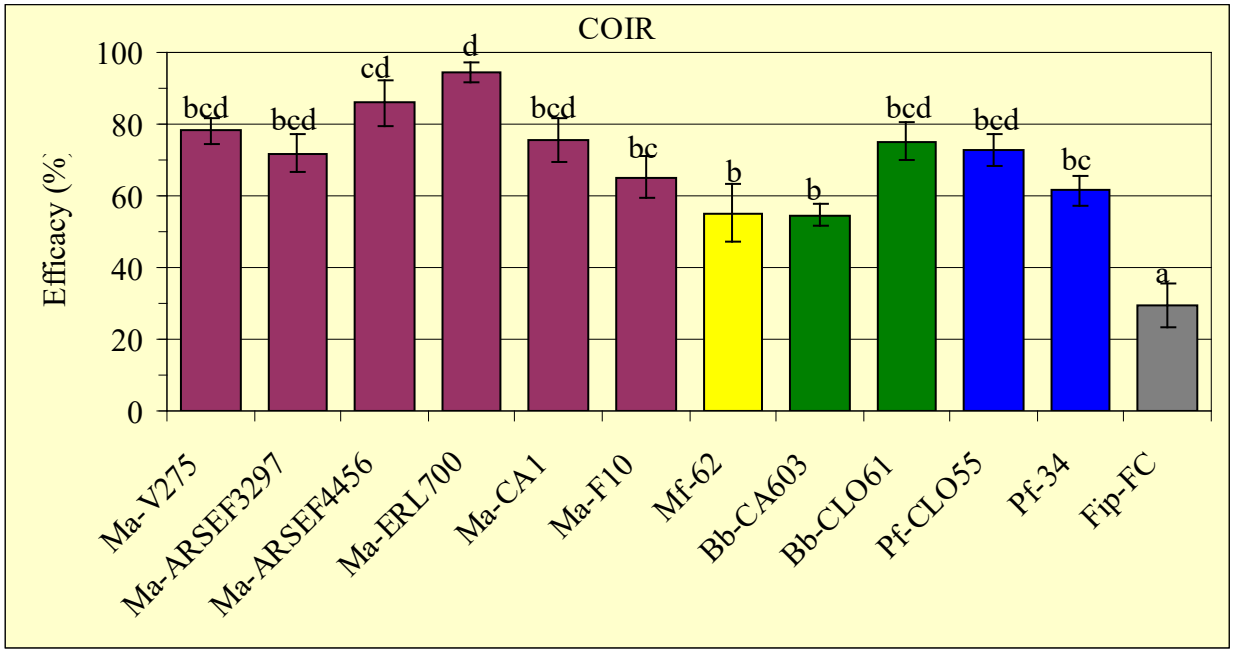

Fig 48. Thrips pupae mortality (% ± SE) in coir growth media. Treatments are same as described in Fig.47.

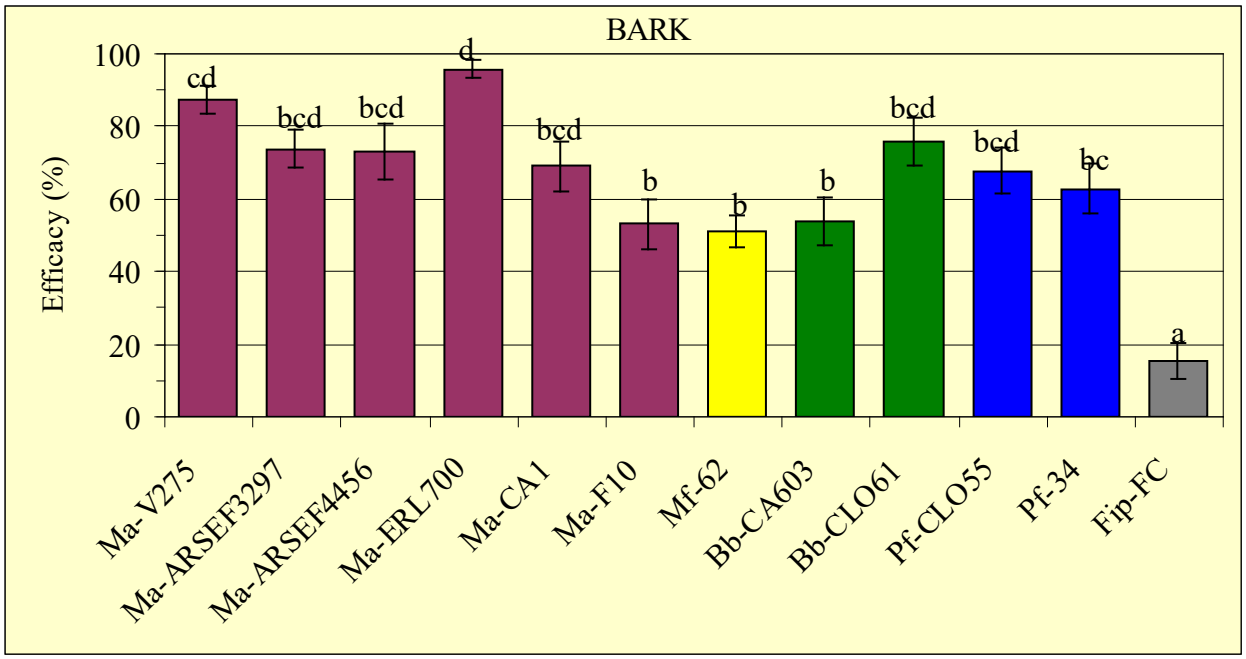

Fig 49. Thrips pupae mortality (% ± SE) in bark growth media. Treatments are same as described in Fig.47.

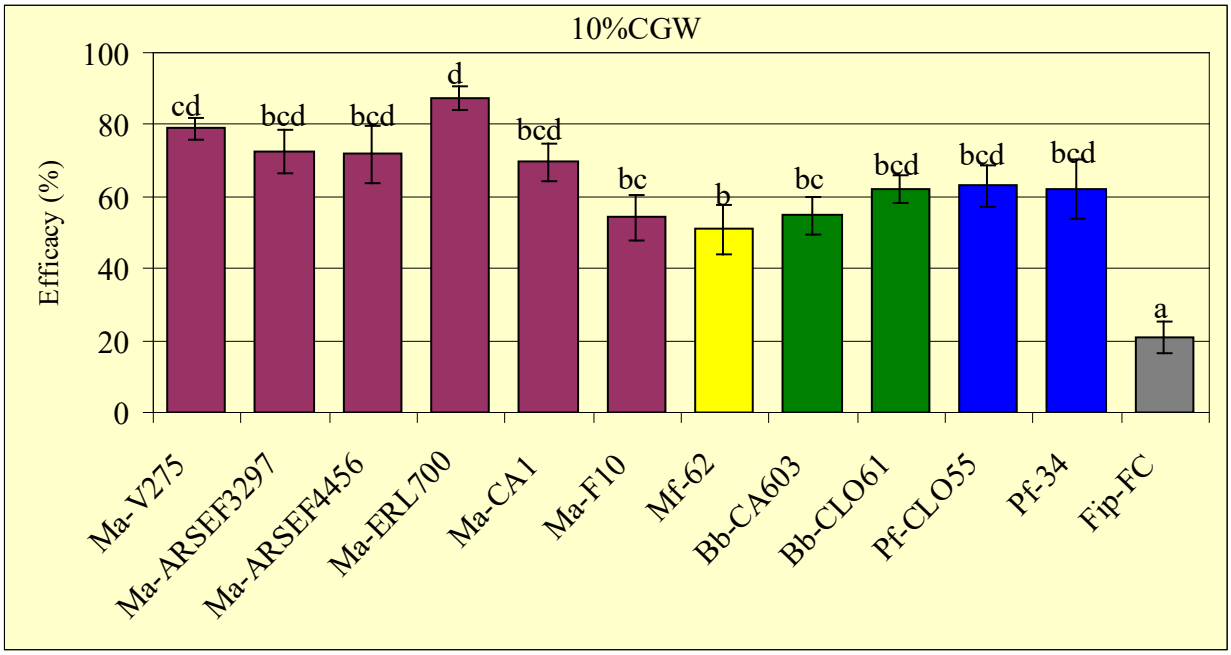

Fig 50. Thrips pupae mortality (% ± SE) in 10% CGW growth media. Treatments are same as described in Fig.47.

