Project title:	Protected edible crops: biological control of plant diseases using insect pathogenic fungi with dual activity against plant pathogens
Project number:	PE 005
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Report:	Annual report, August 2012
Previous report:	-
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Date project commenced:	1st August 2011
Date project completed (or expected completion date):	31st July 2013

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Three commercially available biological control agents and three novel products have been identified that have the potential to control tomato powdery mildew.

Background

Plant pathogens are a significant constraint on the production of protected edible crops. At the same time, growers are under considerable pressure to reduce their use of synthetic chemical fungicides as a result of new legislation plus the increasing demand from supermarkets for produce with zero detectable pesticide residues. However, alternative control agents are currently in short supply. The overall aim of this project is to investigate commercial biocontrol agents based on insect pathogenic fungi as potential control agents of plant pathogens. Although these agents are normally used against insect pests in Integrated Pest Management (IPM) programmes, research done outside the UK has suggested that some insect pathogenic fungi can also have activity against plant diseases.

Summary

In this first year of the project, a method was developed for measuring the effect of candidate insect pathogenic fungi on tomato powdery mildew. The method involved applying powdery mildew spores to tomato leaflets maintained in a Petri dish, and then applying a known concentration of the control agent. The effect of the agent was measured in terms of its ability to control the growth of the powdery mildew on the leaflet and also to reduce the production of powdery mildew spores. This method allowed a range of agents to be evaluated under controlled and reproducible conditions.

Laboratory experiments were then done to evaluate three commercial “bio-insecticides” against tomato powdery mildew. These were: the fungal species *Lecanicillium muscarium* strain 19.79, which was used as the commercial product Mycotal (Koppert BV), *Beauveria bassiana*, ATCC 74040 which was used as the product Naturalis L (Belchim) and two biological products not yet approved for use on protected tomatoes HDC F122 and HDC F123. In addition, we evaluated three other novel agents: (i) the bacterial biopesticide Serenade (based on *Bacillus subtilis* QST 713, Agraquest Ltd); (ii) HDC F124 (also not yet approved for protected tomatoes); (iii) Thiovit (800 g/kg sulphur, Syngenta). All the agents were used at the manufacturers’ recommended concentrations. Two sets of experiments

were done. In the first, Mycotal was evaluated alongside Serenade, HDC F124 and Thiovit. In the second, Mycotal was compared against HDC F123 and Naturalis.

All of the tested agents controlled tomato powdery mildew in the laboratory experiment. For the first set of experiments, the treatments reduced the sporulation of powdery mildew by 77% (Mycotal), 63% (Serenade), 94% (HDC F124) and 98% (Thiovit). In the second set of experiments, the treatments reduced the sporulation of powdery mildew by 94% (Mycotal), 75% (HDC F122), 93% (Naturalis) and 92% (HDC F123).

These are encouraging results, although additional research will be required to evaluate the control agents under conditions that more closely reflect the conditions occurring on a crop scale.

Research was also done to develop a laboratory method to evaluate the effect of insect pathogenic fungi against *Pythium* and *Rhizoctonia*, and in developing a method for coating tomato seed with spores of insect pathogenic fungi. These will be used to quantify the effect of seed applications of *Beauveria bassiana* on *Pythium* and *Rhizoctonia* diseases of tomato later in the project.

Financial Benefits

It is difficult to comment on the financial benefits given the early nature of results. However any new method that would allow growers to reduce their reliance on synthetic chemical fungicides for the control of powdery mildew and damping off diseases would be financially beneficial at a time when the availability of chemical pesticides is declining, and when growers are under increasing pressure to produce crops with zero detectable pesticide residues.

Action Points

No actions are being recommended until the project has been completed.

SCIENCE SECTION

Introduction

Growers of protected edible crops are under significant pressure to reduce usage of synthetic chemical fungicides. One solution is to use biological control agents of fungal diseases as a partial or total replacement for fungicides. Biocontrol agents include microbial antagonists, competitors, plant growth promoters and elicitors of induced systemic resistance. Unfortunately, there is a shortage of authorised biocontrol products within the UK for disease control. A novel way forward could be to use biological control agents that already have authorisation for use on protected crops, but which are currently used to control invertebrate pests rather than plant pathogens. For this project, we are particularly interested in the potential of insect pathogenic fungi to help control powdery mildew and damping off.

Insect pathogenic fungi are being used by growers in the UK and EU as biocontrol agents of a range of arthropod pests. A total of 5 different species of insect pathogenic fungi are either registered for use already in the UK, are undergoing UK registration or have potential for EU/ UK registration: *Beauveria bassiana*, *Isaria fumosorosea*, *Lecanicillium muscarium*, *Lecanicillium longisporum*, and *Metarhizium anisopliae*. They are all members of the fungal taxonomic order Hypocreales. Biopesticide products based on the fungi include HDC F122, Naturalis (based on *B. bassiana*), Mycotal (based on *L. muscarium*), and HDC F123.

In the last few years, research conducted in the USA and Canada has been published that challenges previously held conceptions about hypocrealean insect pathogenic fungi. These fungi were traditionally thought of solely as insect pathogens, however it is now apparent that some strains have additional 'econutritional modes'. These include being able to grow as endophytes (i.e. colonise plant tissue without causing damage to the plant) and the ability to parasitize other fungi (Vega *et al*., 2008). This shift in our understanding was reviewed in 2009 by an international team of scientists including Dr Dave Chandler at Warwick (Vega *et al*., 2009).

