

**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:** Final report, October 2013

**Previous report:** Annual report, August 2012

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**Date project commenced:** 1<sup>st</sup> August 2011

**Date project completed (or expected completion date):** 31<sup>st</sup> September 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

Mycotal, Naturalis L and a coded bioinsecticide (HDC F123) have been shown to have the potential to control tomato powdery mildew in experimental systems. The coded bioinsecticide (HDC F123) also has potential to control cucumber powdery mildew. Cultures from the biocontrol agents Naturalis L and HDC F123 gave a small but significant level of control of *Pythium* on tomato when applied to seed.

## 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 was to investigate commercial biocontrol agents based on insect pathogenic fungi as potential control agents of plant pathogens. A number of biopesticides, based on insect pathogenic fungi, are being sold in the UK and / or mainland Europe for insect pest control as part of Integrated Pest Management (IPM) programmes. However, research done outside the UK has suggested that some insect pathogenic fungi can also have activity against plant diseases. We wanted to find out whether insect pathogenic fungi available as commercial biopesticides have potential for biocontrol of powdery mildew and damping off diseases.

## Summary

Laboratory experiments were done to evaluate three commercial bioinsecticides 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 agents: (i) the bacterial biopesticide Serenade ASO (based on *Bacillus subtilis* QST 713, Agraquest Ltd); (ii) HDC F124 (also not yet approved for protected tomatoes); (iii) Thiovit Jet (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 ASO, HDC F124 and Thiovit Jet. In the second, Mycotal was compared against HDC F123 and Naturalis L. 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 ASO), 94% (HDC F124) and 98% (Thiovit Jet). 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). An experiment was then done to measure the effect of Mycotal, HDC F122, Naturalis L, HDC F123, and the biofungicide AQ10, against tomato powdery mildew applied to whole tomato plants. In this case, the treatments reduced the sporulation of powdery mildew by 65% (Mycotal), 94% (HDC F122), 76% (Naturalis L), 70% (HDC F123) and 73% (AQ10). A laboratory experiment was also conducted to evaluate commercial bioinsecticides against cucumber powdery mildew. Here, HDC F123 reduced the sporulation of cucumber powdery mildew by 82%, but the other treatments tested (Mycotal, HDC F122, and Naturalis L) did not give significant control. An experiment was done to determine whether applying a drench of the bioinsecticides to the roots of tomato plants gave control of tomato mildew inoculated on the leaves. In this case, none of the treatments gave control (HDC F122, Naturalis L, Mycotal, HDC F123).

Research was also done to develop a laboratory method to evaluate the effect of insect pathogenic fungi against *Pythium*, and to develop a method for coating tomato seed with spores of insect pathogenic fungi. Two of the treatments (HDC F123 and Naturalis L) gave a small (15%) but statistically significant amount of control of *Pythium* when applied this way.

The results obtained with biocontrol of tomato and cucumber powdery mildew are encouraging, and suggest that using bioinsecticides sprayed onto the leaf surface have potential as a new form of control. Additional research will be required to determine the best timing of application, to evaluate the control agents under conditions that more closely reflect commercial production, and to investigate their use as part of Integrated Pest and Disease Management.

### **Financial Benefits**

It is difficult to comment on the financial benefits given that this work was done mainly as a set of laboratory experiments. 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**

The biopesticides investigated here are approved for use on protected crops, or are undergoing evaluation through SCEPTRE. It is too early to recommend using them now as control agents of powdery mildew, but growers should be aware that these products have potential as a new type of control agent.

## 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 were 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. They are commonly referred to as biopesticides. A total of 5 different species of insect pathogenic fungi are either registered for use already in the UK, are undergoing UK registration, are registered for use in other countries of the EU, or have potential for EU/ UK registration: *Beauveria bassiana*, *Isaria fumosorosea*, *Lecanicillium muscarium*, *Lecanicillium longisporum*, and *Metarhizium brunneum* (previously known as *Metarhizium anisopliae* but has recently been reclassified). They are all members of the fungal taxonomic order Hypocreales. In this project, we investigated four different biopesticides based on insect pathogenic fungi. Two of these products are commercially available already: Naturalis L (based on *B. bassiana*) and Mycotal (based on *L. muscarium*). The other two products are not currently approved for use on protected tomato crops and hence have been assigned the code numbers HDC F122 and HDC F123. Towards the end of the project, we also obtained an isolate of *B. bassiana* from Dr Bonnie Ownley

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 more complex life histories and can exhibit 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* spp. 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.

The project had 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.

As the project progressed, the results we obtained meant that we deviated slightly from the project plan as written in the grant proposal. In Objective 1, we used a different bioassay

method from that written in the grant proposal, which enabled us to evaluate more candidate control agents than originally planned. In Objective 3, we did not get high levels of infection in tomato seedlings with the isolate of *Rhizoctonia* that we were using, so we diverted resource into *Pythium* experiments.

## **Materials and methods**

### Insect pathogenic fungi

We used four different commercial fungal biopesticides in the project. Depending on the experiment, they were used either as the formulated product, or we used them as fungal cultures grown from the products on agar based media. Stock cultures of the fungal isolates were stored on porous plastic beads in liquid nitrogen vapour (Chandler, 1994). Laboratory cultures were grown on Sabouraud dextrose agar (SDA) slopes and maintained in a refrigerator at 4°C for up to six months. Subcultures for laboratory experiments were prepared on SDA from the slope cultures and incubated at 23°C for 10-12 d in the dark and powdered spores collected from the plates. Because the formulated products could, in theory, perform differently from the cultured fungi (the formulants used in the products could affect their efficacy for example), we refer to the cultured fungi by the code numbers used in the Warwick Crop Centre culture collection, rather than using the name of the product. The fungi used were as follows:

- *Lecanicillium muscarium*, used as the product Mycotal. The culture collection code for this fungus is 19.79.
- *Beauveria bassiana* used as the product Naturalis L. The culture collection code for this fungus is 432.99.
- HDC coded product HDC F122. The culture collection code for this fungus is 433.99.
- HDC coded product HDC F123. The culture collection code for this fungus is 275.86.

A fifth fungus, *Beauveria bassiana* strain 11.98, which has been investigated by Ownley and coworkers (U. Tennessee, USA) was included as a control agent of plant pathogens. Delays in accessing this strain meant that it was not included in Experiment 2.1.

### **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, *Oidium 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 20°C with supplemental heating at 18°C and vented at temperatures greater than 22°C. 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  $\text{ml}^{-1}$  in sterile 0.01% Tween 20.

A culture of the causative agent of cucurbit powdery mildew (*Sphaerotheca fusca*) was obtained from a natural UK cucumber crop infection in 2013 (courtesy of Derek Hargreaves, DHHC Ltd). It was maintained on young cucumber plants (cv. Burpless Tasty Green, Thompson and Morgan (UK) Ltd) in a controlled environment room at 20°C and a 16:8 hour L: D lighting regime. Cucumber plants were inoculated on a weekly basis when approximately 3 -4 weeks old by tapping heavily infested leaves over the cucumber plant for approximately twenty seconds. Spore suspensions for experiments were prepared as described above.

### 1.1 Development of a laboratory bioassay to measure the susceptibility of tomato powdery mildew (*Oidium neolycopersici*) to control agents

In this part of the project, different laboratory bioassay methods were investigated to quantify the ability of insect pathogenic fungi and other agents to control the growth of *O. neolycopersici*. The bioassay method was developed to allow a greater number of treatments to be assessed over a shorter period of time. The work was done in two parts. In part one, a range of 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. 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 *Oidium neolycopersici*

In part two, we selected two methods which showed the most promise and investigated them further. These were as follows: Method 1 = leaf discs (2.7cm 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. These methods were used in the rest of the study.