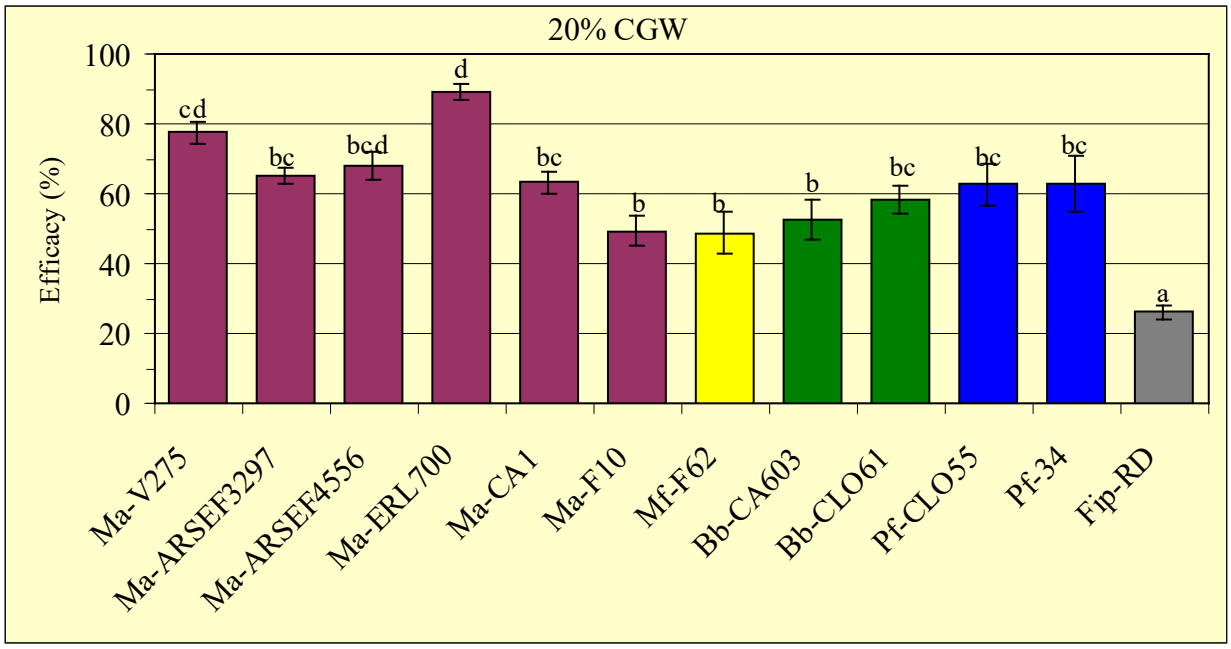

Fig 51. Thrips pupae mortality (% ± SE) in 20% CGW growth media. Treatments are same as described in Fig.47.

On average >50% of adults emerging from *M. anisopliae* treatments developed symptoms of mycosis. Post emergence infection was not observed in adults from the *B. bassiana* or *P. fumosoroseus* treatments (data not shown). Adult emergence rates from untreated media varied significantly in different growth media. Thrips survival was higher in bark than peat, as evidenced by the higher emergence rates. Thrips emergence was lowest in coir, 10% and 20% CGW (Fig. 52).

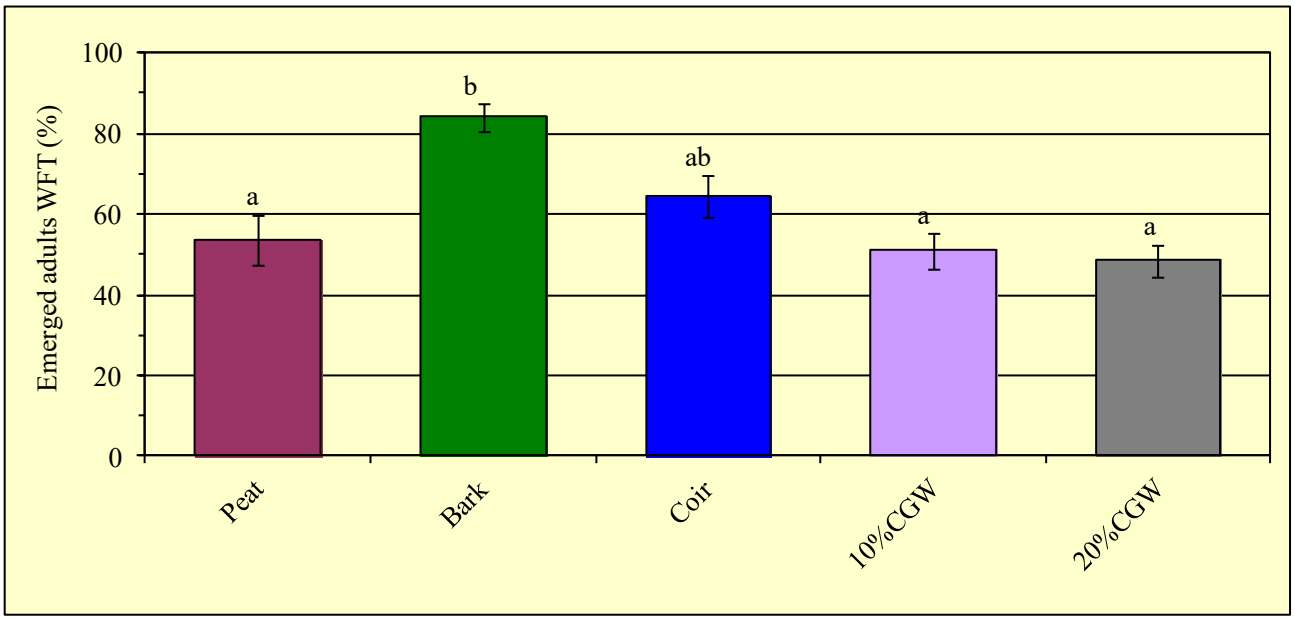

Fig 52. Adult thrips emergence from untreated growing media 11 days post inoculation. Means (± SE) with same letter are not significantly different by Tukey's test ($P < 0.05$).

Glasshouse experiments

Thrips mortality levels varied significantly among treatments ($P < 0.001$) and different growing media ($P < 0.007$). However, interactions between the treatments and growing media were not significantly different.

Thrips mortality levels of between 78-85% were obtained when *M. anisopliae* V275 was applied as a drench, or 84-91% when premixed in the different growing media. There was no significant difference in mortality levels when V275 was applied alone or in combination with reduced rates of imidacloprid or fipronil in all growing media (Figs. 53-57).

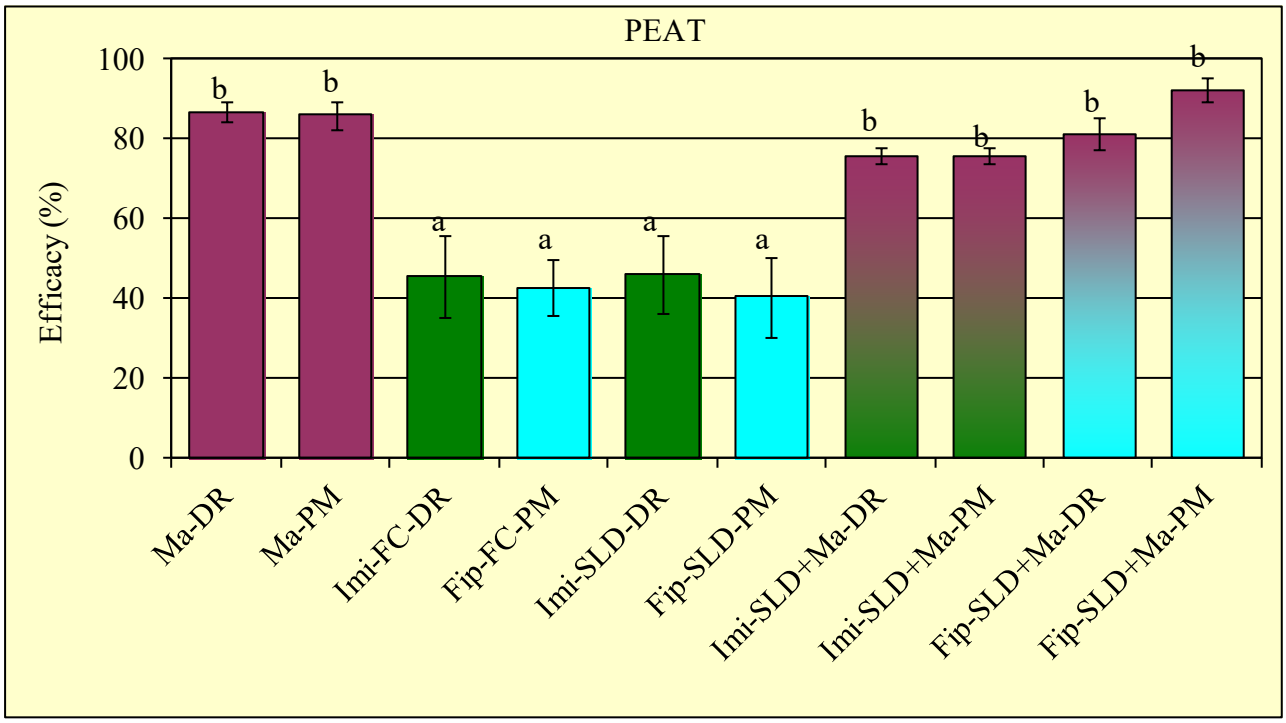

Fig 53. Thrips pupae mortality (mean % ± SE) in peat growth media treated with *M. anisopliae* V275 (1 × 10^{10} conidia/l medium) alone, a reduced rate or the recommended dose of imidacloprid or fipronil alone, or a combination of *M. anisopliae* and reduced rate of each insecticides. Ma-DR: *M. anisopliae* applied as drench, Ma-PM: *M. anisopliae* premixed, Imi-FC-DR: Imidacloprid applied as a drench at the recommended dose, Fip-FC-PM: Fipronil premixed at the recommended dose, Imi-SLD-DR: Imidacloprid applied as a drench at the reduced rate, Fip-SLD-PM: Fipronil premixed at the reduced rate, Imi-SLD+Ma-DR: Imidacloprid used at the reduced rate with *M. anisopliae* applied as a drench, Imi-SLD+Ma-PM: Imidacloprid used at the reduced rate with *M. anisopliae* premixed, Fip-SLD+Ma-DR: Fipronil used at the reduced rate with *M. anisopliae* applied as a drench, Fip-SLD+Ma-PM: Fipronil used at the reduced rate with *M. anisopliae* premixed. Means (± SE) with same letter (12 days post inoculation) are not significantly different by Tukey's test ($P < 0.05$).

