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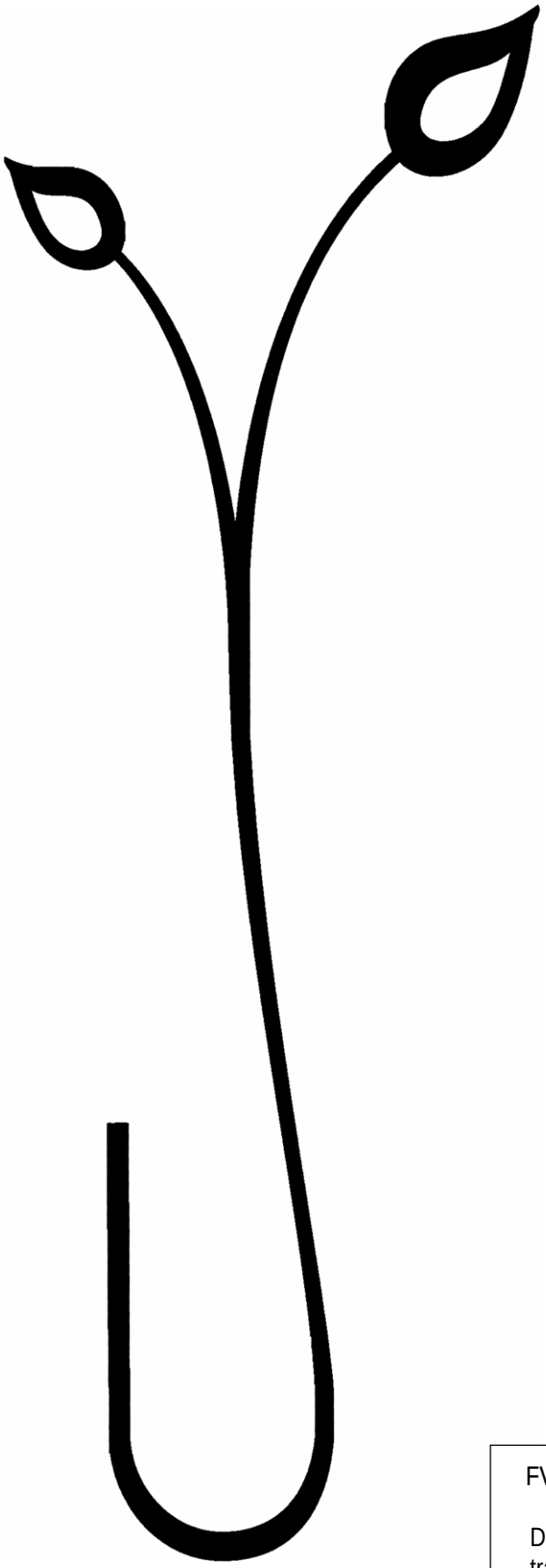

Research Report

FV 352

Disease management in organic brassica seed and transplants

Annual Report 2009-10

Project title:	Disease management in organic Brassica seed and transplants
Project number:	FV352
Project leader:	Dr John Clarkson (Warwick HRI) Dr Steven Roberts (Plant Health Solutions) (Joint Leaders)
Report:	Annual report, May 2010
Previous report:	None
Key staff:	Anita Scruby
Location of project:	Warwick HRI, Wellesbourne and Garden Organic, Coventry
Project coordinator:	Mr Roger White
Date project commenced:	1st June 2009
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Key words:	Brassica, transplants, disease, control, organic, plant raising, compost, microbial inoculants, *Pythium, Rhizoctonia, Alternaria, Phoma*

The results and conclusions in this report are based on a series of experiments 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.

Dr J Clarkson
Principle Investigator
Warwick HRI

Signature ..... Date .....