Both *B. bassiana* and *Lecanicillium* have been shown to have potential as biological control agents of plant disease. For example, a strain of *B. bassiana* was shown to endophytically colonise tomato plants when applied as a seed dressing and gave protection to seedlings against *Rhizoctonia solani* and *Pythium myriotylum* (Ownley *et al*., 2008). *Beauveria bassiana* applied to the roots of cotton seedlings conferred protection against *Xanthomonas*

axonopodis inoculated onto leaves (Griffin *et al*., 2006). There is evidence that *B. bassiana* protected the plant through induced systemic resistance in much the same way as plant growth-promoting rhizobacteria (Ownley *et al*. 2010). Meanwhile, *L. muscarium*, used as Mycotal, significantly controlled cucurbit powdery mildew (*Sphaerotheca fusca*) on melon leaves in laboratory experiments. A related species, *L. longisporum* significantly reduced the number of powdery mildew (*Sphaerotheca fuliginea*) spots on cucumber leaves in a laboratory bioassay and in a greenhouse bench experiment (Kim *et al.,* 2007; 2010). Finally, researchers in Oregon have shown that *Metarhizium anisopliae* can grow in the root zone of ornamental plants (Bruck, 2005). In Canada, *Metarhizium* was shown to promote the growth of field maize when spores of the fungus were applied as a seed treatment (Kabaluk & Ericsson, 2007). The mechanism of this effect on plant growth is unknown. However, other microorganisms that colonize the root zone and promote plant growth – such as *Trichoderma* – can protect plants against diseases by inducing systemic resistance and / or by outcompeting plant pathogens in the root zone.

The question now is whether these fungal strains can be used for the biological control of diseases affecting UK protected crops. The pathogens affecting UK crops are different to those affecting production in N America, while UK production and environmental conditions are also very different. In the current project, we are investigating the potential of strains of *L. muscarium*, *B. bassiana* and HDC F123 to control powdery mildew and damping off.

The project has three Objectives as follows:

- Objective 1. Quantify the effect of foliar sprays of the insect pathogenic fungus *Lecanicillium longisporum* on powdery mildew on tomato.
- Objective 2. Determine the potential of *B. bassiana*, applied as a root drench to tomato plants, to control powdery mildew.
- Objective 3. Quantify the effect of seed applications of the insect pathogenic fungus *Beauveria bassiana* on *Pythium* and *Rhizoctonia* diseases of tomato.

Materials and methods

Objective 1: Use of L. muscarium as a biological control agent of powdery mildew

The aim of Objective 1 was to investigate the use of the fungal biopesticide Mycotal (Koppert BV), based on *Lecanicillium muscarium* strain 19.79, as a biological control agent of powdery mildew.

1.1 Preparation of pathogen inoculum

A culture of the causative agent of tomato powdery mildew, *Odium neolycopersici,* which originated from a natural UK tomato crop infection in the early 1990s, was maintained on young tomato plants (cv. Espero, Pinetree de Ruiter Seeds Ltd., UK) in a greenhouse, at 20ºC with supplemental heating at 18ºC and vented at temperatures greater than 22ºC. A 16:8 hour L: D lighting regime was maintained with supplementary lighting (400 W high pressure sodium lamps) used between November and March and shading with thermal screens applied throughout the summer period. Tomato plants were inoculated on a weekly basis when approximately 3-4 weeks old (ca. 30 cm high). This was done by tapping heavily infested leaves over the tomato plant for approximately twenty seconds. Spore suspensions for experiments were prepared by agitating heavily infested leaflets from the culture in 0.01% sterile Tween 20 solution (BDH Laboratory Supplies, UK). Spores were counted using an Improved Neubauer haemocytometer and suspensions prepared at concentrations ranging from 10^3 to 10^8 spores ml^{-1} in sterile 0.01% Tween 20.

1.1 Development of a laboratory bioassay to measure the susceptibility of powdery mildew to novel control agents

In this part of the project, a laboratory bioassay was developed to quantify the ability of insect pathogenic fungi and other novel agents to control the growth of *O. neolycopersici*. This bioassay was different to the one that we proposed to use in the grant application. It was developed to allow a greater number of treatments to be assessed over a shorter period of time.

Several potential bioassay systems were investigated (Figure 1). These were all based on keeping detached tomato leaflets under controlled conditions. The leaflets were then inoculated with a suspension of *O. neolycopersici* spores, before being treated with candidate control agents. A range of different set ups were investigated, primarily to determine whether (i) control leaf material remained in a good condition during the period of the bioassay, and (ii) leaf material inoculated with *O. neolycopersici* supported good growth of the pathogen. The systems investigated included detached leaflets with petioles

maintained in water/ nutrient solutions, or leaflets/leaf discs floated in water / nutrient solution in Petri dishes. Some of the methods investigated had issues of water loss and subsequent leaf and pathogen death, contamination and poor pathogen growth.

Figure 1: Candidate systems investigated for a bioassay of novel control agents against powdery mildew

The two methods which showed the most promise were as follows: Method 1 = leaf discs (7cm cut from detached leaflets using a cork borer) placed on water agar; Method 2 = the "double Petri dish" assay, which consisted of two stacked Petri plates (15cm diameter) with a hole between them through which the petiole of a leaflet placed in the upper plate was immersed in water or nutrient solution in the lower plate. Both of these methods have the added benefit in that they could both be used to investigate control of pathogens on leaf material from other crops such as cucumber.

1.2 Pilot experiments to determine O. neolycopersici inoculums levels

A series of experiments were done to determine the effect of different *O. neolycopersici* inoculum rates on the levels of disease developing under bioassay conditions. Three week old tomato leaflets were sprayed until run off (approximately 2ml per leaflet) using a handheld trigger spray (Arco, UK) with spore suspensions ranging from 10^3 to 10^7 spores ml^{-1}. Inoculated leaflets were left for one hour to dry before being transferred to either the double Petri dish chambers or used to cut leaf discs and transferred to the leaf disc chamber as described previously. The bioassay chambers were maintained within a controlled environment room at 20ºC; 16:8 L: D; 83µmol. The chambers were observed

daily for onset of disease symptoms and after 10 days the leaflets were visually scored for disease and spore production assessed. Disease severity was rated on a scale of 0-5 (Table 1) based on a visual estimation of the % leaf area covered with powdery mildew.

Table 1. Disease level scoring system used in bioassays of novel control agents against powdery mildew

Score	Percentage surface area infected with powdery mildew
0	0
1	1-20
2	21-40
3	41-60
4	61-80
5	81-100

Spore production by *O. neolycopersici* was assessed by placing each leaflet in a 50ml Duran Bottle containing 10ml of sterile 0.01% Tween 20 solution and shaking briefly by hand. The spore suspensions were transferred to a 20ml Universal bottle and 0.5ml of aniline blue dye solution added. Spores were counted using an Improved Neubauer haemocytometer. Two spore counts were made per Universal bottle.