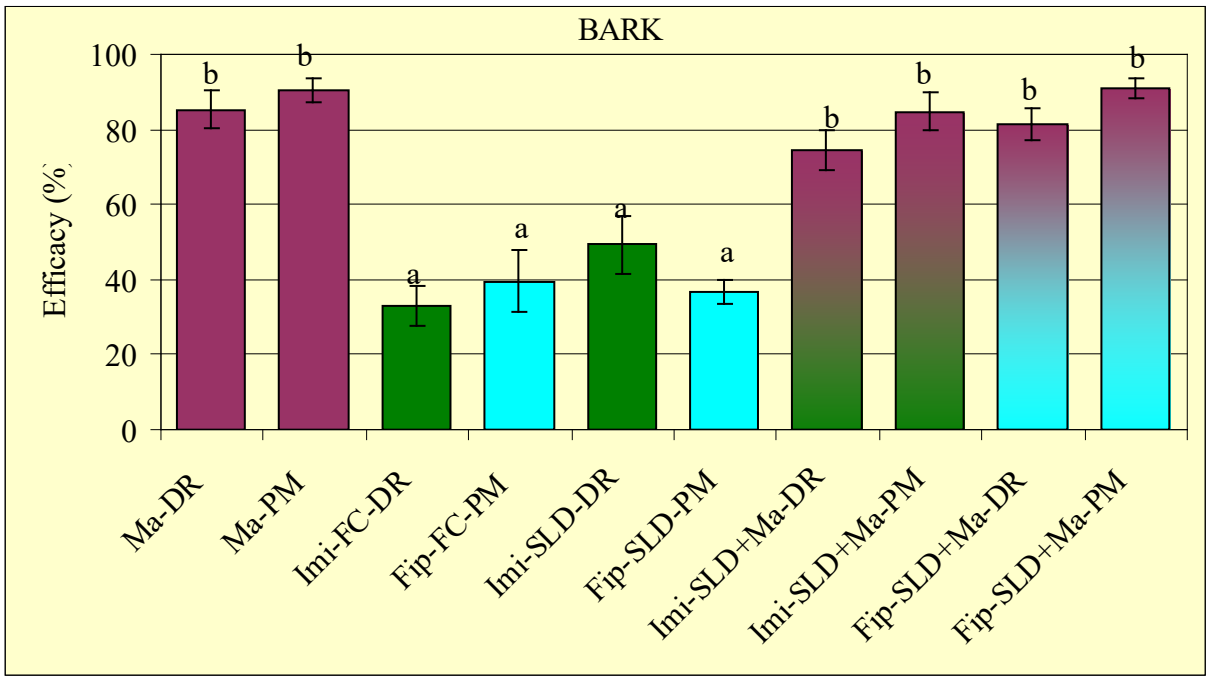

Fig 54. Thrips pupae mortality (% ± SE) in bark growth media. Treatments are same as described in fig. 53.

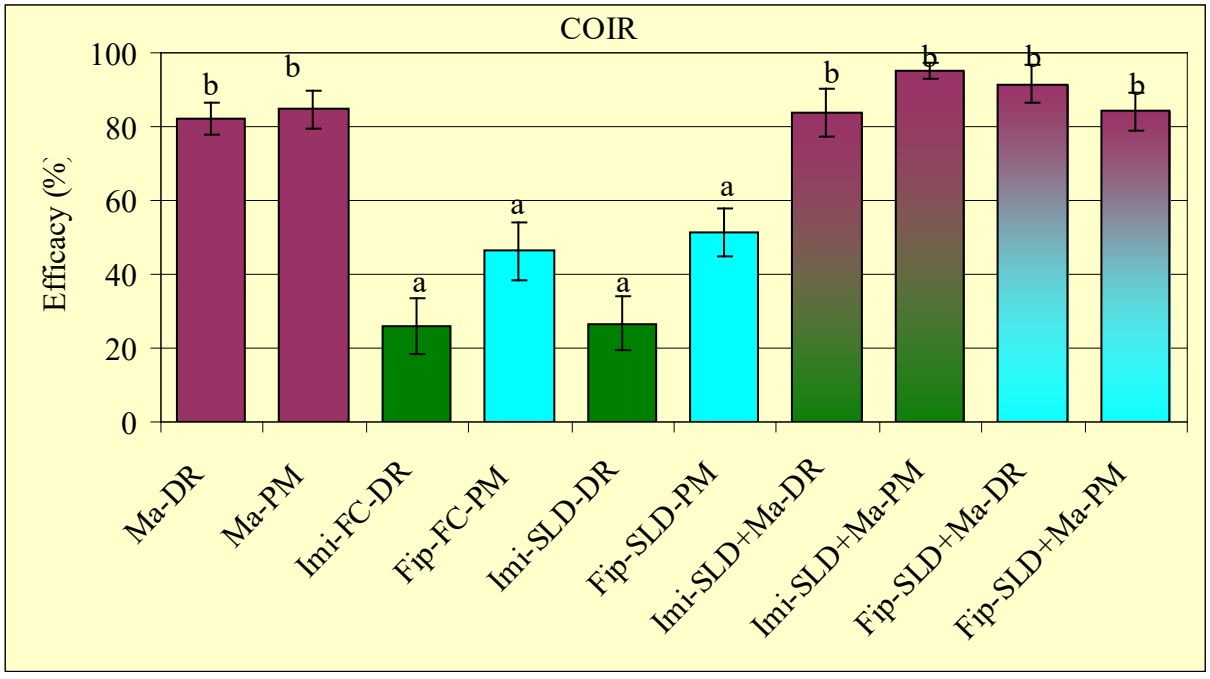

Fig 55. Thrips pupae mortality (% ± SE) in coir growth media. Treatments are same as described in fig.53.

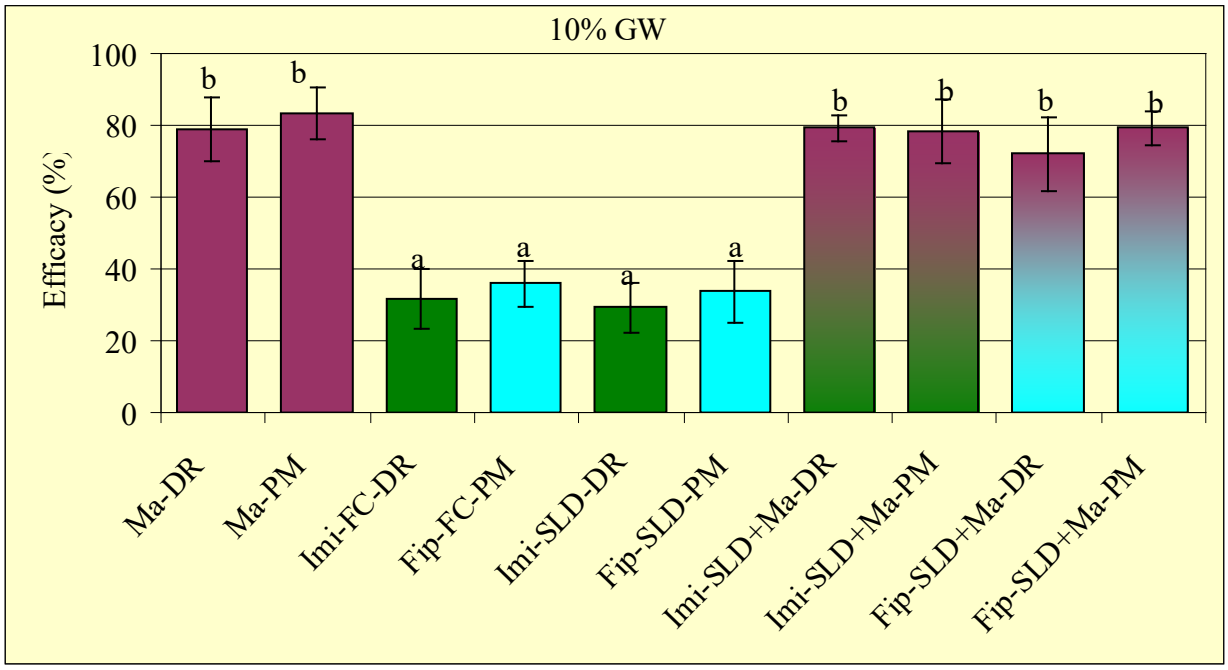

Fig 56. Thrips pupae mortality (% ± SE) in 10% CGW growth media. Treatments are same as described in Fig.53.