### 1.2 Pilot experiments to determine tomato powdery mildew (*Oidium neolycopersici*) inoculum levels

A series of experiments was done to determine the effect of different *O. neolycopersici* inoculum rates on the levels of disease developing under bioassay conditions. This allowed us to select the most appropriate amount of inoculum to use in later experiments. 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  $\text{ml}^{-1}$ . 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 20°C; 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 control agents against tomato powdery mildew (*Oidium neolycopersici*)

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 treatments on tomato powdery mildew (*Oidium neolycopersici*) growth and spore production

In this part of the project, the effect of *L. muscarium* on *Oidium neolycopersici* growth and spore production was quantified. *Lecanicillium muscarium* was used as the commercial product Mycotal (Koppert BV). In addition, we evaluated three other agents: (i) the bacterial biopesticide Serenade ASO (based on *Bacillus subtilis*, Agraquest Ltd); (ii) HDC F124; (iii) Thiovit Jet (800 g/kg sulphur, Syngenta). Using the double Petri dish chamber described above, three week old tomato leaflets were sprayed until just before 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 ASO, HDC F124 or Thiovit Jet (Table 2). The treatments were made up to the manufacturers recommendations and applied until just

before 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 20°C; 16:8 L: D; 83µmol. The leaflets 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	<i>Lecanicillium muscarium</i> (10 <sup>10</sup> cfu/g)
Serenade ASO	<i>Bacillus subtilis</i> (10 <sup>9</sup> cfu/g)
HDC F124	-
Thiovit Jet	Sulphur (800g/kg)

1.4 Experiment to determine the effect of *Lecanicillium muscarium*, *Beauveria bassiana* and HDC F123 on tomato powdery mildew (*Oidium neolycopersici*) growth and spore production

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<sup>6</sup> spores ml<sup>-1</sup>. Inoculated leaflets were left for one hour to dry before being treated with one of four different fungal biopesticides: Mycotal, Naturalis L, HDC F122 or HDC F123, each applied at a rate of 10<sup>7</sup> spores ml<sup>-1</sup> (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 20°C; 16:8 L:D; 83µmol. The leaflets were observed daily for onset of disease symptoms and after 10 days they 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	<i>Lecanicillium muscarium</i> ( $1 \times 10^{10}$ cfu/g)
Naturalis L	<i>Beauveria bassiana</i> ( $2.3 \times 10^7$ cfu/ml)
HDC F122	( $3.7 \times 10^{10}$ cfu/g)
HDC F123	( $9 \times 10^{11}$ cfu/kg)

1.5 Experiment to determine the effect of dose of *Lecanicillium muscarium* and HDC F122 on tomato powdery mildew (*Oidium neolycopersici*) growth and spore production

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  $\text{ml}^{-1}$ . Inoculated leaflets were left for one hour to dry before being treated with either Mycotal or HDC F122 at four different rates ( $10^4$  to  $10^7$  spores  $\text{ml}^{-1}$ ). The treatments (Table 4) were applied until just before 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 20°C; 16:8 L:D; 83 $\mu\text{mol}$ . The leaflets were observed daily for onset of disease symptoms and after 10 days they were visually scored for disease and spore production assessed. There were three replicate bioassay chambers per treatment.

**Table 4.** Treatments used in experiment 1.5

Treatment	Active ingredient	Rate
Mycotal	<i>Lecanicillium muscarium</i> ( $1 \times 10^{10}$ cfu/g)	$10^4$ , $10^5$ , $10^6$ , $10^7$ spores $\text{ml}^{-1}$
HDC F122	( $3.7 \times 10^{10}$ cfu/g)	$10^4$ , $10^5$ , $10^6$ , $10^7$ spores $\text{ml}^{-1}$

1.6 Experiment to determine the effect of *Lecanicillium muscarium* and other treatments on *Oidium neolycopersici* growth and spore production on whole tomato plants

In Experiment 1.3, 1.4 and 1.5, the entomopathogenic fungus *L. muscarium* and other treatments gave good levels of control of *O. neolycopersici* (see Results section, below). Therefore, it was decided to conduct an additional experiment to evaluate these on a larger spatial scale. Three week old tomato plants were sprayed until run off (approximately 30ml per plant) using a handheld trigger spray (Arco, UK) with *O. neolycopersici* at a concentration of  $10^6$  spores  $\text{ml}^{-1}$ . Inoculated plants were left for one hour to dry before being treated with either Mycotal, Naturalis L, HDC F122 or HDC F123 at a rate of  $10^7$

spores ml<sup>-1</sup> (Table 5). The treatments were applied until just before run off (approximately 30ml per leaflet) using a handheld trigger spray (Arco, UK) as before. The plants were left for a further one hour to dry before being transferred to a controlled environment room at 20°C; 16:8 L:D; 83µmol. The leaflets were observed daily for onset of disease symptoms and after 10 days they were visually scored for disease and spore production was assessed. There were three replicate plants per treatment and the bioassay was done on two separate occasions.

**Table 5.** Treatments used in experiment 1.6

Treatment	Active ingredient
Mycotal	<i>Lecanicillium muscarium</i> (1 x 10 <sup>10</sup> cfu/g)
Naturalis L	<i>Beauveria bassiana</i> (2.3 x 10 <sup>7</sup> cfu/ml)
HDC F122	(3.7x10 <sup>10</sup> cfu/g)
HDC F123	(9 x 10 <sup>11</sup> cfu/kg)
AQ10	<i>Ampelomyces quisqualis</i> (5 x 10 <sup>9</sup> spores/g)

### 1.7 Experiment to determine the effect of *Lecanicillium muscarium*, *Beauveria bassiana* and HDC F123 on cucurbit powdery mildew (*Sphaerotheca fusca*) growth and spore production

The aim of this experiment was to evaluate *L. muscarium* and other entomopathogenic fungi against cucurbit powdery mildew. Using the leaf disc assay described above, leaf discs (2.7cm) were cut, using a cork borer, from detached leaves and sprayed until run off (approximately 0.5ml per disc) using a handheld trigger spray (Arco, UK) with *S. fusca* at a concentration of 10<sup>6</sup> spores ml<sup>-1</sup>. Inoculated leaf discs were left for one hour to dry before being treated with either, Mycotal, Naturalis L, HDC F122 or HDC F123 at a rate of 10<sup>7</sup> spores ml<sup>-1</sup> (Table 6). The treatments were applied until just before run off (approximately 0.5 ml per leaf disc) using a handheld trigger spray (Arco, UK) as before. The leaf discs were left for a further one hour to dry before being transferred to Petri dishes containing water agar. The bioassay chambers were maintained within a controlled environment room at 20°C; 16:8 L:D; 83µmol. The leaf discs were observed daily for onset of disease symptoms and after 14 days the leaf discs were visually scored for disease and spore production assessed. There were three leaf discs per Petri dish. The treatments each

consisted of six replicate Petri dishes (= 18 leaf discs) while the controls consisted of 12 replicated dishes (= 36 leaf discs). The bioassay was done on two separate occasions.

**Table 6.** Treatments used in experiment 1.7

Treatment	Active ingredient
Mycotal	<i>Lecanicillium muscarium</i> ( $1 \times 10^{10}$ cfu/g)
Naturalis L	<i>Beauveria bassiana</i> ( $2.3 \times 10^7$ cfu/ml)
HDC F122	( $3.7 \times 10^{10}$ cfu/g)
HDC F123	( $9 \times 10^{11}$ cfu/kg)

**Objective 2. Determine the potential of *Beauveria bassiana*, applied as a root drench to tomato plants, to control powdery mildew.**

The aim of this Objective was to investigate the ability of the insect pathogenic fungi to induce systemic resistance to powdery mildew when applied to the root zone.