Dr S J Roberts
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Report authorised by:

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Grower Summary

Headlines

- Incorporation of composted green waste into the growing medium reduced damping-off at low disease pressures.
- Four seed treatments showed promise for the control of *Phoma* and *Alternaria* in Brassicas

Background and objectives

The use of healthy, clean seed and planting material is an important component of effective disease management for plant propagators and is essential for organic growers who have fewer options for disease control. This was highlighted at an HDC/HDRA stakeholder day (Managing Pests, Diseases and Weeds in Organic Vegetable Production, Ryton Organic Gardens 2007) where the importance of seed quality and good disease management was identified by stakeholder discussion groups as a priority area for future research.

Diseases caused by soil and seedborne fungal pathogens such as *Pythium* spp., *Rhizoctonia solani* (damping-off, soil-borne) and *Alternaria* spp., *Phoma* (seedborne) are a major problem for all plant propagators, especially for high volume / high value plants such as vegetable brassicas which have a total production value of ca. £200 million in the UK (Defra statistics, 2007). Additional disease problems may also be created for organic plant raisers through the increasing practice of companion planting (e.g. bird's foot trefoil for management of cabbage root fly) as the species employed are often fodder crops of variable seed quality which can harbour pathogens and reduce the germination and emergence of both companion and crop.

A number of products which claim to have benefits for disease management in organic transplant production are now available in the EU and are marketed as growth promoters, plant strengtheners or crop protection agents. In particular, suppressive microbial inoculants and composts have shown promise for disease control and there is increasing interest in these products in conventional production systems because of the pressure to reduce pesticide use and the potential loss of many active ingredients with the proposed revision of EC directive 91/414.

The aim of this project is to evaluate a range of organically acceptable compost and brassica seed treatments for their efficacy and cost effectiveness in controlling damping-off diseases caused by *Pythium* and *Rhizoctonia* and seedborne diseases caused by *Phoma* and *Alternaria*.

Summary of results and conclusions

Compost treatments

The microbial products Trianum, Prestop, Mycostop, Subtilex, Revive P and green waste compost inoculated with *Trichoderma* (*T. viride* S17A or *T. harzianum* from Trianum) were tested for their efficacy in controlling damping-off of cauliflower seedlings caused by *P. ultimum* and *R. solani* and compared with a fungicide treatment (thiram- treated seed) in multiple experiments. The pathogens were introduced into Bulrush Organic Modular Compost and the microbial treatments added as drenches or granules at the recommended rates. Green waste compost with or without *Trichoderma* was added at 20% v/v. The amended Bulrush compost was dispensed into modules and cauliflower seed (cv. Belot) sown. The number of healthy seedlings was assessed over time.

Damping-off disease pressure varied between experiments but overall there was no consistent or clear benefit from adding the microbial products tested for control of *P. ultimum* or *R. solani.* However, at low disease pressures there was some evidence that green waste with or without *Trichoderma* was beneficial. The thiram-treated seed consistently controlled *P. ultimum* but was less effective against *R. solani* at high disease pressures.

Seed treatments

Hot water, two plant oils (thyme and clove), and five microbial products (Serenade, Mycostop and three experimental products) were assessed for their efficacy in the control of two seed-borne fungal pathogens of brassicas, and improving emergence of the companion plant bird's foot trefoil. Seed was treated at recommended rates and pathogen infestation levels assessed in a standard 2,4-D blotter seed test (brassicas) or freeze-blotter test (bird's foot trefoil). Effects on emergence and disease transmission were assessed by sowing seeds in trays of Bulrush Organic Modular Compost.

Hot Water, Thyme oil, and HDC B0002 gave statistically significant reductions in *Phoma* seed infestation levels, and a greater reduction that the chemical standard Thiram. Hot Water in particular reduced infestation to undetectable levels (i.e. <1.5%). In transmission/emergence tests the proportion of seedlings affected by *Phoma* was significantly reduced by treatment with hot water, thiram, thyme oil and Serenade with hot water having the greatest effect.