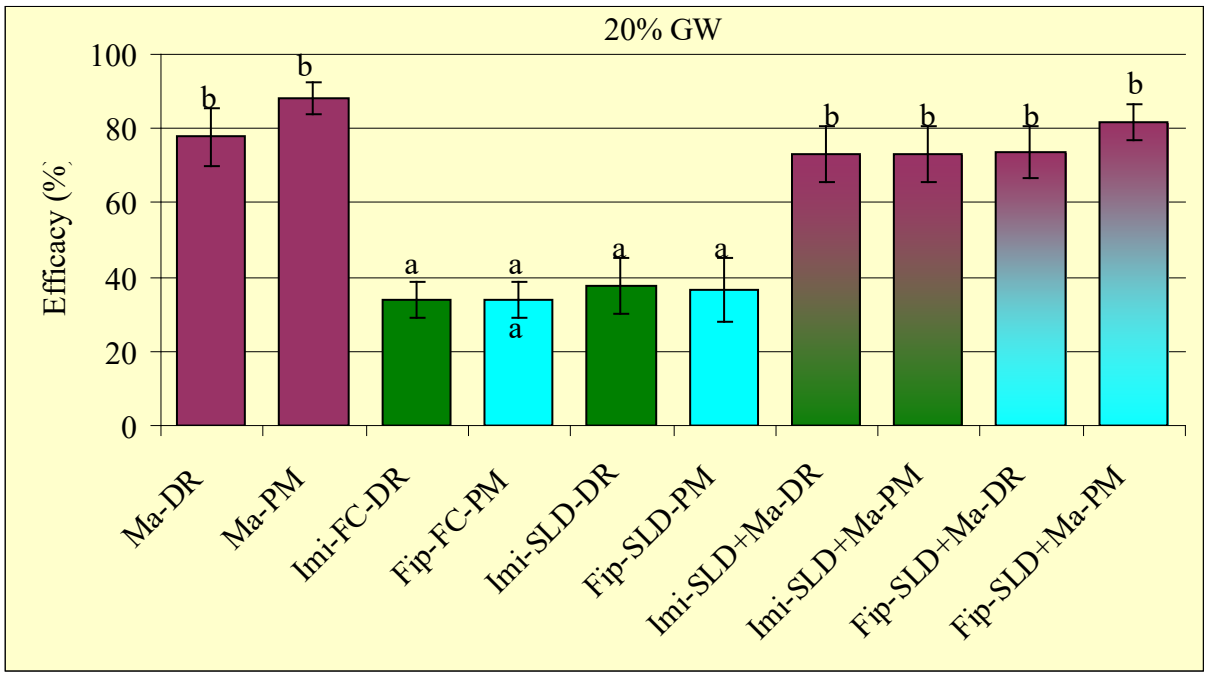

Fig 57. Thrips pupae mortality (% ± SE) in 20% CGW growth media. Treatments are same as described in fig.53.

In the untreated controls, adult emergence varied significantly among different growing media (Fig. 58). Highest thrips emergence rates were observed in bark (84.0% ± 3.4) and coir (64.5% ± 5.2), whereas the lowest emergence rates were observed in peat (53.5% ± 6.5), 10% CGW (51.0% ± 4.5) and 20% CGW (48.5% ± 4.0).

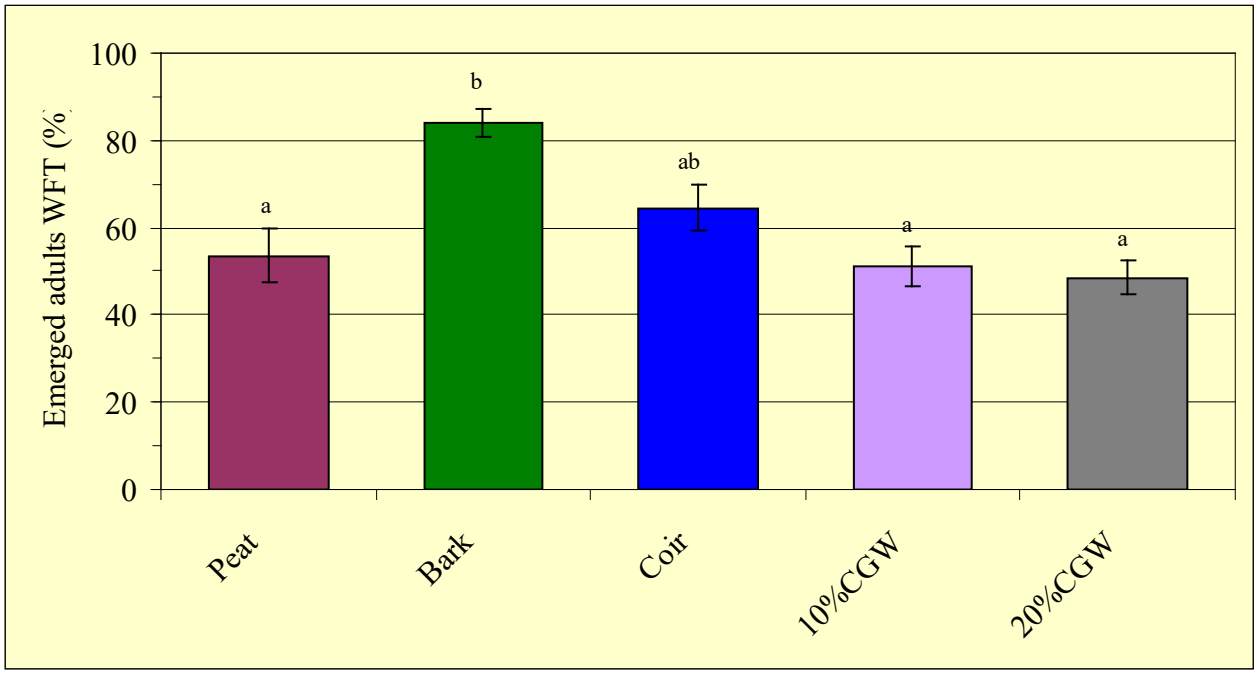

Fig 58. Adult thrips emergence from untreated growing media 12 days post inoculation.

Discussion

All the insect-pathogenic fungi assayed, irrespective of their original host or country of origin, caused higher thrips mortality than fipronil in all of the growing media tested. There is no clear explanation as to why the fungi performed better than the chemical insecticide but we can only presume that thrips prepupae and /or pupae are highly susceptible to fungal infection. We report significantly higher mortality by pre-mixing the fungal pathogens into the growing medium, which suggests that sufficient inoculum is being acquired by larvae as they move into the medium to pupate. A more likely scenario is that freshly moulted prepupae and/or pupae acquire sufficient conidia in the growing media to become infected. Wraight *et al*. (2004) found that by spraying late instar Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) with *Beauveria bassiana*, good control was achieved with high levels of pupal mortality. It is tempting to speculate that insects are more susceptible to fungal infection when undergoing metamorphosis. Indeed, Zacharuk and Tinline (1968) noted that pupae of five Elaterids were highly susceptible to *M. anisopliae* infection. However, it is also possible that the growing media or soils offer a more

conducive environment for infection because they, unlike the crop canopy, are buffered against dramatic fluctuations in temperature and humidity.

Interestingly, we did not observe any additive or synergistic interactions between *M. anisopliae* and reduced rate of insecticides. Imidacloprid is largely utilised as a systemic insecticide and has relatively low efficacy in soil (Elbert *et al*., 1991). However, other workers have demonstrated synergistic effects when using *M. anisopliae* or *B. bassiana* with sub-lethal doses of imidacloprid for control of subterranean pests (Quintela and McCoy, 1998; Jaramillo *et al*., 2005). Limited contact with insecticides in treated media can inhibit their effectiveness. Uneven mixing and/or avoidance of high concentration zones by the insect would limit insect movement and contact with the toxic material. This has been demonstrated for other soil-dwelling insects, with associated impacts on efficacy (Thompson and Brandenburg, 2005; Kepler and Bruck, 2006).

Only a few adult thrips emerged from the various treatments, and half of these ultimately died from mycosis. While it is possible that these adults acquired inoculum following emergence as they moved through the growing medium, the time interval from emergence to death was short. This suggests that infection may have been acquired in the larval stage; larvae that acquired fewer conidia when moving into the growing medium would take longer to die than those contacted by high numbers of conidia as mortality is dose-related. It is possible that these individuals survived through to adulthood but succumbed shortly thereafter.

The type of growing medium used also influences adult emergence. More adults usually emerged from bark media than the other growth substrates tested. The physical characteristics of different media and their different water-holding capacities undoubtedly also play a role in thrips survival. Helyer *et al*. (1995) and Ebssa *et al*. (2001) reported 60-65% adult emergence from untreated compost, suggesting that thrips emergence from compost can be rather low due to suffocation. In media with a more ‘open’ structure, i.e., bark-based, aeration is likely to be greater and it becomes less waterlogged than those of a more compact nature, e.g. peat blends. This open structure is more likely to benefit thrips survival. Bark media also appear to favour better survival and subsequent emergence of black vine weevil (Buxton, 2003) and sciarids (unpublished observations).

Both laboratory and glasshouse trials show that *M. anisopliae* V275 is highly efficacious in controlling thrips pupae in a range of growing media (Ansari *et al*., 2007). The fact that it is also effective in controlling BVW larvae (Shah *et al*., 2007) clearly demonstrate it’s potential for use in integrated pest management programmes.

Trials at Johnsons of Whixley Nurseries, York.