1.3 Experiment to determine the effect of Lecanicillium muscarium and other alternative treatments on Odium neolycopersici growth and spore production

In this part of the project, the effect of *L. muscarium* on *Odium neolycopersici* growth and spore production was quantified. *Lecanicillium muscarium* was used as the commercial product Mycotal (Koppert BV). In addition, we evaluated three other novel agents: (i) the bacterial biopesticide Serenade (based on *Bacillus subtilis*, Agraquest Ltd); (ii) HDC F124; (iii) Thiovit (800 g/kg sulphur, Syngenta). HDC F124 and Thiovit were not part of the original project plan, however because the bioassay methods developed in the project appeared to be operating particularly well, we thought it was worth using them to investigate other treatments. Using the double Petri dish chamber described above, three week old tomato leaflets were sprayed until run off (approximately 2ml per leaflet) using a handheld trigger spray (Arco, UK) with *O. neolycopersici* at a concentration of 10^6 spores ml^{-1}. Inoculated leaflets were left for one hour to dry before being treated with Mycotal, Serenade, HDC F124 or Thiovit (Table 2). The treatments were made up to the

manufacturers recommendations and applied until run off (approximately 2ml per leaflet) using a handheld trigger spray (Arco, UK) as before. The leaflets were left for a further one hour to dry before being transferred to the double Petri dish chambers. The bioassay chambers were maintained within a controlled environment room at 20ºC; 16:8 L: D; 83µmol. The chambers were observed daily for onset of disease symptoms and after 10 days the leaflets were visually scored for disease and spore production assessed as described previously. On each occasion there were five replicate bioassay chambers per treatment and the bioassay was repeated on two occasions.

Table 2: Treatments used in Experiment 1.3.

Treatment	**Active ingredient**
Mycotal	*Lecanicillium muscarium* (10^{10} cfu/g)
Serenade	*Bacillus subtilis* (10^{9} cfu/g)
HDC F124	-
Thiovit	Sulphur (800g/kg)

1.4 Experiment to determine the effect of Lecanicillium muscarium, Beauveria bassiana and HDC F123 on Odium neolycopersici

In Experiment 1.3, the entomopathogenic fungus *L. muscarium* gave good levels of control of *O. neolycopersici* (see Results section, below). Therefore, it was decided to conduct an additional experiment to evaluate a wider range of entomopathogenic fungi against *O. neolycopersici*. Using the double Petri dish assay described above, three week old tomato leaflets were sprayed until run off (approximately 2ml per leaflet) using a handheld trigger spray (Arco, UK) with *O. neolycopersici* at a concentration of 10^{6} spores ml^{-1}. Inoculated leaflets were left for one hour to dry before being treated with either Mycotal, Naturalis, HDC F122 or HDC F123 at a rate of 10^{7} spores ml^{-1}(Table 3). The treatments were made up to the manufacturers recommendations and applied until run off (approximately 2ml per leaflet) using a handheld trigger spray (Arco, UK) as before. The leaflets were left for a further one hour to dry before being transferred to the double Petri dish chambers. The bioassay chambers were maintained within a controlled environment room at 20ºC; 16:8 L:D; 83µmol. The chambers were observed daily for onset of disease symptoms and after 10 days the leaflets were visually scored for disease and spore production assessed. There were five replicate bioassay chambers per treatment and the bioassay was done on two separate occasions.

Table 3. Treatments used in experiment 1.4

Treatment	Active ingredient
Mycotal	*Lecanicillium muscarium* (1 x10^{10} cfu/g)
Naturalis	*Beauveria bassiana* (2.3 x 10^{7} cfu/ml)
HDC F122	(3.7x10^{10} cfu/g)
HDC F123	(9 x 10^{11} cfu/kg)

Objective 3: Ability of insect pathogenic fungi to protect against damping off

The aim of this Objective is to investigate the ability of insect pathogenic fungi to control *Pythium* and *Rhizoctonia* damping off. The Objective was originally scheduled to be done in 2013. However, there was a delay to Objective 1 at the start of the project, when the culture of powdery mildew was transferred to a different glasshouse, causing the culture to "crash". It took several months to restore the culture to sufficient size for experiments. Therefore, during this time, we decided to start work on Objective 3 in order that all milestones would be completed during the lifetime of the project.

3.1 Preparation of pathogen inoculum

Isolates of *Pythium* (Table 4), from the Warwick Crop Centre collection, were grown on potato dextrose agar (PDA) and stored on PDA slopes at 5ºC. Inoculum for experiments was produced by adding five agar plugs from a three day old culture to sterilised (120ºC for 15 minutes on two consecutive days) mixture of Levington F1 compost (60g, 4mm sieved), potato pieces (25g, 2mm^2) and an appropriate amount of water for an overall moisture content of 80% and incubated for two weeks incubation at 18ºC. A *Rhizoctonia solani* culture, from the Warwick Crop Centre collection, known to be pathogenic on oilseed rape was grown on potato dextrose agar (PDA) and stored on PDA slopes at 5ºC. Inoculum for experiments was produced by adding five agar plugs from a seven day old culture to a mixture of wheat bran flakes (8g), sand (195g) and water (35ml) autoclaved at 120ºC for 15 minutes and incubated for two weeks incubation at 18ºC.