All treatments gave a statistically significant reduction in the level of *Alternaria* infestation compared to the untreated control. The greatest reductions were achieved with Hot Water, Thyme oil, clove oil, the microbials Serenade and HDC B0002, and the fungicide Thiram. Again Hot Water reduced infestation to undetectable levels (i.e. <1.5%).

Emergence was relatively poor for the bird's foot trefoil and was not improved by any of the treatments.

Treatment with hot water led to a small but significant increase in damping off. None of the treatments gave a significant reduction in damping-off compared to the untreated controls.

Overall there would appear to be four leading treatments for control of seed-borne disease in Brassicas: hot water, thyme oil, Serenade and HDC B0002. The first three of these treatments have also been tested and shown promise in previous work on both seedborne fungi and bacteria. Currently, hot water is the only treatment that can be legally used, as it does not require approval. Unfortunately its routine application is not without problems and the temperature-time regimes need to be optimised on a per seed-lot basis to ensure maximum efficacy and minimum seed damage. Thyme oil is a natural plant product with broad spectrum disinfectant activity but would require formal approval as a pesticide before it could be used as a commercial seed treatment. Serenade ASO is a microbial product based on a strain of *Bacillus subtilis*. It is currently approved for foliar application to all crops (via a SOLA), but does not have approval as a seed treatment. The manufacturer should be encouraged to seek approval for seed treatment. Experimental product HDC B0002 is also a microbial product and the manufacturer should be encouraged to seek approval as a seed treatment.

Approval status of products

Table 1. Pesticide approval status of the various treatments/products examined in this study

Treatment/Product	**Status**
Compost treatments	
Trianum	Not approved in the UK, listed on Annexe 1 of 91/414.
Prestop	Not approved in the UK, listed on Annexe 1 of 91/414.
Mycostop	Not approved in the UK. Approved in several EU countries.
Subtilex	Not approved.
Revive P	Not approved, but marketed as a 'Microbial Soil Treatment'.
Green Waste	Approval not required.
Seed treatments	
Hot water	Approval not required.
Thiram	Approved as a seed treatment for Brassicas.
Thyme oil	Not approved, Annexe 1 listing in progress?
Clove oil	Not approved, listed on Annexe 1 of 91/414.
Serenade ASO	Not approved for application to seeds. Approved for foliar application to all crops (SOLA).
Mycostop	Not approved in the UK. Approved in several EU countries.
HDC B0001	Experimental product. Not approved.
HDC B0002	Experimental product. Not approved.
HDC B0003	Experimental product. Not approved.

Financial benefits

None to date.

Action points for growers

- Consider incorporation of composted green waste into growing media for Brassica transplants.
- Do not routinely use hot-water treatments without optimisation on a per-seed lot basis to avoid the potential for detrimental effects on emergence.
- Consider supporting an HDC-funded ‘commodity approval’ for thyme oil.
- Contact the manufacturers of the potential microbial seed treatments (Agraquest and Becker Underwood) or their distributors to demonstrate interest in the products.

Science Section

Introduction

Microbial inoculants have previously been shown to control soil and seedborne diseases in experimental systems, but few have been marketed commercially. However, products currently available in the UK which may suppress root rots include Trianum (Koppert), Prestop and Mycostop (Fargro) which contain *Trichoderma harzianum*, *Gliocladium catenulatum* and *Streptomyces griseoviridis* respectively. These beneficial micro-organisms have been shown to reduce damping-off and root rots caused by *Pythium* and other spp. (e.g. Harman, 2000; McQuilken *et al*., 2001; Mohammadi, 1992). More recently, a HortLINK project (HL0176) demonstrated that applying the beneficial fungus *Trichoderma viride* S17A with green waste compost resulted in enhanced field suppression of the soil-borne pathogen *Sclerotium cepivorum* causing *Allium* white rot. Hence, combining microbial and compost treatments may be a promising approach for an effective disease management strategy in Brassica transplants.