Trials conducted at Johnsons of Whixley, nurseries were aimed at demonstrating the efficacy and robustness of *M. anisopliae* against BVW with following specific objectives.

1. To demonstrate *M. anisopliae* against BVW under commercial plant production system.
2. To compare *M. anisopliae* efficacy in two different plant growth media which included Johnson of Whixley's potting mix of peat and bark (75:25) and commercial bark (B&Q).
3. To determine the influence of *M. anisopliae* application method on its efficacy.
4. To compare *M. anisopliae* efficacy with that of Vi-nil (fipronil) and Exemptor (thiacloprid)

Materials and Methods

Fungal inoculum, insecticides and BVW eggs were obtained and applied as described in section 8.1. A mixture of peat and bark (75:25) medium is routinely used at Johnsons and Whixley nurseries; therefore, it was used along with commercial bark based media to compare *M. anisopliae* in these media. Two different plants i.e. *Euonymus fortune*i "Emerald Gold" and *Fuchsia* were kindly provided by Johnsons of Whixley. Plants were arranged in completely randomized block design with each plant adequately spaced to avoid cross contamination. Rest of the procedure and materials were same as used in studies at Swansea University.

Results and Discussion

All treatments were highly efficacious against BVW in potted *Fuchsia* (Fig. 59). Irrespective of its application method, *M. anisopliae* provided over 90% protection against BVW. Efficacy of *M. anisopliae* varied also varied non-significantly with that of Vi-nil and Exemptor. No larvae were observed in any of the *Euonymus* plants. All materials and methods, except for BVW eggs, were the same for *Euonymus* and *Fuchsia* plants, therefore, it is highly likely that the batch of BVW eggs used in *Euonymus* was non-viable. This hypothesis is also supported by the fact that similar sort of results were observed at Bell Brothers nurseries, Boston, where the same batch of BVW eggs was used to infest strawberry plants. Since this problem was observed for the first time during this project, further studies are needed to determine factors causing such loss of viability in BVW eggs.

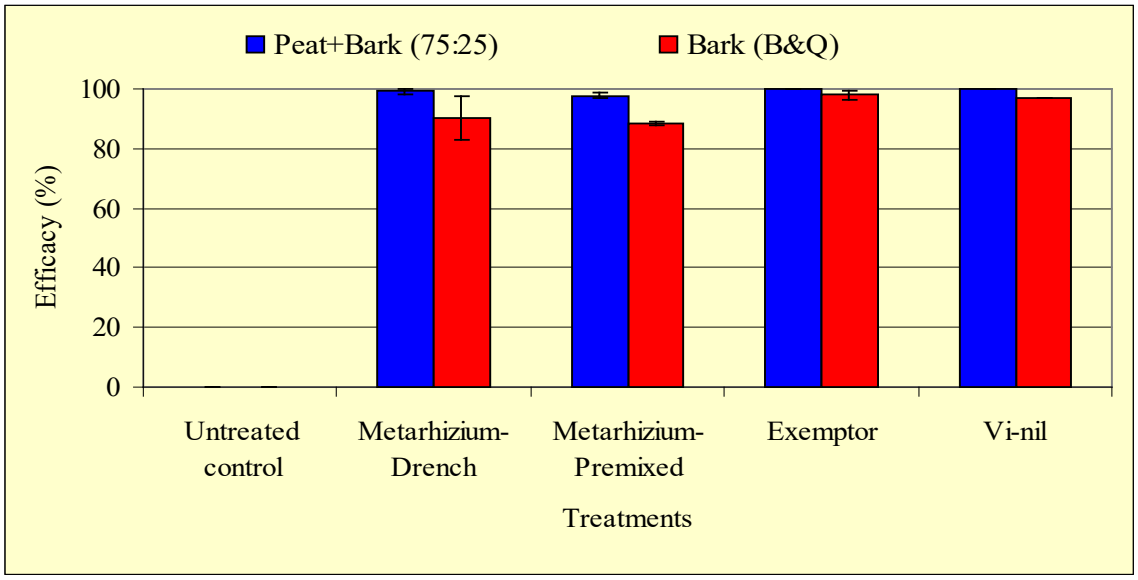

Fig. 59 Efficacy (%) of *M. anisopliae* and insecticides against BVW in *Fuchsia* potted in peat + bark (75:1) or bark alone. The treatments are listed below. No treatment (control), *M. anisopliae* applied as drench or premixed in media, Thiacloprid (Exemptor) and Fipronil (Vi-Nil) premixed at the recommended dose.

Trials at Delfland Nurseries, March, Cambridgeshire

Our earlier studies showed that *M. anisopliae* V275 was compatible with entomopathogenic nematodes, the objective of this trial was demonstrate that these two biocontrol agents could be used together to eradicate overwintering BVW larvae.

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.

Experimental procedure

Young strawberry plants (c.v. Elsanta) were 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 five 3rd-instar BVW larvae 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 and Discussion

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. 60). 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. 61).

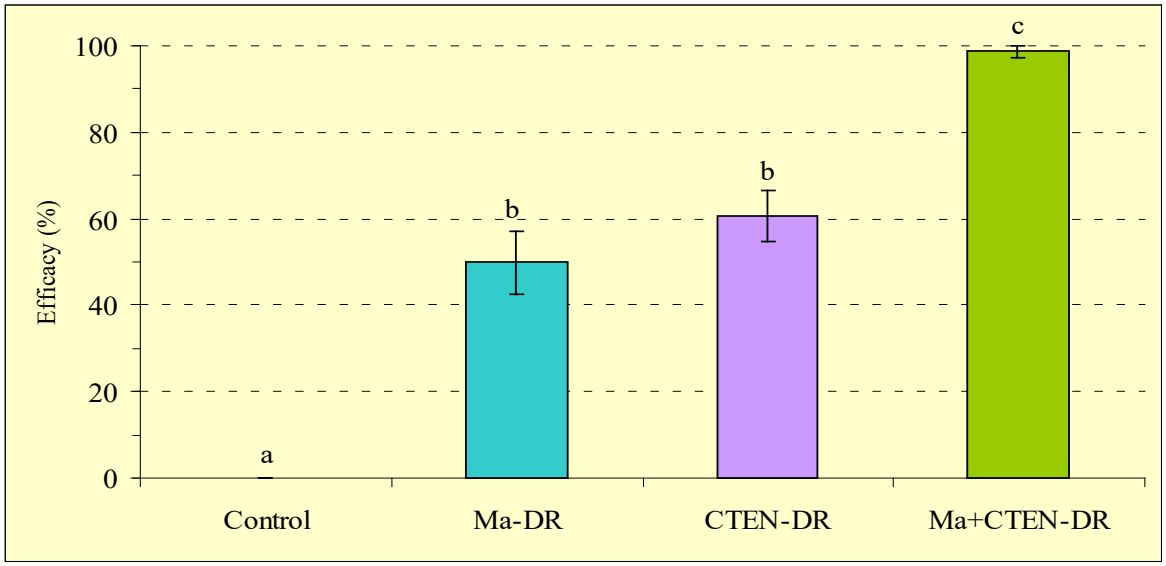

Fig. 60. Efficacy (%) of *M. anisopliae* and cold tolerence nematode (CTEN) against BVW in strawberry growbags. Treatments are listed below. No treatment (control), *M. anisopliae* applied as a drench (MaDR), nematodes applied as a drench (CTEN-DR), nematodes and fungus applied as a drench (Ma+CTEN-DR). Each strawberry plant was infested with five 3rd instar BVW larvae and destructively assessed 10 weeks post inoculation. Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.001$).

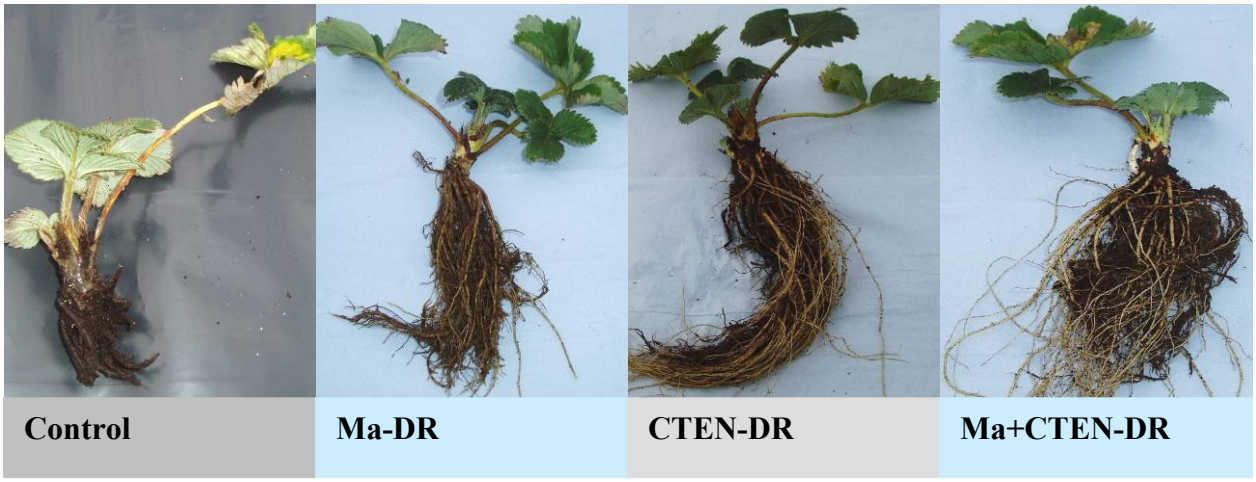

Fig. 61. Comparison of the root system of strawberry plants exposed to the following treatments: No treatment (control), *M. anisopliae* applied as a drench (MaDR), Nematodes applied as a drench (CTEN-DR), Nematodes and fungus applied as a drench (Ma+CTEN-DR). Each strawberry plant was infested with five 3rd instar BVW larvae and destructively assessed 10 weeks post inoculation.