**2.1 Experiment to determine the effect of root drenches of *Beauveria bassiana* and other treatments on *Oidium neolycopersici* growth and spore production.**

The roots of six week old tomato plants were drenched (25mls per plant) with either Mycotal, Naturalis L, HDC F122 or HDC F123 (Table 7) at a rate of  $10^8$  spores  $\text{ml}^{-1}$  either four days prior to or at the same time as powdery mildew inoculation. Powdery mildew, at a concentration of  $10^6$  spores  $\text{ml}^{-1}$ , was sprayed onto the leaves and stems of the plants until just before run off (approximately 30ml per plant) using a handheld trigger spray (Arco, UK), as described previously. The plants were left for hour to dry before being transferred to a controlled environment room at 20°C; 16:8 L:D; 83 $\mu\text{mol}$ . The plants were observed daily for onset of disease symptoms and after 10 days the leaflets were visually scored for disease and spore production assessed.

**Table 7.** Treatments used in experiment 2.1

Treatment	Active ingredient
Mycotal	<i>Lecanicillium muscarium</i> ( $1 \times 10^{10}$ cfu/g)
Naturalis L	<i>Beauveria bassiana</i> ( $2.3 \times 10^7$ cfu/ml)
HDC F122	( $3.7 \times 10^{10}$ cfu/g)
HDC F123	( $9 \times 10^{11}$ cfu/kg)

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

The aim of this Objective was to investigate the ability of insect pathogenic fungi to control *Pythium* and *Rhizoctonia* damping off.

#### **3.1 Preparation of pathogen inoculum**

For this Objective we used isolates of *Pythium* and *Rhizoctonia* that were held within the Warwick Crop Centre collection of plant pathogens. We had a total of three isolates of *Pythium* and one isolate of *Rhizoctonia*. The isolates of *Pythium* (Table 8), from the Warwick Crop Centre collection, were grown on potato dextrose agar (PDA) and stored on PDA slopes at 5°C. Inoculum for experiments was produced by adding five agar plugs from a three day old culture to sterilised (120°C for 15 minutes on two consecutive days) mixture of Levington F1 compost (60g, 4mm sieved), potato pieces (25g, 2mm<sup>2</sup>) and an appropriate amount of water for an overall moisture content of 80% and incubated for two weeks at 18°C. The *Rhizoctonia solani* culture, which was reported to be pathogenic on oilseed rape, was grown on potato dextrose agar (PDA) and stored on PDA slopes at 5°C. 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 120°C for 15 minutes and incubated for two weeks incubation at 18°C.

**Table 8.** Plant pathogens used in Objective 3

<b>Isolate number</b>	<b>Pathogen species (as recorded in the culture collection database)</b>	<b>Host</b>	<b>Origin</b>
1	<i>Pythium aphanidermatum</i>	cabbage	Chris Gilligan, U. Cambridge
2	<i>Pythium ultimum</i> (var. <i>ultimum</i> )	cabbage	Chris Gilligan, U. Cambridge
3	<i>Pythium</i> spp.	Pepper	Tim O'Neil, ADAS
4	<i>Rhizoctonia solani</i>	Oilseed rape	Amanda Bennett, Warwick HRI

#### **3.2 Molecular characterisation of Pythium isolates**

Research was done to identify the *Pythium* isolates to species level using DNA sequence analysis. The isolates were recorded in the culture collection as being *Pythium*

*aphanidermatum*, *Pythium ultimum*, and *Pythium* spp. We assumed that these identifications had been done on the basis of morphological criteria using mycological identification keys. However, it is not unheard of for fungal isolates to be misidentified using taxonomic methods that rely on fungal morphology, and hence it was decided to use a DNA based method as a check. PDA liquid shake cultures (100ml in 250ml conical flask) were inoculated with 3 agar plugs per flask of each of the *Pythium* isolates (Table 8), and maintained in an orbital shaking incubator in darkness at 18°C 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 -20°C 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 94°C for 2 min, annealing 55°C 30s; (b) 35 cycles of extension 72°C 30s denaturing 94°C for 30 s, annealing 55°C 30s; (c) final extension conditions of 72°C 5 min. PCR products were then separated on a 1.5% agarose gel at 6V.cm<sup>-1</sup> 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). A BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) was then used together with ITS primers 1 and 4 to generate forward and reverse products. Nucleotide sequences were characterized using 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 against others deposited within the NCBI DNA database (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>).

### 3.3 Pilot experiment to determine inoculum levels for laboratory bioassays

Pilot experiments were done to determine the effect of different inoculum rates of the *Pythium* (0- 60% v/v) and *Rhizoctonia* isolates (0- 20% v/v) (Table 8) on the emergence of healthy tomato seedlings. This was done in order to (i) identify an isolate that was pathogenic to tomato and (ii) to calculate a suitable level of inoculum to use in bioassays of the effectiveness of candidate biocontrol agents (we wanted to use *Pythium* or *Rhizoctonia* inoculum levels that resulted in 50% seedling emergence in the bioassays). 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 inocula were mixed thoroughly with the Levington F1 compost

before dispensing into modules. The modules were maintained in a controlled environment room at 20°C; 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 20°C for 2 days. The number of colony forming units per g inoculums (CFU/g) was then calculated.

### 3.4 Development of seed coating methodology

For this experiment we used fungal conidia produced from cultures of entomopathogenic fungi grown on an agar based medium, as opposed to using the formulated fungal biopesticide Isolates of insect pathogenic fungi (Table 9) were grown on Sabouraud Dextrose agar (SDA) at 23°C, 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<sup>7</sup>, 10<sup>8</sup> and 10<sup>9</sup> spores ml<sup>-1</sup>. 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 class II laminar air flow cabinet. Treated seeds were stored at 4°C prior to use. The number of conidia adhering per seed was 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 20°C. Germination was assessed after 7 days.

**Table 9.** Fungal isolates used in experiment 3.4

Isolate <sup>†</sup>	Species	Country of origin
432.99	<i>Beauveria bassiana</i>	USA
433.99	<i>HDC F122</i>	USA
19.79	<i>Lecanicillium muscarium</i>	UK
275.86	<i>HDC F123</i>	Germany

<sup>†</sup>code number for the isolate used in the Warwick Crop Centre culture collection of entomopathogenic fungi.

### 3.5 Experiment to investigate the ability of insect pathogenic fungi to control *Pythium* and *Rhizoctonia* damping off.

The aim of this experiment was to quantify the effect of entomopathogenic fungi, applied as seed treatment to tomato, against damping off. Using the system described above (section 3.4), tomato seeds were coated with fungal spores ( $10^9$  spores  $\text{ml}^{-1}$ ) in a water and Tween suspension and stored at 4°C prior to use. *Pythium lutarium* (*Pythium* Isolate 3) was cultured as described in section 3.1 and mixed thoroughly with Levington F1 compost (20% v:v ratio) maintained at 80% water content before being dispensed into 35-cell modules (15.5ml volume, cut from 345 module trays). Seeds (Table 10) were sown into modules (one seed per cell) of *Pythium* inoculated compost and controls (which consisted of compost with no *Pythium*), and maintained in a controlled environment room at 20°C; 16:8 L:D; 83µmol and watered from below. There were four different types of experimental treatment: (i) uncoated seeds planted in *Pythium*-free compost; (ii) uncoated seeds planted in compost containing *Pythium*; (iii) seeds coated with fungal biocontrol agents and planted in *Pythium*-free compost; (iv) seeds coated with fungal biocontrol agents and planted in compost containing *Pythium*. 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 level was quantified by suspending in SDW and serially diluting triplicate 1g samples, then spreading 100µl aliquots onto the surface of PDA plates and incubating at 20°C for 2 days. The number of colony forming units per g inoculums (CFU/g) was then calculated as described previously. There were three replicate modules per treatment.