During an EC funded project (STOVE) in which Plant Health Solutions was a partner a number of physical, microbial, resistance inducing and natural products were examined for their efficacy as seed treatments against a number of seedborne host/pathogen combinations. The hosts included Brassicas and pathogens included *Alternaria* spp. and a *Phoma* sp. The resistance inducing products were generally unsuccessful while physical treatments were very effective and are already used/or being developed commercially by a number of seed companies. Promising results were also obtained for a number of the microbial agents, which included bacteria (*Bacillus subtilis* strains, *Streptomyces griseoviridis*, *Pseudomonas chlororaphis*) and fungi (*Clonostachys rosea* =*Gliocladium roseum*).

This aim of this project will therefore be to evaluate a range of microbial / green waste compost treatments for control of damping-off caused by *P. ultimum* and *R. solani* and organically acceptable Brassica seed treatments for control of *Phoma* and *Alternaria* diseases.

Compost Treatments

Materials and Methods

Preparation of pathogen inoculum

A *Pythium ultimum* (var. *ultimum*) culture known to be pathogenic on Brassica seedlings was obtained from Chris Gilligan (University of Cambridge), grown on potato dextrose agar (PDA) and stored on PDA slopes at 5°C. Inoculum for experiments was produced by adding eight agar plugs from a 7-day-old culture to a sterilised (121°C for 60 minutes on two consecutive days) mixture of Bulrush modular organic compost (250 ml, 4 mm sieved), potato pieces (25 g, 4 mm^2) and an appropriate amount of water for an overall moisture content of 70%. After 2 weeks incubation at 18°C, the inoculum was harvested by sieving (2 mm) before use in experiments.

A *Rhizoctonia solani* culture from the WHRI collection known to be pathogenic on oilseed rape was grown on PDA and stored on PDA slopes at 5°C. Inoculum for experiments was produced by adding five agar plugs from a 7-day-old culture to a mixture of wheat bran flakes (8 g), sand (195 g) and water (35 ml water) autoclaved at 121°C for 15 minutes. After 2 weeks incubation at 18°C, the inoculum was harvested by sieving (2mm) before use in experiments.

In some experiments the pathogen inoculum of both *P. ultimum* and *R. solani* was quantified by suspending in SDW and serially diluting triplicate 1 g samples, then spreading 0.1 ml aliquots onto the surface of PDA plates and incubating at 20°C for 7 days. The number of colony forming units per g inoculum (CFU/g) was then calculated.

Preparation of microbial treatments

Trichoderma viride S17A from the WHRI culture collection was grown on PDA at 18°C for three weeks after which spore suspensions (approx 10^8 spores /ml) were obtained by adding 20 ml sterile distilled water (SDW) to the cultures and scraping gently with a spatula. This spore suspension (5 ml) was then used to inoculate wheat bran flakes (12 g with 30 ml water in 250 ml flasks) which had been autoclaved at 121°C for 15 minutes. The inoculated wheat bran was incubated for 3 days at 20°C before use. Prestop, Mycostop, Subtilex powders and Revive P suspension were added to water at the appropriate rate before being added to Bulrush compost. Trianum granules were added directly to the compost at the appropriate rate. Rates of product application are shown in Table 1.

Table 1. Rates of application for microbial treatments

Product	**Microbial organism**	**Recommended rate of application**
Trianum	*Trichoderma harzianum*	750 g m^3 compost
Prestop	*Gliocladium catenulatum*	500 g m^3 compost
Mycostop	*Streptomyces griseoviridis*	1.4 g m^3 compost
Subtilex	*Bacillus subtilis*	2.3 g m^3 compost
Revive P	*Bacillus subtilis*	100 ml concentrate m^3 compost

Preparation of **Trichoderma** *inoculated green waste compost*

Spore suspensions of *T. viride* S17A (5 ml) were used to inoculate 150 g sterile rye grain (Sylvan Spawn Limited, Peterborough, UK) in honey jars (approx. volume 320 ml) and incubated for 14 days at 18°C. The colonised grain was then used to inoculate green waste compost (4 mm sieved, Organic Recycling Ltd) at a rate of 2% w/w. For the *T. harzianum* product, Trianum, granules were added directly to green waste compost at a rate of 2% w/w and incubated as for S17A. The amount of *Trichoderma* in the green waste composts was quantified by serial dilution and plating as described for the pathogen inoculum.