Table 4. Plant pathogens used in Objective 3

Isolate	Pathogen species	Host	Origin
1	*Pythium aphanidermatum*	cabbage	Chris Gilligan, University of Cambridge
2	*Pythium ultimum* (var. *ultimum*)	cabbage	Chris Gilligan, University of Cambridge
3	*Pythium* spp.	Pepper	Tim O'Neil, ADAS
4	*Rhizoctonia solani*	Oilseed rape	Amanda Bennett, Warwick HRI

3.2 Molecular characterisation of Pythium *isolates*

PDA liquid shake cultures (100ml in 250ml conical flask) were inoculated with 3 agar plugs per flask of each of the *Pythium* isolates (Table 4), and maintained in an orbital shaking incubator in darkness at 18ºC and 200rpm for five days. Mycelium was harvested after five days by filtration through muslin, rinsed with sterile water, blotted dry then freeze dried and stored at -20ºC until required. DNA was extracted from 100mg freeze dried mycelium using a GenElute plant genomic DNA miniprep kit (Sigma-Aldrich, Poole, UK). The concentration of DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Fungal DNA (1ng) was amplified by PCR using ITS primers 1 and 4 (White *et al*., 1990). The thermocycler conditions were as follows: (a) Initial denaturing 94ºC for 2 min, annealing 55ºC 30s: (b) 35 cycles of extension 72ºC 30s denaturing 94ºC for 30 s, annealing 55ºC 30s; (c) final extension conditions of 72ºC 5 min. PCR products were then separated on a 1.5% agarose gel at $6V.cm^{-1}$ for 1h and visualised using Gel Red staining and exposure to UV light. PCR products were purified using a QIAquick PCR product purification kit (Qiagen, Crawley, UK) then a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) was used together with ITS primers 1 and 4 to generate forward and reverse products. Sequence data was produced by an ABI 3130xl genetic analyser (Applied Biosystems, Warrington UK). These sequences were compared and consensus versions were constructed. A multiple sequence alignment programme (MegAlign, DNASTAR Inc., Madison, USA) was used to compare these sequences and others downloaded from DNA databases available on the internet (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

3.3 Pilot experiment to determine inoculums levels for laboratory bioassays

Pilot experiments were done to determine the effect of different pathogen inoculum rates of the *Pythium* (0- 60% v/v) and *Rhizoctonia i*solates (0- 20% v/v) (Table 3) on the emergence of healthy tomato seedlings to identify an isolate that was pathogenic to tomato and so that appropriate levels could be used in subsequent experiments to determine the effectiveness of insect pathogenic fungi as control agents of damping off. All experiments were set up in 35-cell modules (15.5ml volume, cut from 345 module trays) using Levington F1 compost maintained at 80% water content and sown with tomato cv Espero (one seed per cell). The pathogen inoculums were mixed thoroughly with the Levington F1 compost before dispensing into modules. The modules were maintained in a controlled environment room at 20ºC; 16:8 L:D; 83µmol and watered from below. The number of seedlings emerged with two fully expanded cotyledons and also the number of healthy seedlings was recorded every two days over a period of two weeks. The pathogen inocula of both *Pythium* and *R. solani* were quantified by suspending in SDW and serially diluting triplicate 1g samples, then spreading 100µl aliquots onto the surface of PDA plates and incubating at 20ºC for 2 days. The number of colony forming units per g inoculums (CFU/g) was then calculated.

3.4 Development of seed coating methodology

Isolates of insect pathogenic fungi (Table 5) were grown on Sabouraud Dextrose agar (SDA) at 23ºC, in the dark and after 14 days spores were harvested in 10ml of sterile 0.05% Triton X-100 by agitating gently with a sterile spreader. The conidia were enumerated using an Improved Neubauer haemocytometer, and suspensions adjusted to 10^7, 10^8 and 10^9 conidia ml^{-1}. The suspensions were centrifuged and the supernatant removed and the spore pellet resuspended in 1ml of either 2% methyl cellulose suspension or water containing 25µl of Tween 20 and mixed with 1g of tomato seeds. Seeds were stirred until the coating appeared uniform and left to air dry overnight in a safety cabinet. Treated seeds were stored at 4ºC prior to use. Conidial rates for application to seeds were determined by adding 1 treated seed to 1ml of 0.05% Triton X-100, which was then vortex mixed, serially diluted and aliquots were plated onto Sabouraud dextrose agar + Dodine (SDAd). The plates were incubated at 20 ± 2°C, in the dark, for 5-7 days and the number of colonies per plate was counted. The effect on seedling emergence was determined by placing treated seeds (10 per isolate/spore concentration) on damp filter paper within a Petri dish and the seeds maintained in the darkness at 20ºC. Germination was assessed after 7 days.

Table 5. Fungal isolates used in experiment 3.4

Isolate[†]	Species	Host/Substrate	Collection site
432.99[a]	*Beauveria bassiana*	*Anthonomus grandis*	USA
433.99[b]	*HDC F122*	*Bemisia* sp.	USA
19.79[c]	*Lecanicillium muscarium*	*Trialeuroides vaporariorum*	UK
275.86[d]	*HDC F123*	*Cydia pomonella*	Germany

[†]Isolate number in the Warwick crop centre culture collection

(a) Active ingredient in the mycopesticide 'Naturalis' (Belchim Crop Protection UK).

(b) Isolate forms the active ingredient in the proprietary mycopesticide 'HDC F122'.

(c) Isolate forms the active ingredient in the proprietary mycopesticide 'Mycotal' (Koppert BV, Netherlands)

(d) Isolate forms the active ingredient in the proprietary mycopesticide 'HDC F123'.

Results

Objective 1: Use of L. muscarium as a biological control agent of powdery mildew

1.2 Pilot experiments to determine O. neolycopersici inoculums levels

Both of the bioassay systems investigated allowed disease development to be observed daily. The disease development showed a similar pattern regardless of the bioassay chamber. Disease levels ranged from 0 to 3.3 x 10^5 spores per cm^2 in the double Petri dish chambers and 0 to 1.98 x 10^5 spores per cm^2 in the leaf disc chambers (Figure 2). Visual assessments of disease severity were always higher in the double Petri dish chambers compared with in the leaf disc chambers despite similar number of spores per cm^2 being recorded. Leaf quality was higher in the double Petri dish assay and so this chamber was chosen for further experiments.