The experiment was repeated on a second occasion using a reduced number of treatments. On this occasion, we did not include seeds coated with fungal biocontrol agents and planted in *Pythium*-free compost, as there was no evidence that the seed coat affected seed germination in the absence of *Pythium*. This enabled us to use six replicate modules per treatment.

**Table 10.** Fungal isolates used in experiment 3.5

Isolate <sup>†</sup>	Species	Country of origin
432.99	<i>Beauveria bassiana</i>	USA
433.99	HDC F122	USA

11.98	<i>Beauveria bassiana</i>	USA
19.79	<i>Lecanicillium muscarium</i>	UK
275.86	HDC F123	Germany

<sup>†</sup>Isolate number in the Warwick crop centre culture collection

### 3.6 Isolation of insect pathogenic fungi from tomato seedlings.

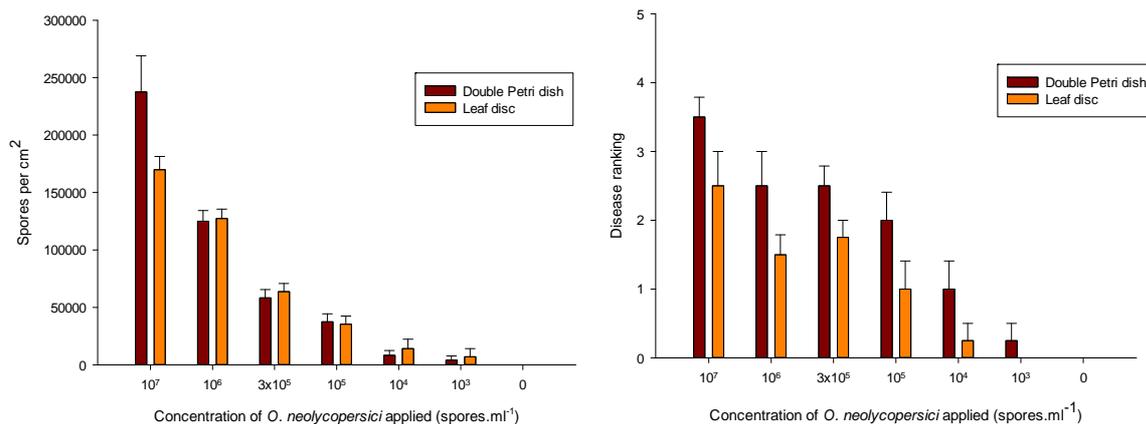
*Tomato seed* coated with fungal spores ( $10^9$  spores ml<sup>-1</sup>) in a water and Tween suspension, as described previously (section 3.4) were grown in Levington F1 compost. After 21 days, intact seedlings were surface sterilised by immersion for one minute in 95% ethanol, then transferred to a 20% sodium hypochlorite solution for three minutes, and placed in 95% ethanol for one minute. The seedlings were then rinsed in sterile deionized water and allowed to dry in a laminar flow hood. Surface sterilised seedlings were aseptically cut into 2 to 3mm pieces for root and stem sections; leaves were cut into 3 to 7 mm pieces before being placed on selective medium. Plates were incubated at 22°C, in darkness for 3 weeks and then observed for fungal growth.

## Results

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

#### 1.2 Pilot experiments to determine *O. neolycopersici* inoculum levels

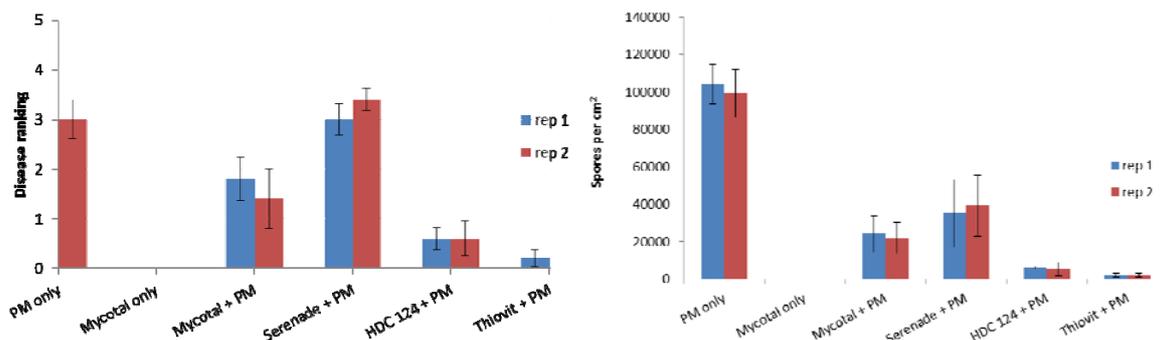
In experiment 1.2 we selected two bioassay systems to take forward: Method 1 (a leaf disc bioassay) and Method 2 (the “double Petri dish” bioassay). Both of the bioassay systems allowed disease development to be observed daily. The disease development showed a similar pattern regardless of the bioassay chamber. Disease levels, measured as spores of *O. neolycopersici* produced per cm<sup>2</sup> on tomato leaves, ranged from 0 to  $3.3 \times 10^5$  spores per cm<sup>2</sup> in the double Petri dish chambers and 0 to  $1.98 \times 10^5$  spores per cm<sup>2</sup> 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<sup>2</sup> 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 *Oidium 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 treatments on *Oidium neolycopersici* growth and spore production

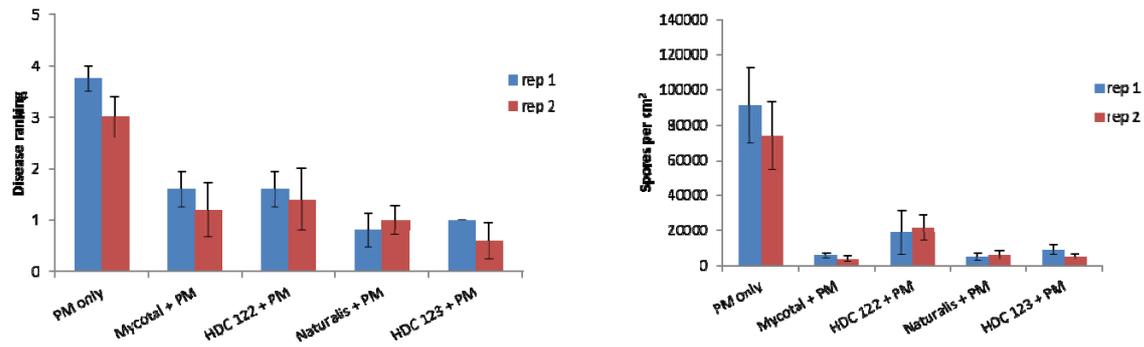
All of the treatments reduced the level of powdery mildew developing on the leaf (Figure 3). In the first repeat of the experiment (which we refer to as “rep 1”), the disease development in the untreated controls on individual replicate leaflets ranged from  $7.5 \times 10^4$  to  $1.04 \times 10^5$  spores per cm<sup>2</sup> at 10 days post inoculation. When the experiment was repeated (rep 2), it ranged from  $6 \times 10^4$  to  $1.4 \times 10^5$  spores per cm<sup>2</sup>. For the experimental treatments, disease development on individual replicate leaflets ranged from 0 to  $5 \times 10^4$  (Mycotal),  $1 \times 10^4$  to  $1.15 \times 10^5$  (Serenade ASO), 0 to  $2 \times 10^4$  (HDC F124) and 0 to  $5 \times 10^3$  spores per cm<sup>2</sup> (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 ASO), 94% (HDC F124) and 98% (Thiovit Jet). Analysis of variance on the data for spores per cm<sup>2</sup> indicated that all treatments were significantly different ( $p < 0.05$ ) 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 ASO, 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 *Oidium 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 per occasion 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 *Oidium neolycopersici*