Setting up experiments

All experiments were set up in 35-cell modules (15.5 ml volume, cut from 345 module trays) using Bulrush modular organic compost maintained at 75% water content and sown with cauliflower cv. Belot (one seed per cell). Before testing any treatments, preliminary experiments were done to determine the effect of different pathogen inoculum rates of *P. ultimum* (0-23% v/v) and *R. solani* (0-5% v/v) on the number of healthy seedlings so that appropriate levels could be used in subsequent experiments. The pathogen inoculum was mixed thoroughly with the Bulrush compost before dispensing into modules. After these preliminary experiments, the microbial treatments and green waste compost inoculated with *Trichoderma* (either *T. viride* S17A or Trianum containing *T. harzianum*) were tested for their ability to control damping-off caused by either *P. ultimum* or *R. solani*. Fungicide treated seed (thiram) was also included as a control. The microbial treatments were all applied in an appropriate volume of water to maintain the Bulrush modular organic compost at 75% water content and were well-mixed with the compost and the pathogen inoculum before dispensing into modules and cauliflower seed sown. The *Trichoderma* inoculated green waste compost was added to the Bulrush compost at a rate of 20% v/v. Modules were placed in a glasshouse compartment maintained at 15°C constant with watering via capillary matting and seep hoses from below. There were four replicate 35 cell modules per treatment. The number of healthy seedlings that emerged and survived was assessed approximately every two days. A summary of the treatments and pathogen inoculum rate in each experiment is shown in Table 2.

Table 2. Summary of experiments and treatments

	Experiment code and pathogen inoculum rate (v/v)					
	P1	R1	P2	R2	P3	R3
Treatment[1]	*P. ultimum* 7%	*R. solani* 0.1%	*P. ultimum* 7%	*R. solani* 0.05%	*P. ultimum* 5% & 3%	*R. solani* 0.15% & 0.08%
Uninoc control	☐	☐	☐	☐	☐	☐
Fungicide	☐	☐	☐	☐	☐	☐
Trianum only	☐	☐	☐	☐	☐	☐
Prestop	☐	☐	☐	☐	☐	☐
Mycostop	☐	☐	☐	☐	☐	☐
Subtilex	☐	☐	☐	☐	☐	☐
Revive P	☐	☐	☐	☐	☐	☐
T. viride S17A					☐	☐
GW only					☐	☐
GW Trianum					☐	☐
GW T. viride S17A					☐	☐

[1] GW = green waste compost, Fungicide = thiram treated seed

Results and Discussion

Pathogen inoculum levels

The initial experiments to establish appropriate inoculum levels for *P. ultimum* showed that for uninoculated compost 22-26 healthy cauliflower seedling seedlings (from a possible 35) survived at the end of the experiment compared with 4-14 seedlings for a pathogen amendment rate of 5% v/v and 22-26 at 0.5-1.0% (Table 3). For *R. solani*, there were 20-25 healthy seedlings for the uninoculated compost compared with 4-5 at amendment rates of 0.01-0.02% (Table 3). It was noted that *P. ultimum* caused pre-emergence damping-off and hence those seedlings that did emerge were usually healthy and survived. In contrast, *R. solani* predominantly caused post-emergence damping-off with most seedlings dying over a period 7-21 days post-emergence.