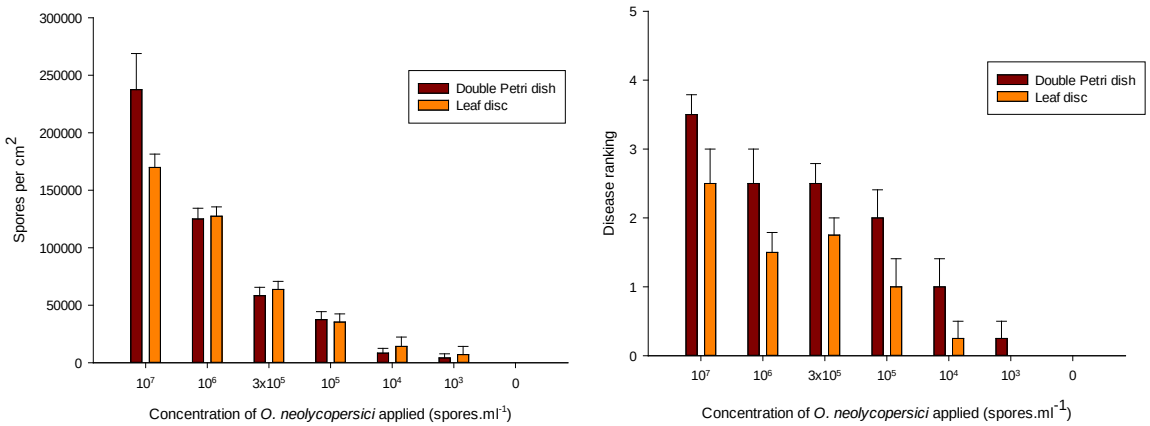

Figure 2: The effect of concentration of spore inoculum on the sporulation of *Odium neolycopersici* when maintained in the double Petri dish bioassay chamber or within the leaf

disc bioassay chamber ten days post inoculation. The error bars represent the standard error of the mean.

1.3 Experiment to determine the effect of Lecanicillium muscarium and other alternative treatments on Odium neolycopersici growth and spore production

All of the treatments reduced the level of powdery mildew developing on the leaf (Figure 3). In the first run of the experiment (which we refer to as "rep 1"), the disease development in the untreated controls on individual replicate leaflets ranged from 7.5 x 10^4 to 1.04 x 10^5 spores per cm^2 at 10 days post inoculation. When the experiment was repeated, it ranged from 6 x 10^4 to 1.4 x 10^5 spores per cm^2. For the experimental treatments, disease development on individual replicate leaflets ranged from 0 to 5 x 10^4 (Mycotal), 1 x 10^4 to 1.15 x 10^5 (Serenade), 0 to 2 x10^4 (HDC F124) and 0 to 5 x 10^3 spores per cm^2 (Thiovit) at 10 days post inoculation. In comparison to the untreated controls the treatments reduced the sporulation of powdery mildew by 77% (Mycotal), 63% (Serenade), 94% (HDC F124) and 98% (Thiovit). Analysis of variance on the data for spores per cm^2 suggests that all treatments were significantly different from controls but not from each other. Two of the Serenade treated leaflets showed the same level of infection as the controls. Visual assessments of disease severity corresponded to the sporulation counts. At 10 days post inoculation 41-60% of the leaflet of untreated inoculated controls was infected with powdery mildew. No reduction in powdery mildew infected areas was observed in leaflets treated with Serenade, but treatment with Mycotal reduced the powdery mildew infected area to 21-40%, and treatment with both HDC F124 and Thiovit reduced the powdery mildew infected area to 0-20%. No mycoparasitism was observed.

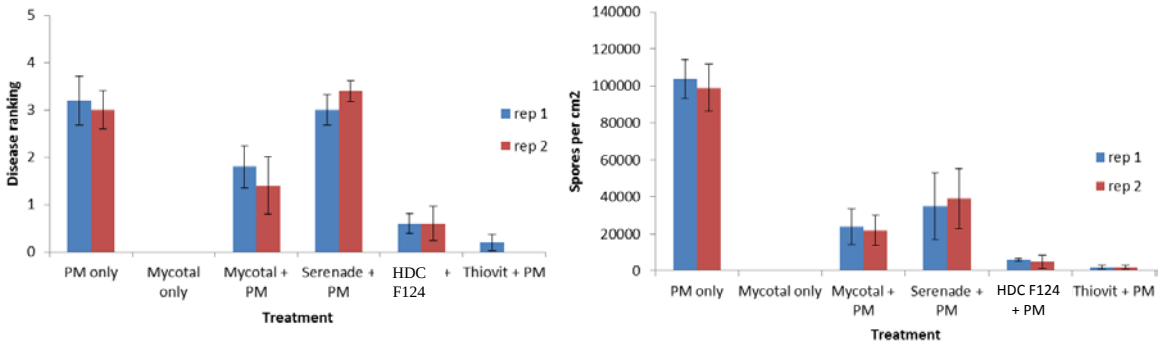

Figure 3: The effect of biopesticide and fungicide treatments on the disease severity and the sporulation of *Odium neolycopersici* when maintained in the double Petri dish bioassay chamber. Data were obtained at ten days post inoculation. The data presented are the mean of five replicate leaflets per treatment and the error bars represent the standard error

of the mean. The graphs show the results obtained for the two occasions on which the experiment was done, i.e. repeat 1 (= rep 1) and repeat 2 (rep 2).

1.4 Experiment to determine the effect of Lecanicillium muscarium, Beauveria bassiana, HDC F122 and HDC F123 on Odium neolycopersici

All of the treatments reduced the level of powdery mildew developing on the leaf (Figure 4). Disease development, at 10 days post inoculation, on the five replicate leaflets ranged from 4.5 x 10^4 to 1.75 x 10^5 spores per cm^2 (repeat 1) and 4 x 10^4 to 1.55 x 10^5 spores per cm^2 (repeat 2) on the untreated controls. In the treated chambers, disease development, on the five replicate leaflets, ranged from 0 to 1 x 10^4 (Mycotal), 0 to 7.5 x 10^5 (HDC F122), 0 to 1.5 x10^4 (Naturalis) and 0 to 1 x 10^4 spores per cm^2 (HDC F123). Results were variable within treatments with some replicate leaflets having no control on powdery mildew whereas others gave good control with the same treatment. This may be due to small differences in the environmental conditions within each chamber affecting the growth of the insect pathogenic fungi. In comparison to the untreated controls the treatments reduced the overall sporulation of powdery mildew by 94% (Mycotal), 75% (HDC F122), 93% (Naturalis) and 92% (HDC F123). Analysis of variance on data on spores per cm^2 indicates all treatments were significantly different from controls but not from each other. At 10 days post inoculation 41-60% of the leaflet of untreated inoculated controls was infected with powdery mildew. Treatment with Mycotal, HDC F122, Naturalis and HDC F123 all reduced the powdery mildew infected area to 0-20% (Figure 4). There was no evidence of any mycoparasitism of powdery mildew form the biopesticide treatments.