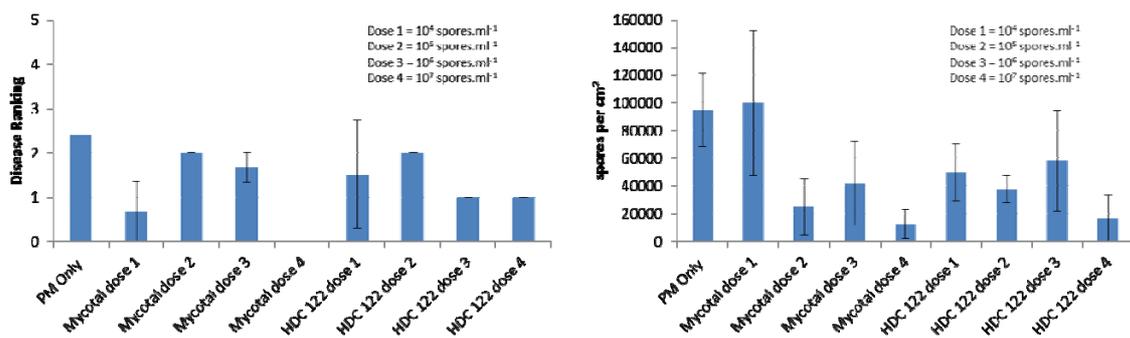
All of the treatments reduced the level of powdery mildew developing on the leaf (Figure 4). Disease development, at 10 days post inoculation, on the five replicate leaflets ranged from  $4.5 \times 10^4$  to  $1.75 \times 10^5$  spores per  $\text{cm}^2$  (repeat 1) and  $4 \times 10^4$  to  $1.55 \times 10^5$  spores per  $\text{cm}^2$  (repeat 2) on the untreated controls. In the treated chambers, disease development, on the five replicate leaflets, ranged from 0 to  $1 \times 10^4$  (Mycotal), 0 to  $7.5 \times 10^5$  (HDC F122), 0 to  $1.5 \times 10^4$  (Naturalis) and 0 to  $1 \times 10^4$  spores per  $\text{cm}^2$  (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 L) and 92% (HDC F123). Analysis of variance on data on spores per  $\text{cm}^2$  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 Mycotol, HDC F122, Naturalis L 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 from the biopesticide treatments.



**Figure 4:** The effect of isolates of HDC F122, Naturalis L (*B. bassiana*), Mycotal (*L. muscarium*) and HDC F123 on the disease severity and sporulation of *Oidium 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”)

1.5 Experiment to determine the effect of dose of *Lecanicillium muscarium* and HDC F122 on *Oidium neolycopersici* growth and spore production

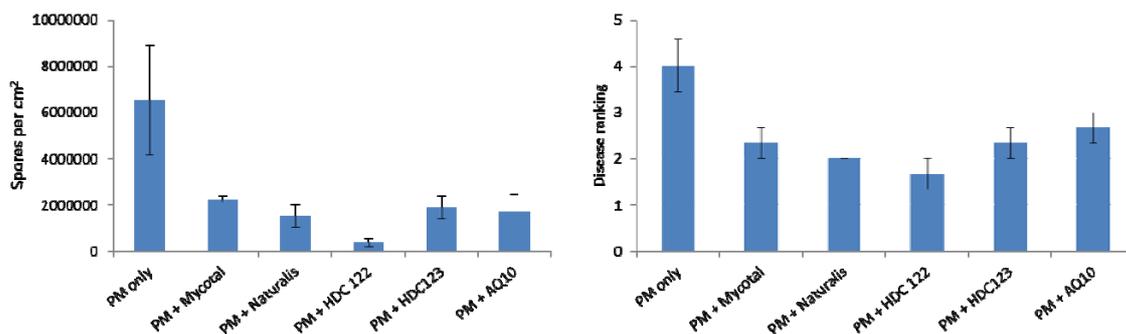
Both treatments reduced the level of powdery mildew developing on the leaf (Figure 5). Disease development, at 10 days post inoculation, on the three replicate leaflets ranged from  $2.5 \times 10^4$  to  $1.75 \times 10^5$  spores per  $\text{cm}^2$  on the untreated controls. In the treated chambers, disease development ranged from 0 to  $2 \times 10^5$  (Mycotal) and 0 to  $1.2 \times 10^5$  (HDC F122) spores per  $\text{cm}^2$ . There was a dose response for Mycotal, in terms of the effect of the biocontrol agent on the number of spores of *O. neolycopersici* produced per unit area of leaf (Figure 5). However no dose response was apparent for HDC F122 (Figure 5). Results were variable within treatments, particularly at the lower doses, with some replicate leaflets having no control on powdery mildew whereas others gave good control with the same treatment. At 10 days post inoculation 41-60% of the leaflet of untreated inoculated controls was infected with powdery mildew. Treatment with Mycotal had little effect on the powdery mildew infected area but treatment with the higher concentrations of HDC F122 reduced the powdery mildew infected area to 0-20% (Figure 5). There was no evidence of any mycoparasitism of powdery mildew from the biopesticide treatments.



**Figure 5:** The effect of different concentrations of the fungal biopesticides Mycotal (*L. muscarium*) and HDC F122 on the disease severity and sporulation of *Oidium neolycopersici* when maintained in the double Petri dish bioassay chamber ten days post inoculation. The error bars represent the standard error of the mean.

#### 1.6 Experiment to determine the effect of *Lecanicillium muscarium* and other treatments on *Oidium neolycopersici* growth and spore production on whole plants

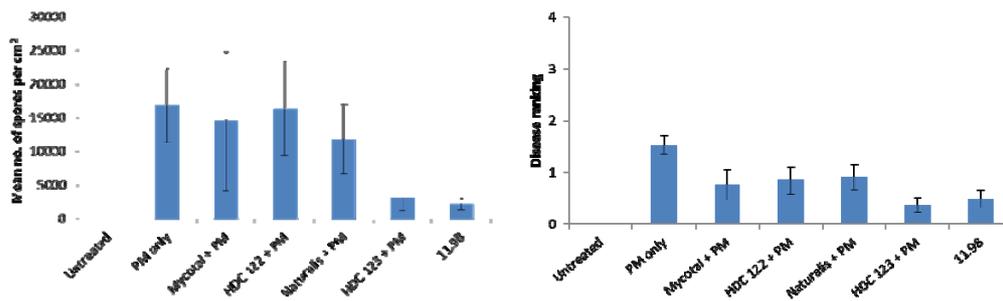
All of the treatments reduced the level of powdery mildew developing on the plant (Figure 6). Disease development, at 10 days post inoculation, on the three replicate plants ranged from  $3.2 \times 10^6$  to  $1.1 \times 10^7$  spores per plant on the untreated controls. Disease development on the replicated treated plants ranged from 1 to  $7 \times 10^5$  (HDC F122),  $5.5 \times 10^5$  to  $2.2 \times 10^5$  (Naturalis)  $2.1$  to  $2.5 \times 10^6$  (Mycotal),  $9 \times 10^5$  to  $2.6 \times 10^6$  (HDC F123) and  $3 \times 10^5$  to  $2.8 \times 10^6$  (AQ10) spores per plant. Results were variable within treatments with some 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 the crop canopy 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% (HDC F122), 76% (Naturalis L), 73% (AQ10), 70% (HDC F123) and 65% (Mycotal). At 10 days post inoculation 61-80% of the leaflet of untreated inoculated controls was infected with powdery mildew. Treatment with Mycotal, HDC F122, Naturalis L and HDC F123 all reduced the powdery mildew infected area to less than 50%% (Figure 6). There was no evidence of any mycoparasitism of powdery mildew from the biopesticide treatments.