Table 3. Effect of pathogen inoculum amendment rate on the percentage of healthy seedlings

P. ultimum				***R. solani***			
Experiment 1		Experiment 2		Experiment 1		Experiment 2	
% rate v/v	% healthy seedlings	% rate v/v	% healthy seedlings	% rate v/v	% healthy seedlings	% rate v/v	% healthy seedlings
0.0	74.2	0.0	62.9	0.00	71.4	0.00	57.1
0.5	54.2	1.0	68.6	0.02	11.4	0.01	14.3
1.0	40.0	3.0	48.6	0.10	0	0.10	0
5.0	11.4	5.0	40.0	0.20	2.9	0.20	2.9
10.0	0			2.00	0		
23.0	0			5.00	0		

In the first experiments testing the microbial treatments (Table 2) a 7% v/v inoculum rate was chosen for *P. ultimum* (experiment P1) and 0.1% for *R. solani* (experiment R1). However, it was clear from the results from these and subsequent experiments that the number of healthy plants in the inoculated control treatments was difficult to predict and that there was no clear relationship with the v/v rate of pathogen inoculum applied. Therefore, pathogen inoculum was quantified by dilution plating before use in the second round of experiments where *P. ultimum* was applied at 7% v/v resulting in 1.8×10^4 pathogen CFU/module cell of compost (experiment P2) and *R. solani* at 0.05% v/v resulting in 1.8×10^3 CFU/module (experiment R2). In the third round of experiments it was decided that two rates of inoculum would be examined at the same time for each pathogen with the aim of assessing the effect of the microbial treatments under different disease pressures and overcoming inconsistencies in relation to pathogen inoculum rate and number of healthy plants in the inoculated control treatments. Hence 3 and 5% v/v inoculum rates were used for *P. ultimum* corresponding to 9×10^2 and 7.7×10^3 CFU/module (experiment P3) and 0.08% and 0.15% v/v rates used for *R. solani* which corresponded to 105 and 560 CFU/module (experiment R3).

Microbial inoculum levels

All the microbial products were applied at the recommended rates (see Table 1) for the first two rounds of experiments (P1, P2, R1, R2). Before the next series of experiments, the concentration of microbial inoculum was quantified by series dilution of suspensions in water for comparison with the expected quantities indicated by the manufacturers. The results indicated that the Prestop and Trianum products had fewer CFU than expected by a factor of 100 and 10 respectively (Table 4) and hence in the third round of experiments (P3, R3) application rates were increased by a factor of 10. The concentration of *Trichoderma* in the green waste inoculated with Trianum and *T. viride* S17A was greater than expected based on previous work and was therefore at an adequate level for the experiments

Table 4. Concentration of microbial inoculum in treatment products and in inoculated green waste compost.

Product[1]	**Observed (CFU/g)**	**Expected (CFU/g)**
Prestop	9.1×10^5	1.0×10^8
Trianum	1.6×10^7	1.5×10^8
Mycostop	6.0×10^7	1.0×10^8
Subtilex	2.2×10^9	5.5×10^{10}
Revive P	1.3×10^9	-
GW Trianum	6.7×10^6	$> 1 \times 10^5$
GW S17A	8.3×10^7	$> 1 \times 10^5$

[1] GW = green waste compost

Effect of microbial treatments

The results from the first round of experiments (P1, R1) indicated that none of the microbial treatments had any effect on damping-off caused by *P. ultimum* or *R. solani* (Figures 1a and b). An average of 88-91% healthy seedlings survived for un-inoculated compost compared with 43% of seedlings for *Pythium*- and 6% seedlings for *Rhizoctonia*-inoculated compost. Microbial treatments resulted in 9-43% healthy seedlings for *Pythium* compared with 6-14% healthy seedlings for *Rhizoctonia.* The thiram treated seed was effective against *P. ultimum* (97% healthy seedlings) but less efficient against *R. solani* (23% healthy seedlings).

In the second round of experiments (P2, R2), again none of the microbial treatments had any effect on damping-off (Figures 1c and d). An average of 71-83% healthy seedlings survived for compost not infested with either pathogen compared with 0% seedlings for *Pythium*- and 3% of seedlings for *Rhizoctonia*-inoculated compost. Microbial treatments resulted in 0-3% healthy seedlings for *Pythium* compared with 6-14% healthy seedlings for *Rhizoctonia.* Again the thiram treatment was more effective against *P. ultimum* (97% healthy seedlings) compared with *R. solani* (3% healthy seedlings).