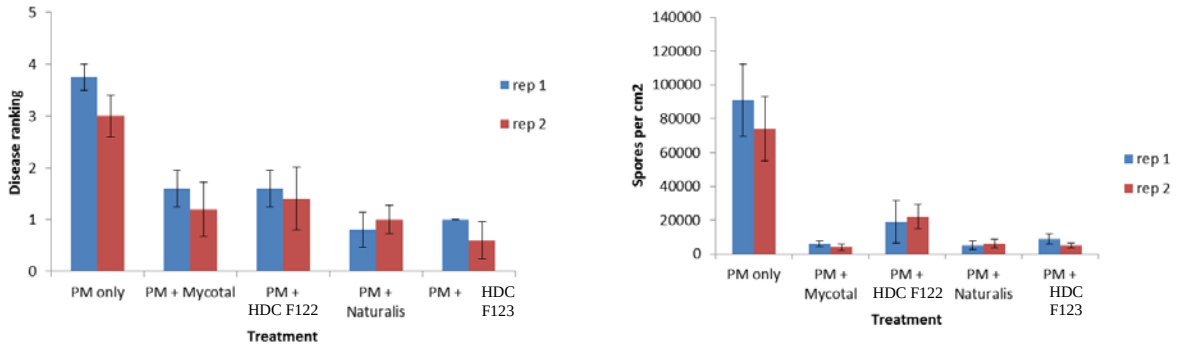

Figure 4: The effect of isolates of HDC F122, *Naturalis (B. bassiana), Mycotal (L. muscarium) and* HDC F123 on the disease severity and sporulation of *Odium neolycopersici* when maintained in the double Petri dish bioassay chamber ten days post inoculation. The error bars represent the standard error of the mean. The terms “rep 1”

and “rep 2” refer to the first and second occasions on which the experiment was done (“rep” = “repeat”)

3.2 Molecular characterisation of Pythium isolates

The three *Pythium* isolates used in this study were molecularly characterised as *P. ultimum* and *P. lutarium* (also known as *P.diclinum*). The isolate that was previously identified as *P. aphanidermatum* was molecularly characterised as *P. ultimum.*

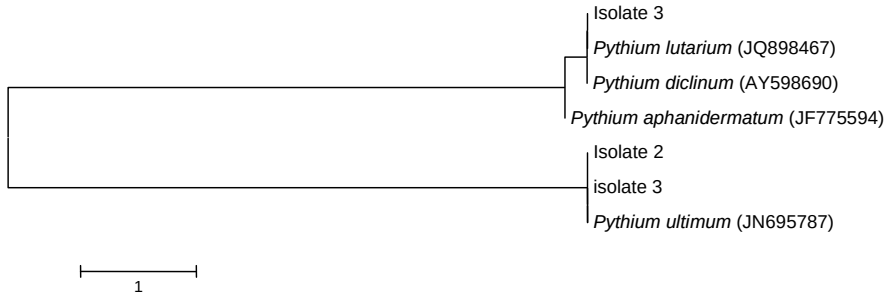

Figure 5. Unrooted phylogenetic tree showing the relationship of the *Pythium* isolates used in this study

3.3 Pilot experiment to determine inoculums levels for laboratory bioassays

The initial experiments to establish appropriate inoculum levels showed that for uninoculated compost, 33 - 35 healthy tomato seedlings (from a possible 35) survived at the end of the experiments (Table 6; Figures 5 and 6). In experiment 1 only a pathogen amendment rate of 25% v/v showed any reduction in seeding survival (29-33 survived from a total of 35). Therefore the experiment was repeated with higher concentrations of inoculum in experiment 2. There was no clear relationship between the v/v rate of pathogen inoculum applied and the number of healthy seedlings in experiment 2 which was thought to be as a result of the low pathogenicity of this isolate to tomato seedlings. Two further *Pythium* isolates were examined in experiments 3 and 4. Both were more pathogenic than *P. ultimum* with only 19-23 and 10-20 healthy seedlings out of a possible 35 emerging for *P. aphanidermatum* and *Pythium* spp. at amendment rates of 15-30%. Similarly, the isolate of *R. solani* examined was not particularly pathogenic to tomato seedlings with only 3-6 seedlings being affected from damping off from a possible 35. In contrast, to *Pythium* infected seedlings, *R. solani* predominantly caused post emergence damping off with most seedlings dying over a period of 7-14 days post-emergence. Future experiments will use the *Pythium* isolate from experiment 4 which has been subsequently molecularly characterised as *P. lutarium* (also known as *P. diclinum*) at a rate of 20%.