Trials at Wallings Nursery, Essex

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 over wintering BVW larvae.

Experimental procedure

Young strawberry plants (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 five 3rd-instar BVW larvae 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. 62). 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. 63). All the larvae recovered from *M. anisopliae* treated plants ultimately died of fungal infection suggesting slow progression of infection at low temperatures.

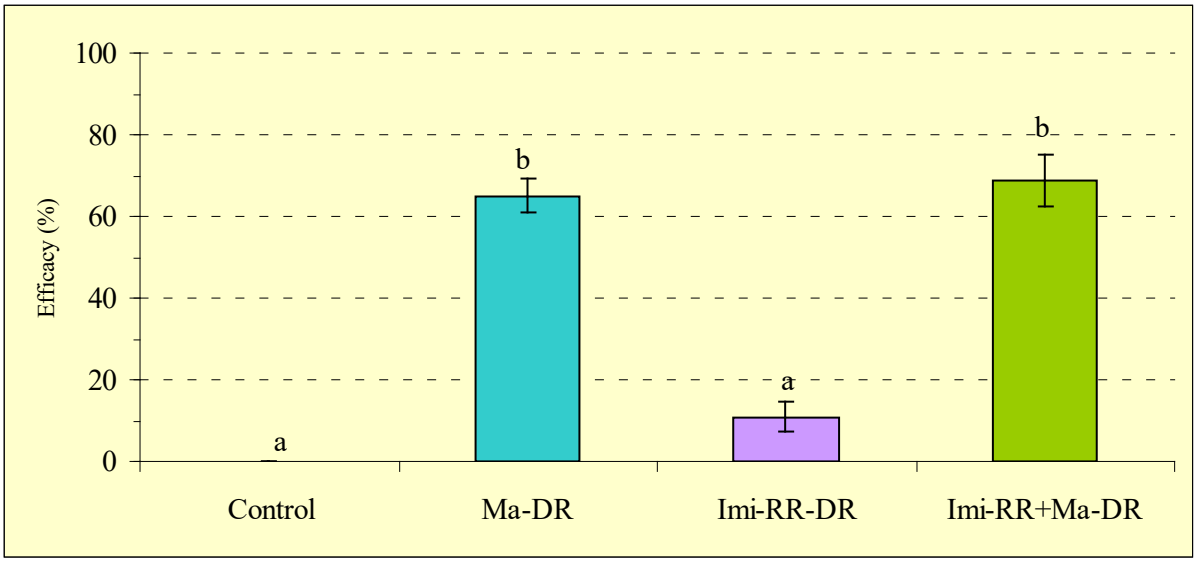

Fig. 62. Efficacy (%) of *M. anisopliae* and insecticide against BVW in strawberry growbags. Treatments are listed below. No treatment (control), *M. anisopliae* applied as a drench (Ma-DR), imidacloprid applied as drench at the reduced rate (Imi-RR-DR), combined application of fungus and reduced rate of insecticide applied as a drench (Imi-RR+Ma-DR). Each strawberry plant was infested with five 3rd instar BVW larvae and destructively assessed 10 weeks post inoculation. Bars displaying same letter vary non-significantly (Tukey's test, $P < 0.001$).

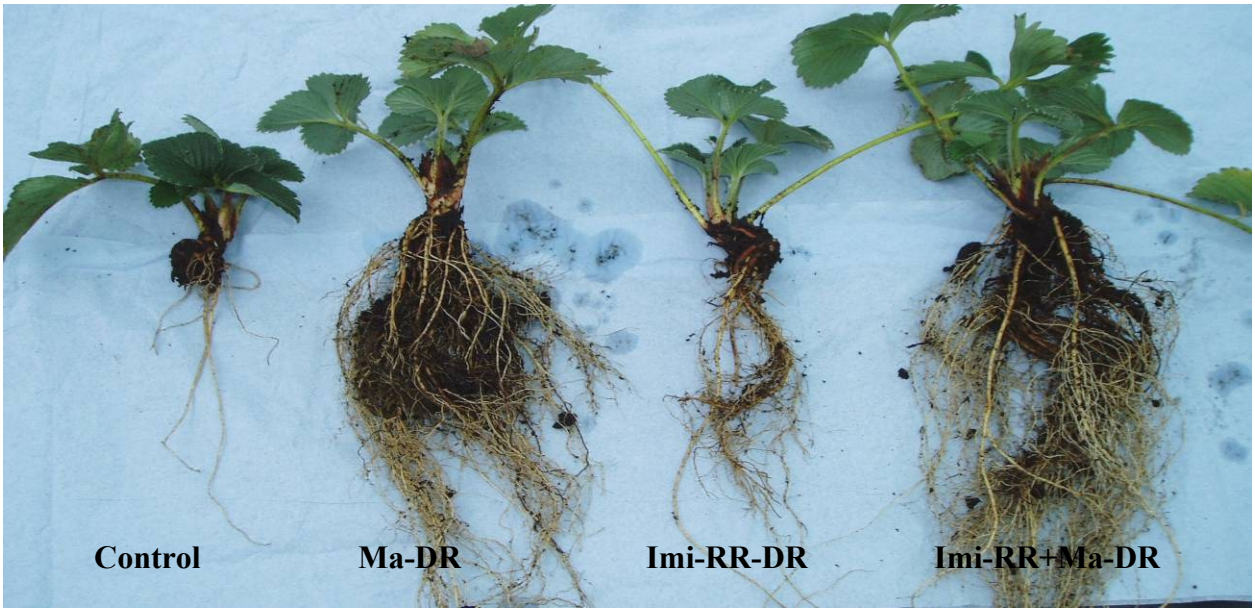

Fig. 63. Comparison of the root system of strawberry plants exposed to the following treatments. From left: No treatment (control), *M. anisopliae* applied as a drench (Ma-DR), Imidacloprid applied as drench at the reduced rate (Imi-RR-DR), combined application of fungus and reduced rate of insecticide applied as a drench (Imi-RR+Ma-DR). Each strawberry plant was infested with five 3rd instar BVW larvae and destructively assessed 10 weeks post inoculation.

The grower trials demonstrated that the efficacy of *M. anisopliae* was independent of plant growth media and plant species. These trials also showed its robustness under different environmental and cultural conditions. Furthermore, *M. anisopliae* worked synergistically with entomopathogenic nematodes in eradicating over-wintering BVW larvae. These findings will reassure growers that *M. anisopliae* is efficacious and could easily be used in combination with their existing pest control strategies or as a replacement to chemicals which are no longer available or effective.

Technology Transfer section:

1. <u>Industrial relevance and plans for future commercial exploitation</u>

A. There is clearly a market for the insect-pathogenic fungus *Metarhizium anisopliae*. It offers a benign alternative to chemical pesticides which are being withdrawn (e.g. Fipronil was withdrawn recently) or to which the pests have developed resistance. Strain V275 has shown to be highly efficacious against black vine weevil larvae and western flower thrips pupae in a wider a range of ornamentals and strawberries.

Steps are currently underway to commercialise *M. anisopliae* V275.

i. Dossier was submitted November 2005 by two companies to place it on the Annex 1 list. This is still under evaluation in the EU.
ii. Koppert (a member of this project) and another company outside the consortium have shown an interest in registering V275 in the UK.
iii. Swansea University has ORETO status now, therefore, any efficacy data generated could be used to help register *M. anisopliae* in the UK
iv. Negotiations are underway with the company outside the consortium to generate efficacy data to help with registration of V275 in the UK. Koppert and the other company will produce different products.
v. Following the positive results of the project, Koppert is committed to the commercialisation of *M. anisopliae* in the UK and will continue working with Dr Butt in the development of this fungus. Koppert has identified a number of critical steps to be taken in developing *M. anisopliae* and have, therefore, agreed to fund further work at Swansea University (until March 2009) to provide commercially sensitive information regarding commercialisation of *M. anisopliae*.
vi. Swansea University will maintain its link with consortium members to address fundamental and applied aspects linked with commercialization of *M. anisopliae* in UK. This “in house” work will be done by December 2008 with support from undergraduate students (summer projects).
vii. Swansea University with Koppert have been developing an advisory leaflet for growers. This leaflet would be produced in collaboration with HDC. It will explain how best to use V275 for control of BVW larvae and WFT pupae in horticultural growing media. UK growers will be better prepared in utilising the *Metarhizium*-based product as soon as it reaches the market. BCA producers will benefit for the rapid uptake of the product.

B. Industrial partners are interested in exploiting project outputs. For example, Bord na Mona will consider how to exploit the approaches used for the microbial study to determine the role of microbes in composting or for quality control purposes.