In the third round of experiments (P3, R3), using the different pathogen inoculum rates and also including the green waste treatments there was less damping-off in the *Pythium* experiment at either inoculum rate than previously observed, with the inoculated control having 63% healthy seedlings for both 3% and 5% pathogen inoculum levels compared with 69% healthy seedlings for compost without *P. ultimum.* Under this low disease pressure situation, the green waste treatments either with or without *Trichoderma* resulted in 77-89% healthy seedlings for both pathogen inoculum levels. Prestop, Mycostop, Subtilex and Revive P also resulted in high numbers of healthy seedlings (77-80%) at the 3% *Pythium* inoculum level. Further statistical analysis in Year 2 of the project will establish whether these results are significant. Despite the low disease pressure, the Trianum treatment (without green waste) resulted in only 11% healthy seedlings suggesting that it may have a phytotoxic effect. This was confirmed in a later experiment where the addition of this product in the absence of either *P. ultimum* or *R. solani* resulted in only 25% seedling germination.

In the *Rhizoctonia* experiment (R3), the 0.15% pathogen inoculum rate resulted in 26% healthy seedlings in the inoculated control compared to 89% seedlings for compost without *R. solani*. None of the treatments, with the exception of green waste only (43% healthy seedlings), *T. viride* S17A (43% healthy seedlings), and the thiram treated seed (94% healthy seedlings), resulted in increased seedling survival compared to the inoculated control. In contrast, the 0.08% pathogen rate resulted in 74% healthy seedlings compared to 94% in the compost without *R. solani* and hence disease pressure was much lower. In this situation, green waste only and green waste (86% healthy seedlings), *T. viride* S17A (94% healthy seedlings) and Revive P (94% healthy seedlings) resulted in increased seedling survival.

Overall, there was no clear evidence that applying any of the microbial or green waste treatments consistently had a benefit in combating damping-off on cauliflower seedlings. A key problem in assessing the treatments was the variation in disease pressure between experiments despite attempts to control and quantify pathogen inoculum. For *P. ultimum*, experiment P1 represented a high disease pressure situation but there was some difficulty in establishing a realistic lower disease pressure situation as in experiment P3, the damping-off levels were probably too low. However, in this latter experiment there was some indication that some of the green waste treatments (with or without *Trichoderma*) were beneficial. A further experiment is planned to confirm this. For *R. solani*, experiment R3 represented appropriate high and low disease pressures and again there was some indication that some of the green waste treatments were beneficial. The results from experiments also suggested that Trianum may have a negative effect on seedling emergence. This requires further confirmation.

The thiram-treated seed gave good control of *Pythium* damping-off at all levels of pathogen inoculum but was only effective against *Rhizoctonia* damping-off at low disease pressure. The use of fungicide-treated seed also had benefits in the absence of pathogen inoculum in some experiments suggesting that the Bulrush Organic Modular Compost had some background pathogen load affecting seedling emergence which

might also explain the relatively low emergence rates for untreated seed used in some of the treatments where pathogen inoculum was not added (68%).

Conclusions

- Damping-off disease pressure varied between experiments but overall there was no consistent or clear benefit from adding the microbial products tested for control of *P. ultimum* or *R. solani.* However, at low disease pressures there was some evidence that green waste with or without *Trichoderma* was beneficial.
- The thiram-treated seed consistently controlled *P. ultimum* but was less effective against *R. solani* at high disease pressures.