**Figure 6:** The effect of isolates of HDC F122, Naturalis (*B. bassiana*), Mycotai (*L. muscarium*), HDC F123 and AQ10 (*A. quisqualis*) on the disease severity and sporulation of *Oidium neolycopersici* on tomato plants ten days post inoculation. The error bars represent the standard error of the mean.

### 1.7 Experiment to determine the effect of *Lecanicillium muscarium*, *Beauveria bassiana* and HDC F123 on cucurbit powdery mildew (*Sphaerotheca fusca*) growth and spore production.

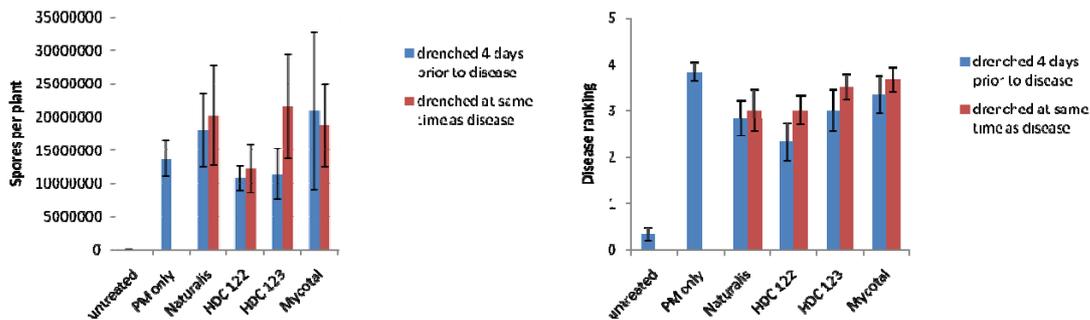
Two of the treatments (HDC F123 and *B. bassiana* 11.98) reduced the level of cucurbit powdery mildew developing on the leaf disc (Figure 7). For the untreated controls, disease development, at 14 days post inoculation, on the 36 replicate leaf discs ranged from 0 to  $1.68 \times 10^5$  spores per  $\text{cm}^2$ . In the treated chambers, disease development on the 18 replicate leaf discs, ranged from 0 to  $1.55 \times 10^5$  (Mycotai), 0 to  $1.08 \times 10^5$  (HDC F122), 0 to  $9.26 \times 10^4$  (Naturalis L), 0 to  $3.49 \times 10^4$  (HDC F123), 0 to  $1.48 \times 10^4$  spores per  $\text{cm}^2$  (*B. bassiana* 11.98). Results were variable within treatments with some replicate leaf discs having no control on cucurbit powdery mildew whereas others gave good control with the same treatment. This may be due to small differences in both the physiological status of the leaf disc or the environmental conditions within each chamber affecting the growth of the insect pathogenic fungi. In comparison to the untreated controls two of the treatments reduced the overall sporulation of cucurbit powdery mildew by 82% (HDC F123) and 87% (*B. bassiana* 11.98). At 14 days post inoculation 21-40% of the leaf disc of untreated inoculated controls was infected with cucurbit powdery mildew. Treatment with Mycotai, HDC F122, Naturalis L, HDC F123 and *B. bassiana* 11.98 all reduced the observed cucurbit powdery mildew infected area to 0-20% (Figure 7). There was no evidence of any mycoparasitism of cucurbit powdery mildew from the biopesticide treatments.



**Figure 7:** The effect of isolates of HDC F122, Naturalis L (*B. bassiana*), Mycotal (*L. muscarium*), HDC F123 and *B. bassiana* 11.98 on the disease severity and sporulation of *Sphaerotheca fusca* when maintained on leaf discs fourteen days post inoculation. The error bars represent the standard error of the mean.

2.1 Experiment to determine the effect of root drenches of *Beauveria bassiana* and other treatments on *Oidium neolycopersici* growth and spore production.

None of the treatments reduced the level of powdery mildew developing on the plant (Figure 8). Disease development, at 10 days post inoculation, on the six replicate plants ranged from  $8.7 \times 10^6$  to  $2.52 \times 10^7$  spores per plant on the untreated controls. Disease development on the plants drenched four days before infection ranged from  $3.2 \times 10^6$  to  $1.7 \times 10^7$  (HDC F122),  $9.9 \times 10^6$  to  $4.1 \times 10^7$  (Naturalis L),  $4 \times 10^5$  to  $7.8 \times 10^7$  (Mycotal) and  $9 \times 10^5$  to  $2.6 \times 10^6$  (HDC F123) spores per plant. Disease development on the plants drenched at the same time as powdery mildew infection ranged from 0 to  $2.2 \times 10^7$  (HDC F122),  $6 \times 10^6$  to  $5.3 \times 10^7$  (Naturalis L),  $4.9 \times 10^6$  to  $4.8 \times 10^7$  (Mycotal) and 0 to  $5.2 \times 10^7$  (HDC F123) spores per plant. Results were variable within treatments with some 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 the crop canopy affecting the growth of the insect pathogenic fungi. At 10 days post inoculation 61-80% of the leaflet of untreated inoculated controls was infected with powdery mildew. Treatment with Mycotal, HDC F122, Naturalis L and HDC F123 resulted in powdery mildew infected areas of less than 60% (Figure 8).

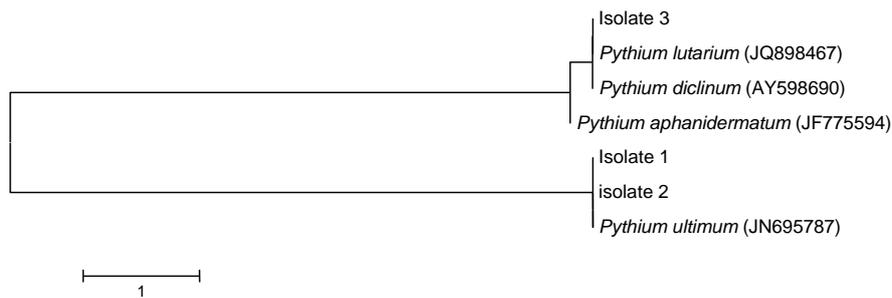


**Figure 8:** The effect of root drenches of isolates HDC F122, Naturalis L (*B. bassiana*), Mycotal (*L. muscarium*) and HDC F123 on the disease severity and sporulation of *Oidium neolycopersici* on tomato plants ten days post inoculation. The error bars represent the standard error of the mean.

### 3.2 Molecular characterisation of *Pythium* isolates

The DNA sequence analysis gave a different species identity to that recorded in the Warwick culture collection database for one of the three *Pythium* isolates:

- *Pythium* isolate 1, recorded in the culture collection database as *Pythium aphanidermatum*, was identified by DNA sequence analysis as *Pythium ultimum*.
- *Pythium* isolate 2, recorded in the culture collection database as *Pythium ultimum*, was confirmed as *P. ultimum* by DNA sequence analysis.
- *Pythium* isolate 3, recorded in the culture collection database as *Pythium* spp., was identified as *P. lutarium* by DNA sequence analysis.



**Figure 9.** Unrooted phylogenetic tree showing the relationship of the *Pythium* isolates used in this study. Numbers in parentheses refer to the nucleotide sequence database code of reference *Pythium* isolates at NCBI.