C. Producers of microbial products e.g. Symbio, Koppert will be able to use our leaching protocols to determine percolation of inoculum through media. The protocols will help in design of better formulations and/or application strategies to minimise leaching of inoculum.

D. Once *M. anisopliae* has been registered, data on synergistic interactions between *M. anisopliae* and EPN or low dose chemicals will help growers reduce input of chemical pesticides, reduce costs for pest control and increase the premium they get for their crops.

E. Growers and industry would benefit in other ways, such as good publicity for reducing or eliminating inputs of chemical pesticides also for reducing exposure of operators and customers to harmful insecticides. Reducing or eliminating chemical inputs will help promote biodiversity which is appealing to consumers.

F. *Metarhizium* will help generate more income for soft fruit growers – since no chemical residues allow for continuous harvesting of crops susceptible to BVW attack (e.g. strawberries, raspberries, blackcurrants).

G. We have shown that *M. anisopliae* can be applied as a drench or premixed into growing media. This gives growers' more choice. Drench applications can be used for spot treatment or to charge untreated growing media. Compost producers like Bord na Mona could sell growing media containing *M. anisopliae* conidia. Advisory leaflets would be produced, with help from industry and HDC, providing advice to growers on how best to deploy *M. anisopliae* in different production systems.

2. <u>Financial benefits</u>

Our findings clearly showed that there is scope for reducing the cost of insect pest control. Once *M. anisopliae* is registered in the UK, growers will be able to reduce the cost of BVW control by:

- Using *M. anisopliae* alone and getting a premium for "organically"produced plants.
- Combining *M. anisopliae* with low dose chemicals or entomopathogenic nematodes, thus allowing each component to be used at a lower application rate (at least 50% and potentially 99%). This results in a corresponding decrease in cost.

Other direct and indirect financial benefits:

- *M. anisopliae* is far superior to chemical pesticides for controlling thrips pupae, thus contributing significantly to IPM programmes for thrips control.
- There are few or no concerns with disposal of spent media because *M. anisopliae* has no environmental risk (unlike chemical pesticides which may leach into groundwater).
- *M. anisopliae* (± entomopathogenic nematodes) will benefit organic growers in many sectors of horticulture (i.e. soft fruit, indoor ornamentals, hardy nursery stock) where BVW and thrips are major pests.
- We have shown that some chemical pesticides can be used at lower than recommended rates (e.g. Vi-nil can control BVW at 1/10th of the recommended rate), thus saving growers money and concomitantly reducing pesticide inputs.

3. Action points for growers

- Lobby industry and PSD to accelerate registration of *M. anisopliae* in the UK.
- Support researchers by helping with registration-related matters. For example, creating groups to generate risk assessment or efficacy data.
- Support researchers to develop protocols which will allow for the rapid uptake of *M. anisopliae,* once the product is registered. These protocols must reveal:

    1. What other pests (e.g. sciarids) could be controlled by *M. anisopliae* providing even more value for money.
    2. How to optimise control of the different pests at reduced cost e.g. incorporation of *M. anisopliae* and efficacy enhancing agent in plant plugs.
    3. Compatibility of *M. anisopliae* with other agents e.g. beneficial predators and parasitoids, fungicides used for disease control
    4. The efficacy of the different application methods: e.g. drip versus overhead irrigation, hydraulic versus electrostatic sprayers.
    5. The efficacy of different formulations – conidia versus conidia on grain (significantly cheaper product)

- Develop workshops to facilitate rapid uptake of products once they reach the market (no time lag).

The protocols and workshops would give growers more insight into the attributes of the biocontrol agents (BCA) and ways to get more “value for money”. .Rapid uptake of the new technologies will ensure growers are competitive in the international arena.

ACKNOWLEDGMENTS

Swansea University acknowledges the support provided by Defra, the Horticultural Development Council, Koppert Biological Systems, Bord na Mona, W. Godfrey & Sons, Johnsons of Whixley, Symbio, and Blue Xylem as part of a Horticulture LINK project (HL0171).

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265-267

Amiri B., Ibrahim L.and Butt T.M. (1999). Antifeedant properties of destruxins and their potential use with the entomogenous fungus *M. anisopliae anisopliae* for improved control of crucifer pests. Biocontrol Sci. Tech. 9:487-498.

Anderson J.P.E. and Domsch K.H. (1978). A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biology and Biochemistry 10: 215-221

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

Ansari, M.A., Shah, F.A., Tirry, L. and Moens, M. (2006). Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control 39: 453-459.

Ansari, M.A., Tirry, L., Vestergaard, S. and Moens, M. (2004). Selection of a highly virulent fungal isolate, *M. anisopliae anisopliae* CLO 53, for controlling *Hoplia philanthus*. J. Invertebr. Patholo. 85:89-96

Ascher, K.R.S. (1993). Nonconventional insecticidal effects of pesticides available from the neem tree, *Aradirachta indica*. Arch. Insect Biochem. Physiol., 22: 433-449.

Ashley, M. W. (2002). Understanding the carbon-fertiliser link. Paper presented at the Anniversary Symposium, Research Station of Crop Culture, 16th October 2002. http://www.lifeforcetm.com/carbon_fertiliser_link.pdf

Blaeser, P., Sengonca, C. and Zegula, T. (2004). The potential use of different predatory bug species in the biological control of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae). J. Pest Sci 77:211-219.

Boucias, D., Stokes, G., Storey, C., G. and Pendland, J.C. (1996). The effect of imidacloprid on the termite *Reticultermers flavipes* and its interaction with the mycopathogen *Beauveria bassaiana*. Pflanzenschutz Nachr. Bayer 49:103-144.

Brownbridge, M., (1995). Prospect for mycopathogens in thrips management. In: Parker, M. Skinner, M., Lewis, T. (eds), thrips biology and management. New York Plenum Press, USA. 281-295.

Bruck, D.J. (2005). Ecology of *M. anisopliae anisopliae* in soilless potting media and the rhizosphere: implications for pest management. Biol. Control. 32:155-163.

Butt, T.M. (1997). Complementary techniques: Fluorescence Microscopy. In: Lacey, L.A (Ed), Manual of techniques in insect pathology. Academic Press, San Diego, USA.355-365.

Butt, T. M. (2002). Use of entomogenous fungi for the control of insect pests, In: Esser, K, Bennett, J. W.(Eds), Mycota, Springer-Verlag, Berlin.111- 134.

Buxton, J. (2003). Vine weevil control in hardy nursery stock. Horticultural Development Council Factsheet 02/03.

Cardinale, M.L. Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C. and Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl Environ Microbiol. 70: 6147-56.

Chandler, D. and Davidson, G. (2005). Evaluation of entomopathogenic fungus *M. anisopliae anisopliae* against soil-dwelling stages of cabbage maggot (Diptera: Anthomyiidae) in glasshouse and field experiments and effect of fungicides on fungal activity. J. Eco.Entomolo. 98; 1856-1862.

Classen, A.T., Boyle, S.I., Haskins, K.E., Overby, S.T. and Hart, S.C. (2003). Community level physiological profiles of bacteria and fungi: plate type and incubation temperatures influences on contrasting soils. FEMS Microbiology Ecology 44: 319-328.

Cowles, R.S. (2004). Impact of azadirachtin on vine weevil (Coleoptera: Curculionidae) Reproduction. Agri. Forest Entomol. 6: 291-294

Cross, J. V. and Burgess, C. M. (1997). Localised insecticide treatment for the control of Vine Weevil larvae (*Otiorhynchus sulcatus)* on field-grown strawberry. Crop Protection 16: 565-574.

Ebssa, L., Borgemeister, C., Berndt, O. and Poehling, H.M. (2001). Efficacy of entomopathogenic nematodes against soil-dwelling life stages of western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae). J. Invertebr. Pathol. 78: 119-127.

Elbert, A., Becker, B., Hartwig, J. and Erdelen, C. (1991). Imidacloprid-einneues systemisches Insektizid. Pflanzenschutz Nachr. Bayer 44: 113-136.

Finney, D.J. (1964). “Probit analysis” Cambridge University Press, London

Fitters, P.F.L., Dunne, R. and Griffin, C.T. (2000). Vine weevil control in Ireland with entomopathogenic nematodes: optimal time and frequency of application. Irish J. Agr. Food Res. 40:199-213

Fukui, R. Fukui, H. and Alvarez, A.M. (1999). Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums. Appl Environ Microbiol. 65: 1020–1028

Gamo, M. and Shoji, T. (1999). A method of profiling microbial communities based on a most-probable-number assay that uses BIOLOG plates and multiple sole carbon sources. Appl. Environ. Microbiol. 65: 4419-4424.

Georgis, R., Koppenhöfer, A.M., Lacey, L.A., Bélair, G., Duncan, L. W., Grewal, P. S., Samish, M., Tan, L., Torr, P. and van Tol, R.W.H.M. (2006). Successes and failures in the use of parasitic nematodes for pest control. Biol. Control 38: 103-123.

Goettel, S. and Inglis, G.D. (1997). Fungi: Hyphomycetes. In: Manual of Techniques in Insect Pathology. Lacey, L.A. (Ed.), Academic Press, San Diego, pp. 213-249.