Table 6. The effect of pathogen inoculum rate on the percentage of healthy seedlings

P. ultimum				*P. aphanidermatum*		*Pythium* spp.		*R. solani*	
Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**		**Experiment 5**	
% rate (v/v)	**% healthy seedlings**	**% rate (v/v)**	**% healthy seedlings**	**% rate (v/v)**	**% healthy seedlings**	**% rate (v/v)**	**% healthy seedlings**	**% rate (v/v)**	**% healthy seedlings**
0	100.00	0	99.0	0	98.1	0	100.0	0	100.0
0.5	94.3	30	20.0	1	91.4	1	91.4	0.1	98.1
1	96.4	40	38.1	5	93.3	5	65.7	1	95.2
5	94.3	50	16.2	10	84.8	10	82.9	5	94.3
10	94.3	60	36.2	15	54.3	15	49.5	10	97.1
15	94.3			20	64.8	20	56.2	15	96.2
20	95.0			30	63.8	30	28.6	20	88.6
25	89.3								

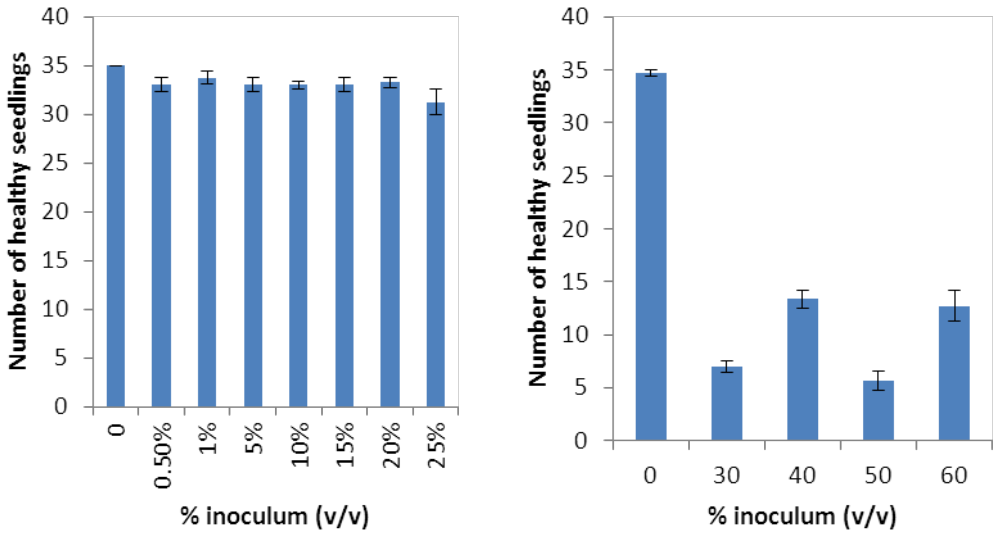

Figure 5. Effect of different inoculum levels of *P. ultimum* on tomato seedlings. The error bars represent the standard error of the mean.

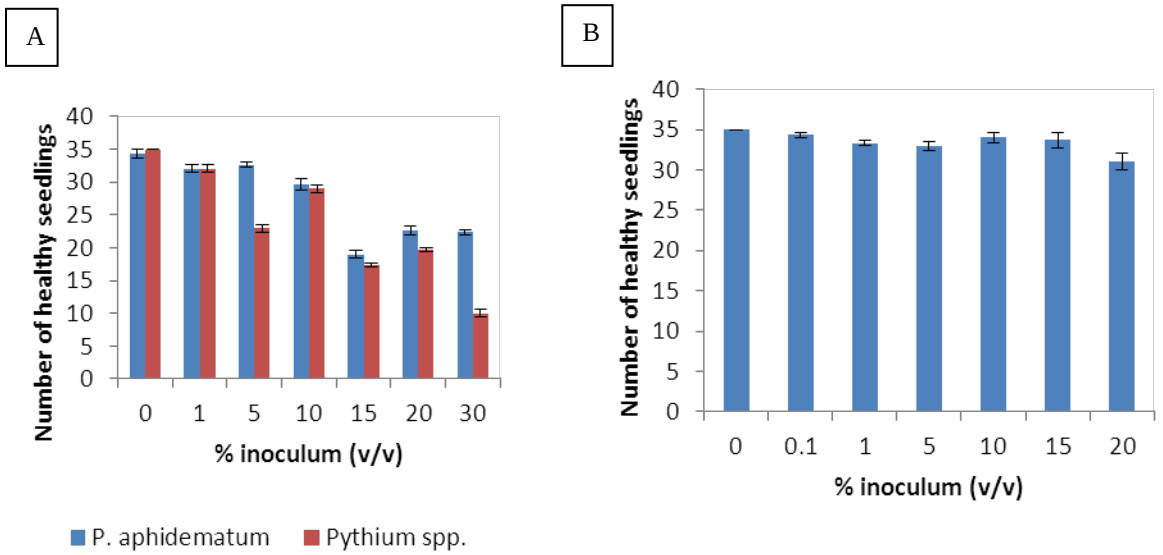

Figure 6. Effect of different inoculum levels of *P. aphidematum* and a *Pythium spp.* (A) and *R. solani* (B) on tomato seedlings. The error bars represent the standard error of the mean.

3.4 Development of seed coating methodology

Seeds coated with the methyl cellulose solution stuck together and spores appeared clumped on the seed surface. Therefore it was decided to dispense with the methyl cellulose solution and coat seeds with fungal spores in a water and Tween suspension. Seeds treated, using this method did not stick together and spores appeared evenly distributed over the seed surface. Seed treated with 1 x 10^7 conidia received 1.6 to 2.02 x 10^5 viable colony forming units per seed (Figure 7). Seed treated with 1 x 10^8 conidia received 4.31 to 6.12 x 10^5 viable colony forming units per seed. Seed treated with 1 x 10^9 conidia received 8.42 to 9.70 x 10^5 viable colony forming units per seed. The results from this experiment will be used to determine the dose rate so that each seed receives a dose rate of 10^6 colony forming units per seed in future experiments.

There was no effect of seed treatment on seed germination. Seed treated with all of the isolate/ concentration combinations examined showed 90% or greater germination (Table 7).