### 3.3 Pilot experiment to determine inoculums levels for laboratory bioassays

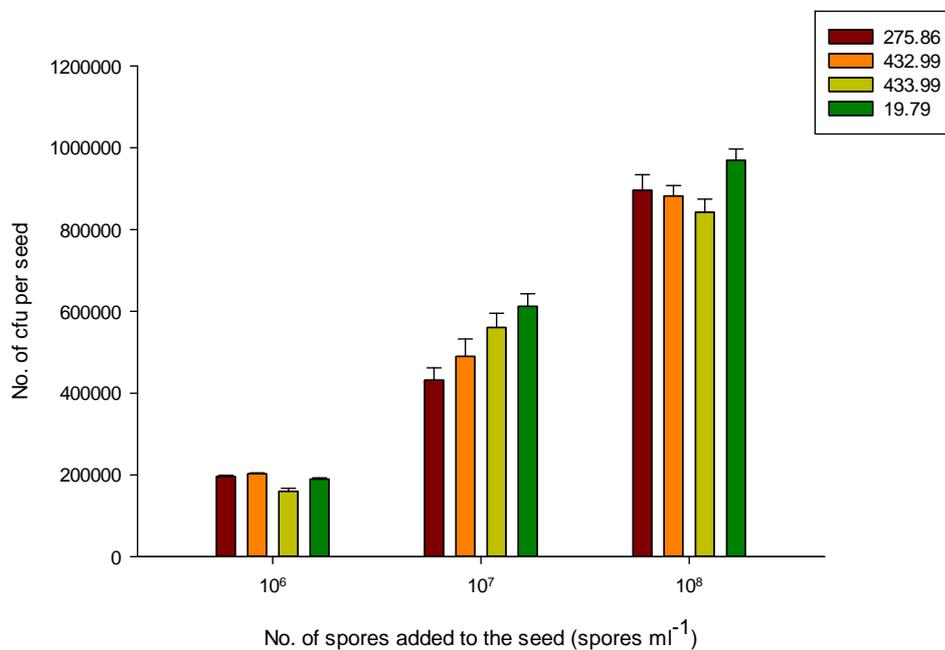
The experiments to establish appropriate inoculum levels showed that for uninoculated compost, 33 - 35 healthy tomato seedlings (from a possible 35) survived at the end of the experiments (Table 11). In experiment 1 (which used *Pythium* isolate 2) only a pathogen amendment rate of 25% v/v showed any 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 (*Pythium* isolates 1 and 3) were examined in experiments 3 and 4. Both were more pathogenic than *Pythium* isolate 2 with only 19-23 and 10-20 healthy seedlings out of a possible 35 emerging for *Pythium* isolate 1 and *Pythium* isolate 3 respectively 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 seedlings dying over a period of 7-14 days post-emergence. For this reason it was decided to stop working with *Rhizoctonia* and subsequent experiments were done with *Pythium* isolate 3 (*Pythium lutarium*) at a rate of 20%.

**Table 11.** The effect of pathogen inoculum rate on the percentage of healthy seedlings

<i>Pythium</i> isolate 2 ( <i>P. ultimum</i> )				<i>Pythium</i> isolate 1 ( <i>P. ultimum</i> )		<i>Pythium</i> isolate 3 ( <i>P. lutarium</i> )		<i>R. solani</i>	
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								

### 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  $1 \times 10^7$  conidia received  $1.6$  to  $2.02 \times 10^5$  viable colony forming units per seed (Figure 10). Seed treated with  $1 \times 10^8$  conidia received  $4.31$  to  $6.12 \times 10^5$  viable colony forming units per seed. Seed treated with  $1 \times 10^9$  conidia received  $8.42$  to  $9.70 \times 10^5$  viable colony forming units per seed. 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 12).



**Figure 10.** The number of colony forming unit (viable spores) recovered from seed treated with different concentrations *HDC F123* (275.86), *Naturalis L* (432.99), *HDC F122* (433.99) and *L. muscarium* (19.79).

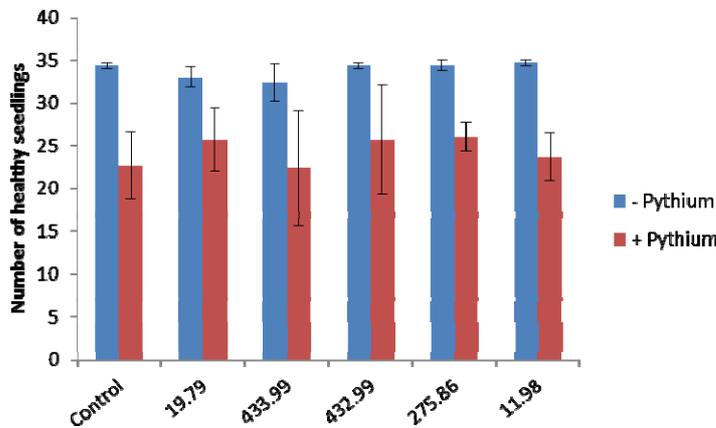
**Table 12.** Percentage germination of tomato seeds coated with different concentrations of different entomopathogenic fungi: Warwick culture collection isolate *B. bassiana* 432.99 (cultured from Naturalis L); isolate 433.99 (cultured from HDC F122); isolate *L. muscarium* 19.79 (cultured from Mycotal); isolate 275.86 (cultured from HDC F123).

Isolate	Species	Spore concentration added	% germination
432.99	<i>Beauveria bassiana</i> (from Naturalis L)	10 <sup>9</sup> spores ml <sup>-1</sup>	100
		10 <sup>8</sup> spores ml <sup>-1</sup>	100
		10 <sup>7</sup> spores ml <sup>-1</sup>	100
433.99	HDC F122	10 <sup>9</sup> spores ml <sup>-1</sup>	100
		10 <sup>8</sup> spores ml <sup>-1</sup>	100
		10 <sup>7</sup> spores ml <sup>-1</sup>	100
19.79	<i>Lecanicillium muscarium</i> (from Mycotal)	10 <sup>9</sup> spores ml <sup>-1</sup>	90
		10 <sup>8</sup> spores ml <sup>-1</sup>	90
		10 <sup>7</sup> spores ml <sup>-1</sup>	100
275.86	HDC F123	10 <sup>9</sup> spores ml <sup>-1</sup>	100
		10 <sup>8</sup> spores ml <sup>-1</sup>	100
		10 <sup>7</sup> spores ml <sup>-1</sup>	100

### 3.5 Experiment to investigate the ability of insect pathogenic fungi to control *Pythium* damping off.

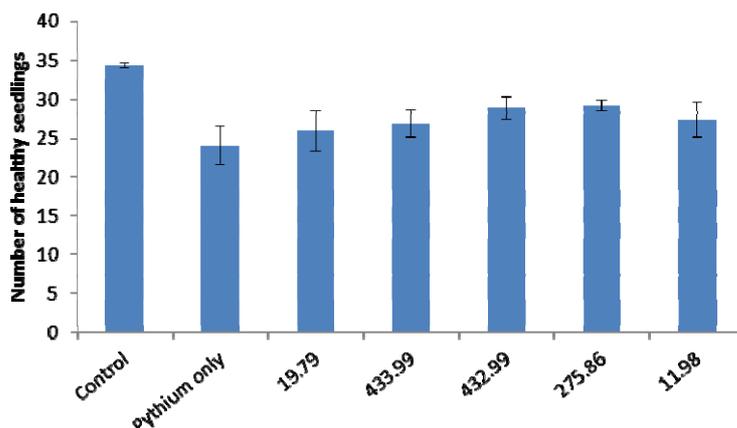
This experiment was done on two occasions. On the first occasion, seed treated with the fungal biocontrol isolates showed 92% or greater germination in untreated compost (Figure 11). Inoculation of the compost with *Pythium* reduced the germination of untreated seed by 34% with 17-30 healthy seedlings out of a possible 35 emerging. This is a higher rate of emergence than observed in pilot experiments (3.3) and possibly indicated a lower level of inoculum in the compost. There was no effect of treating the seed with the biocontrol fungi on the emergence of healthy seedlings in compost containing *Pythium* (Figure 11). However results were very variable between modules, with 17-30 healthy seedlings emerging in the control modules and 9-30 healthy seedling emerging in the treated seed modules, and this natural within-treatment variability is likely to have resulted in low statistical power in the experiment. The experiment was therefore repeated with a higher level of replication (= occasion 2). On this second occasion, inoculation of the compost with *Pythium* reduced the

germination of untreated seed by 30%, with 16-31 healthy seedlings out of a possible 35 emerging compared to 33-35 healthy seedlings in the un-inoculated control (Figure 12).