Harrison, I., Laverty, M. and Sterling, E. (2004). Alpha, Beta, and Gamma Diversity Connexions module: m12147 (http://cnx.org/content/m12147/latest/)

Heinemeyer, O., Insam, H., Kaiser, E.A. and Walenzik, G. (1989). Soil microbial biomass and respiration measurements: An automated technique based on infra-red gas analysis. Plant and Soil 116: 191-195

Herron, G. A. and James, T.M. (2005). Monitoring insecticide in Australian *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) detects fipronil and spinosad resistance. Australian J. Entomol. 44:299-303.

Helyer, N.L., Brobyn, P.N. and Edmondson, R.N., (1995). Control of western flower thrips (*Frankliniella occidentalis* Pergande) pupa in compost. Ann. Appl. Biol. 127:405-412.

Hoitink, H.A.J. and Boehm, M. J. (1999). Biocontrol within the context of soil microbial communities:a substrate-dependent phenomenon. Annual Review of Phytopathology 37:427–46

Hopkins, D.W., Macnaughton, S.J. and O'Donnel, A.G. (1991). A dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. Soil Biology and Biochemistry 23: 217-225

Inglis, D.G., Goettel, M.S., Butt T.M. and Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In: Butt T.M., Jackson, C.W., Magan, N., (Eds), Fungi as biocontrol agents: Progress, Problems and Potential. CABI, Wallingford, UK. 23-69.

Jacobson, R.J., Chandler, D., Fenlon, J. and Russell, K.M. (2001). Compatibility of *Beauveria bassiana* (Balsaamo) Vuillemin with *Amblyseius cucumeris* Oudemans (Acarina: Phytoseiidae) to control *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) on cucumber plants. Biocontrol Sci. Technol. 11:391-400.

Jaramillo, J., Borgemeister, C., Ebssa, L., Gaigl, A., Tobón, R. and Zimmermann, G. (2005). Effect of combined applications of *M. anisopliae anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) strain CIAT 224 and different dosage of imidacloprid on the subterranean burrower bug *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae). Biol. Control. 34: 12-20.

Jensen, S.E. (2000). Insecticides resistance in the western flower thrips, *Frankliniella occidentalis*. Integr. Pest Manag. Review 5: 131-146.

Kakouli-Duuarte, T., Labuschagne, L. and Hague, N.G.M. (1997). Biological control of the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic nematodes (Nematoda : Rhabditida). Ann. Appl. Biol. 131: 11-27.

Kaya, H.K. and Stock, S.P. (1997). Techniques in insect nematology. In: Manual of Techniques in Insect Pathology. Lacey, L.A. (Ed), Academic Press, San Diego, CA, pp. 281-324.

Kepler, R.M. and Bruck, D.J. (2006). Examination of the interaction of the black vine weevil (Coleoptera: Curculionidae) and an entomopathogenic fungus reveals a new tritrophic interaction. Environ. Entomology 35: 1021-1029.

Kirk, W. D.J. and Terry, L. I. (2003). The spread of the western flower thrips *Frankliniella occidentalis* Pergande. Agric. Forest Entomol. 5: 301-310.

Koppenhöfer, A.M., (2000). Nematodes. In: Lacey, L., Kaya, H.K., (Eds.), Field Manual of Technique in Invertebrate Pathology. Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 283-301.

Lasaridi K.E. and Stentiford, E.I. (1996) Respirometric Techniques in the Context of Compost Stability Assessment: Principles and Practice, The Science of Composting part 1, Ed. Marco be Bertoldi, Paolo Sequi, Bert Lemmess, Tiziano Pape. Blackie Academic and professional London. pp. 1-659

LeOra Software (1987). POLO-PC: A user's guide to probit for logit analysis. LeOra software, Berkeley, CA.

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

Lola-Luz T., Downes, M. and Dunne, R. (2005). Control of Black vine weevil *Otiorhynchus sulcatus* (Fabricus) (Coleoptera: Cruculionidae) in grow bags outdoors with nematodes. Agr. forest Entomol. 7: 121-126.

Masaki, M., Ohmura, K. and Ichinohe, F. (1984). Host range studies of the black vine weevil *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae). Appl. Entomol. Zool. 19: 95-106.

McVay, J.R., Gudauskas, R.T. and Harper, J.D. (1977). Effect of *Bacillus thuringiensis* nuclear-polyhdrosis virus mixtures on *Trichoplusia ni* larvae. J. Invertebr. Pathol. 29: 367-372.

Monidini, C. and Insam, H. (2003). Community level physiological profiling as a tool to evaluate compost maturity: a kinetic approach. European J. Soil Biology 39: 141-148

Moorhouse, E.R., Charnley, A.K. and Gillespie, A.T. (1992). A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Ann. Appl. Biol. 121: 431–454.

Moorhouse, E.R., Easterbrook, M.A., Gillespie, A.T. and Charnley, A.K. (1993). Control of *Otiorhynchus sulcatus* (*Fabricius*) (Coleoptera: Curculiondae) larvae on a range of hardy ornamental nursery stock species using the Entomopathogenic fungus *M. anisopliae anisopliae*. Biocontrol Sci. Technol. 3: 63-72.

Mordue (Luntz), A. J., and Blackwell, A., (1993). Azadirachtin: an update. J insect Physiology 39: 903-924.

Musabyimana, R. Saxena, C., Kairu, E.W., Ogol, C.P.K.O., and Khan, Z.R. (2001). Effects of Neem seed Derivatives on behavioural and physiological responses of the *Cosmopolites sordidus* (Coleoptera: Curculionidae) J. Eco. Entomol. 94: 449-454.

Newmeyer, D. (1990). Filtering small quantities of conidial suspensions to remove mycelial fragments. Fungal genetics newsletter 37.

Quintela, E.D. and McCoy, C.W. (1998). Conidial attachment of *M. anisopliae anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) treated with imidacloprid. J. Invertebr. Pathol.72: 220-230.

Ranjard, L. Poly, F., Lata, J.C., Mougel, C., Thioulouse, J. and Nazaret, S. (2001). Characterisation of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl. Environ. Microbiol. 67: 4479-4487

Roditakis, E., Couzin, I.D., Barlow, K., Franks, N.R. and Charnley, A.K. (2000). Improving secondary pick up of insect fungal pathogen conidia by manipulating host behaviour. Ann., Appl. Biol. 137: 329-335.

Schmutterer, H. (1990). Properties and potential of natural pesticides from the neem tree, *Amdirachta indica.* Ann. Rev. Entomol. 3: 27 1-297.

Schnitzer, M. (1982). Organic Matter Characterization 581-594 Methods of soil analysis part 2. Chemical and Microbial Properties Ed. A.L. Page. American Society of Agronomy Madison WI USA. 1-1159

Schnürer J. and Rosswall T (1982) Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Appl. Environ Microbiol 43:1256–1261

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. Biol. Control, 40: 246-252

Shah, F.A., Wang, C-S. and Butt, T. M. (2005). Nutrition influences growth and virulence of the insect-pathogenic fungus *M. anisopliae anisopliae* FEMS Microbiology Letters 251: 259-266.

SPSS (2003) “Statistical product and service solution, system user’s guide” Version 13. Psychology Press Ltd, Publishers, East Sussex, UK.

Thompson, S.R. and Brandenburg R.L. (2005). Tunnelling responses of mole crickets (Orthoptera: Gryllotalpidae) to the entomopathogenic fungus, *Beauveria bassiana*. Environmental Entomology 34: 140-147.

Thomsen L. and Eilenberg J. (2000).Time-concentration mortality of *Pieris brassicae* (lepidoptera :Pieridae) and *Agrotis segetum* (lepidoptera : noctuidae) larvae from different destruxins. Environmental Entomology 29: 1041-1047.

van Lenteren, J.C., Benuzzi, M., Nicoli, G. and Maini, S. (1992). Biological control in protected crops in Europe. In: van Lenteren, J.C. Minks, A.K., Ponti, O.M.B. (eds), Biological Control and Integrated Crop Protection: Towards Environmentally Safer Agriculture. Pudoc Scientific Publishers, Wageningen, the Netherlands, 77-89.

van Tol, R.W.H. M., Bezooijen, J.V. and Ketelaars, T.A.C.M. (1998). Searching behaviour of entomopathogenic nematodes: roots and soil temperature determine success of black vine weevil (*Otiorhynchus sulcatus*) control. Insect Pathogens and Insect Parasitic Nematodes, IOBC Bulletin 21: 187 –191.

van Tol, R.W.H.M., van Dijk, N. and Sabelis, M.W. (2004). Host plant preferences and performance of the vine weevil *Otiorhynchus sulcatus*. Agr. Forest Entomol. 6: 267-278.

Whittaker, R.H.(1972). Evolution and measurement of species diversity. Taxon, 251.

Wraight, S.P. and Ramos, M. (2004). Investigations of Colorado potato beetle mortality following foliar applications of *Beauveria bassiana* [abstract]. Society for Invertebrate Pathology Annual Meeting Proceedings 37, pp. 103.

Zacharuk, R.Y. and Tinline, R.D. (1968) Pathogenicity of *Metarrhizium anisopliae***,** and other fungi, for five Elaterids (Coleoptera) in Saskatchewan. J. Invertebr. Pathol. 12: 294-309.