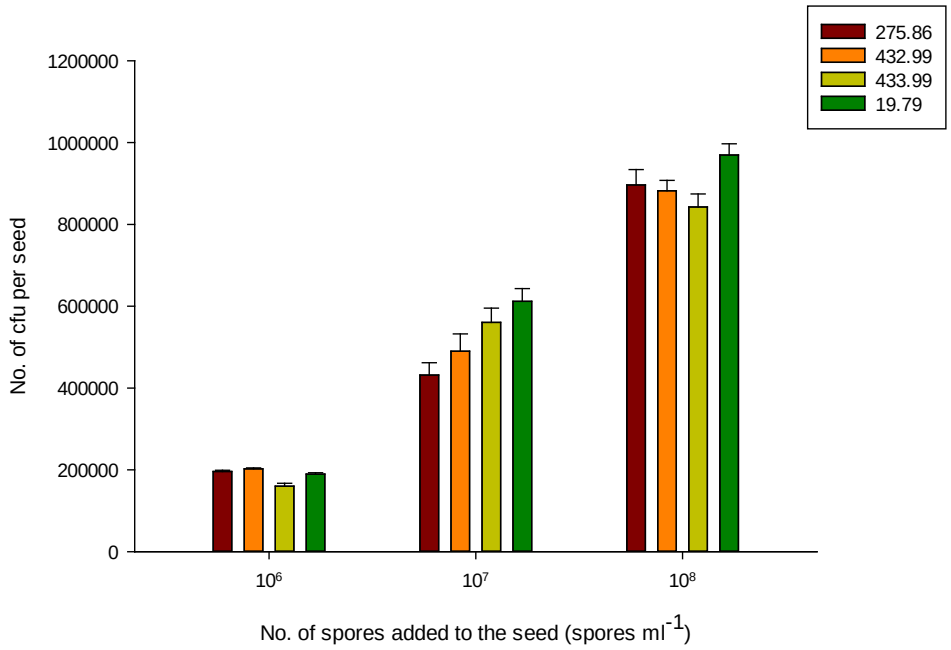

Figure 7. The number of colony forming unit (viable spores) recovered from seed treated with different concentrations *HDC F123* (275.86), Naturalis (432.99), *HDC F122* (433.99) *and L. muscarium* (19.79).

Table 7. Percentage germination of tomato seeds coated with different concentrations *HDC F123* (275.86), *B. bassiana* (432.99), *HDC F122* (433.99) *and L. muscarium* (19.79).

Isolate	Species	Spore concentration added	% germination
432.99	*Beauveria bassiana*	10^9 spores ml^{-1}	100
		10^8 spores ml^{-1}	100
		10^7 spores ml^{-1}	100
433.99	*HDC F122*	10^9 spores ml^{-1}	100
		10^8 spores ml^{-1}	100
		10^7 spores ml^{-1}	100
19.79	*Lecanicillium muscarium*	10^9 spores ml^{-1}	90
		10^8 spores ml^{-1}	90
		10^7 spores ml^{-1}	100
275.86	*HDC F123*	10^9 spores ml^{-1}	100
		10^8 spores ml^{-1}	100
		10^7 spores ml^{-1}	100

Discussion

Control of tomato powdery mildew in the laboratory bioassay

Significant levels of tomato powdery mildew control were obtained in the laboratory bioassay using insect pathogenic fungi (Mycotal, Naturalis and HDC F123) as well the bacterial biofungicide Serenade, and two other "alternative" products, HDC F124 and Thiovit. These results are encouraging, and although additional research will be required to show proof of concept, it is a step forwards in identifying potential new biocontrol agents and other alternatives for powdery mildew control for growers. One of the insect pathogenic fungi investigated in this study, *L. muscarium*, is known from previous work to be able to parasitise plant pathogenic fungi (Askary *et al.* 1998; Kim *et al*., 2007; 2010), which would account for its activity against tomato powdery mildew in the bioassay. *Beauveria bassiana* and *M. anisopliae* are not known to be mycoparasitic and more research is needed to determine their mechanism of action. Previous research with *B. bassiana* indicates that some strains are able to inhibit the growth of plant pathogenic fungi through competition (Ownley *et al.* 2004) and / or antibiosis (Renwick *et al.* 1991; Reisenzein & Tiefenbrunner 1997; Bark *et al.* 1996; Veseley & Koubova 1994; Lee *et al.* 1999). *Beauveria bassiana* is known to produce secondary metabolites with antimicrobial properties that are believed to be involved in preventing colonisation of *B. bassiana*–infected insects (Zimmerman, 2007). *Beauveria bassiana*, *M. anisopliae* and *L. muscarium* produce a range of secondary metabolites and enzymes, including chitinases, which are deployed during the infection and colonisation of insect tissue, but which could also have fungistatic or fungicidal properties

The levels of control of tomato powdery mildew with the insect pathogenic fungi observed in the bioassay were all high, which is encouraging given that all treatments were applied at the manufacturers' recommended concentrations. Further research is required to identify the minimum spore concentration needed for powdery mildew control. The control agents were also applied shortly after the powdery mildew was applied to leaves: in future work it would be worth investigating how control is affected by the time of application of the insect pathogenic fungi relative to the powdery mildew (including applications of the insect pathogenic fungi before and after the powdery mildew application). There is also a need to quantify the efficacy of the treatments at greater spatial scales, starting with whole plant bioassays, and moving progressively towards crop scale experiments done under commercially realistic growing conditions.

Unfortunately, the tomato powdery mildew culture being used in the project "crashed" when it had to be moved to a new glasshouse compartment. This caused a delay in Objective 1

while the culture was restored. To prevent slippage in milestones further down the line, work was started on Objective 3, which included development of bioassays with *Pythium* and *Rhzoctonia*, and in developing a method for coating tomato seed with spores of insect pathogenic fungi. These will be used to quantify the effect of seed applications of *Beauveria bassiana* on *Pythium* and *Rhizoctonia* diseases of tomato later in the project.

Conclusions

Three commercial biopesticides based on insect pathogenic fungi (Mycotal, Naturalis and HDC F123) that have potential for control of tomato powdery mildew have been identified. Three other products - Serenade, HDC F124 and Thiovit – also have potential for tomato powdery mildew control. Further work is required to determine the levels of control achievable with these agents under experimental conditions that more realistically reflect the conditions occurring within commercial crop production.

Knowledge and Technology Transfer

Biopesticides offer dual control (PE005: Protected edible crops: biological control of plant diseases using insect pathogenic fungi with dual activity against plant pathogens). HDC News **185**: 13 – 15.

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