**Figure 11.** The effect of seed treatments of isolates 19.79 (Mycotal), 433.99 (HDC F122), 432.99 (Naturalis L), 275.86 (HDC F123) and 11.98 on tomato seedlings – occasion 1. The error bars represent the standard error of the mean.

All of the treatments had significantly ( $P < 0.001$ ) fewer healthy seedlings emerging than the *Pythium*-free control. In compost treated with *Pythium*, two of the biocontrol treatments (seeds inoculated with fungal biocontrol isolates 275.86 and 432.99) had significantly ( $P < 0.001$ ) more healthy seedlings emerging compared to untreated seeds, with 23-27 and 19-32 healthy seedlings out of a possible 35 emerging respectively. Seed treatment with these two isolates resulted in a 15% improvement in seedling emergence.



**Figure 12.** The effect of seed treatments of isolates HDC F122, *B. bassiana* isolate 432.99 (cultured from Naturalis L), *L. muscarium* isolate 19.79 (cultured from Mycotal), fungal isolate 275.86 (cultured from HDC F123) and *B. bassiana* isolate 11.98 on tomato seedlings. This was the second occasion on which this experiment was done (referred to as occasion 2 in the text). The error bars represent the standard error of the mean.

### 3.6 Isolation of insect pathogenic fungi from tomato seedlings.

Only *Beauveria bassiana* (isolate 433.99 and 11.98) were isolated from tomato seedling tissue. From 25 seedlings, surface sterilised prior to plating, three seedlings showed evidence of endophytic growth from leaf tissue (Table 13).

Table 13. Summary of re-isolations of entomopathogenic fungi from coated tomato seed

<b>Isolate</b>	<b>Species</b>	<b>Root</b>	<b>Stem</b>	<b>Leaf</b>
19.79	<i>Lecanicillium muscarium</i>	N	N	N
433.99	HDC F122	N	N	Y
432.99	<i>Beauveria bassiana</i>	N	N	N
275.86	HDC F123	N	N	N
11.98	<i>Beauveria bassiana</i>	N	N	Y

## Discussion

We observed significant levels of tomato powdery mildew control in the laboratory bioassay with detached leaflets using insect pathogenic fungi (Mycotal, Naturalis L, HDC F122 and HDC F123) as well the bacterial biofungicide Serenade, and two other products, HDC F124 and Thiovit Jet. We obtained good levels of control of tomato powdery mildew on whole tomato plants with Mycotal, Naturalis L, HDC F122 and HDC F123, as well as the biofungicide AQ10. We also found good control of cucumber powdery mildew in a laboratory bioassay with HDC F123 and *B. bassiana* 11.98. These results are encouraging, and although additional research will be required to show proof of concept, it is a significant step forwards in identifying potential new biocontrol agents and other alternatives for powdery mildew control for growers.

The fact that cucumber powdery mildew was controlled by a smaller number of fungal biopesticides than tomato powdery mildew is an important finding. It could mean that cucumber powdery mildew is generally less amenable to biological control, or it could mean that the insect pathogenic fungi we tested have different specificities, which might then be useful in understanding the mechanism of action of the fungal biocontrol agents. When the bioassays were being done, the leaf material was routinely observed under a binocular microscope to look for evidence of mycoparasitism. No mycoparasitism was observed, however it may have been that these observations were not sufficient to identify the mechanism of action, and we would most likely need to run more detailed experiments in future. 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), so it is likely to have done the same against tomato powdery mildew in the bioassays used in this project. The other species of insect pathogenic fungi studied here 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 when used at the manufacturers' recommended concentrations, which is encouraging. 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 experiments done in experimental greenhouse compartments and then moving on to experiments done in commercially grown crops. Because some of the fungal biopesticides are already approved for use on tomato crops, it would be possible to use them in experiments on a nursery without needing an experimental permit or having to destroy the crop afterwards.

We observed no control of tomato powdery mildew when the fungal biopesticides Mycotal, Naturalis L, HDC F122 or HDC F123 were applied as drenches to the roots of tomato plants. The fungal biopesticide HDC F123 has been reported in the scientific literature to be able to colonize the rhizosphere, which could be a possible method for induction of systemic resistance to pathogens in the plant: however our data suggests this is unlikely. *Beauveria bassiana* isolate 11.98, applied as a root drench to cotton seedlings, has been reported in the literature to give control of *Xanthomonas axonopodis* inoculated onto cotton leaves as a result of induced systemic resistance (Griffin *et al.*, 2006; Ownley *et al.* 2010). Unfortunately, because of the long time taken to finalise the Material Transfer Agreement for *B. bassiana* 11.98 between the Universities of Warwick and Tennessee, we weren't able to include it in our experiment against tomato powdery mildew.

We had evidence of a small but statistically significant amount of control of *Pythium lutarium* on tomato using spores of the insect pathogenic fungal isolates 275.86 (which were cultured from HDC F123) and 432.99 (cultured from Naturalis L) which were applied as a seed coating. The *Pythium* experiments were straightforward to conduct but we observed a high level of variability between replicates of the same treatment. This may have been caused by replicate trays receiving different amounts of *Pythium* inoculum (possibly as a result of an uneven / clumped distribution of *Pythium* in the compost). We chose a level of *Pythium* inoculum intended to give 50% seedling emergence in the controls, which is a logical choice in terms of detecting biocontrol activity but is also likely to give the largest within-treatment variation in seedling emergence. *Beauveria bassiana* 11.98 was reported to give control of *Pythium myriotylum* when applied to tomato seed, with control occurring as a result of the endophytic colonization of the seedling by *B. bassiana* (Ownley *et al.*, 2008). We observed

no control of *Pythium lутarium* with *B. bassiana* 11.98 in our bioassay and there was no evidence of endophytic colonization. It is possible that our seed coating method did not facilitate plant colonization by *B. bassiana* 11.98, or it could be that the variety of tomato used was not susceptible to colonization by *Beauveria*. It would be worth investigating different seed application systems to identify ways of allowing endophytic colonization by *B. bassiana* 11.98, which may also increase the efficacy of other fungal isolates (particularly 275.86 and 432.99, the two fungal isolates which gave significant control of *Pythium* in our bioassay).

## Conclusions

- Three commercial biopesticides based on insect pathogenic fungi (Mycotal, Naturalis L and HDC F123) that have potential for control of tomato powdery mildew have been identified. The product HDC F124 also has potential to control tomato powdery mildew control whilst Serenade ASO and Thiovit Jet, already used in commercial programs had some effect on the disease helping to verify the bioassay systems used.
- The commercial biopesticide HDC F123 was found to have potential for control of cucumber powdery mildew. The insect pathogenic fungus *Beauveria bassiana* 11.98, which is not used as a commercial biopesticide, also gave significant control of cucumber powdery mildew in the laboratory bioassay used in this project.
- Cultures of two of the fungal biopesticides - 275.86 (cultured from HDC F123) and 432.99 (cultured from Naturalis L) – gave small but significant amounts of control of *Pythium lутarium* when applied as a coat to tomato seeds.
- Further work is required to determine the levels of control of tomato and cucumber powdery mildew achievable with these agents under experimental conditions that more realistically reflect commercial crop production. Research to optimize the timing of application, and to investigate mode of action against powdery mildew, is also warranted. Research to improve the seed application method might also be worthwhile.

## 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.

Chandler, D. (2012). Can we use insect pathogenic fungi to control plant pathogenic fungi? Presentation at the AAB conference Advances in Biological Control & IPM, 16-17 October 2012.

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