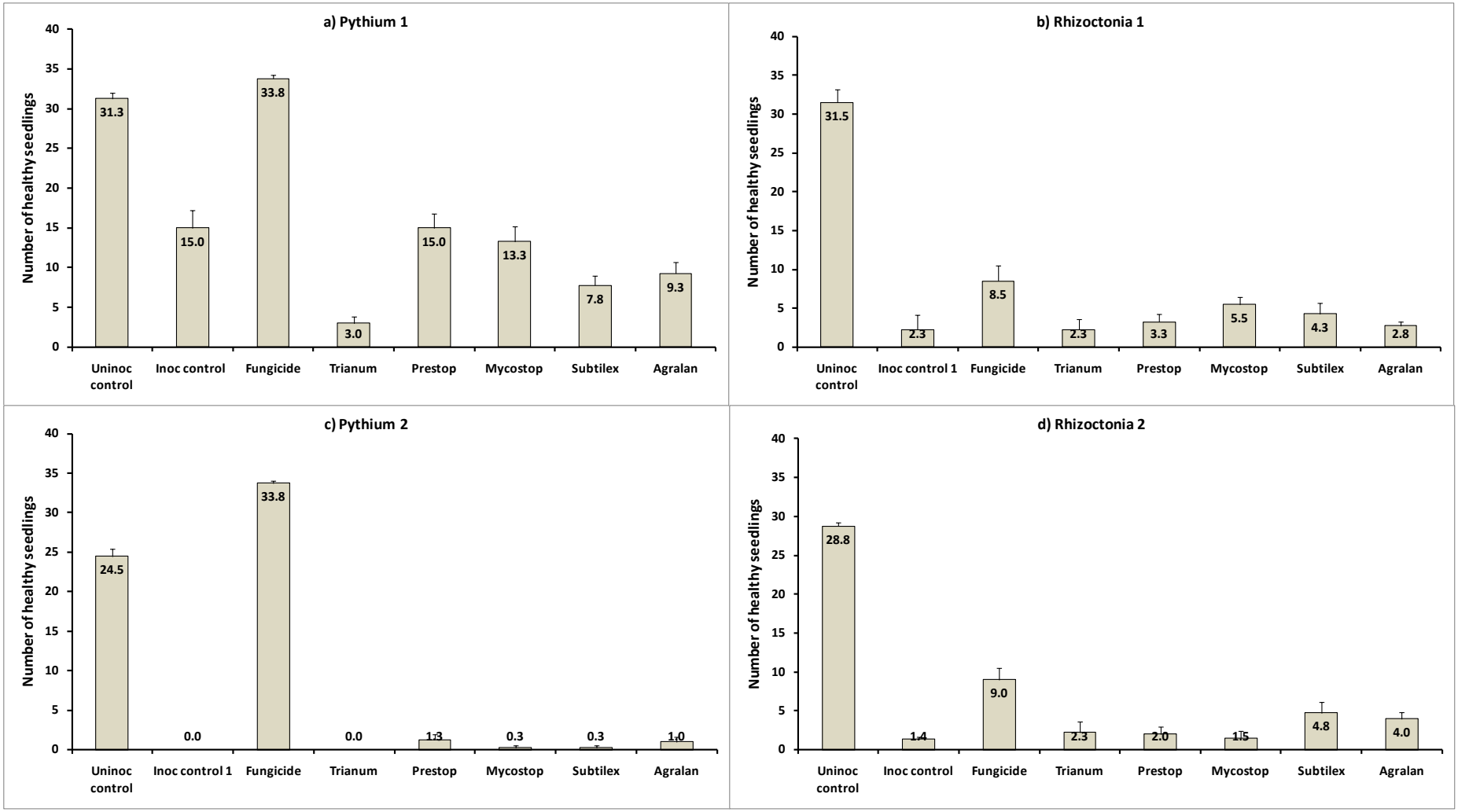

Figure 1. Effect of microbial treatments on damping-off of cauliflower seedlings due to *P. ultimum* (a, c) and *R. solani* (b, d) in first and second round of experiments. Numbers of healthy seedlings are means of four replicates of 35. Error bar = standard error of the mean. Uninoc control = no pathogen, Inoc control = pathogen only, Fungicide = thiram treated seed.

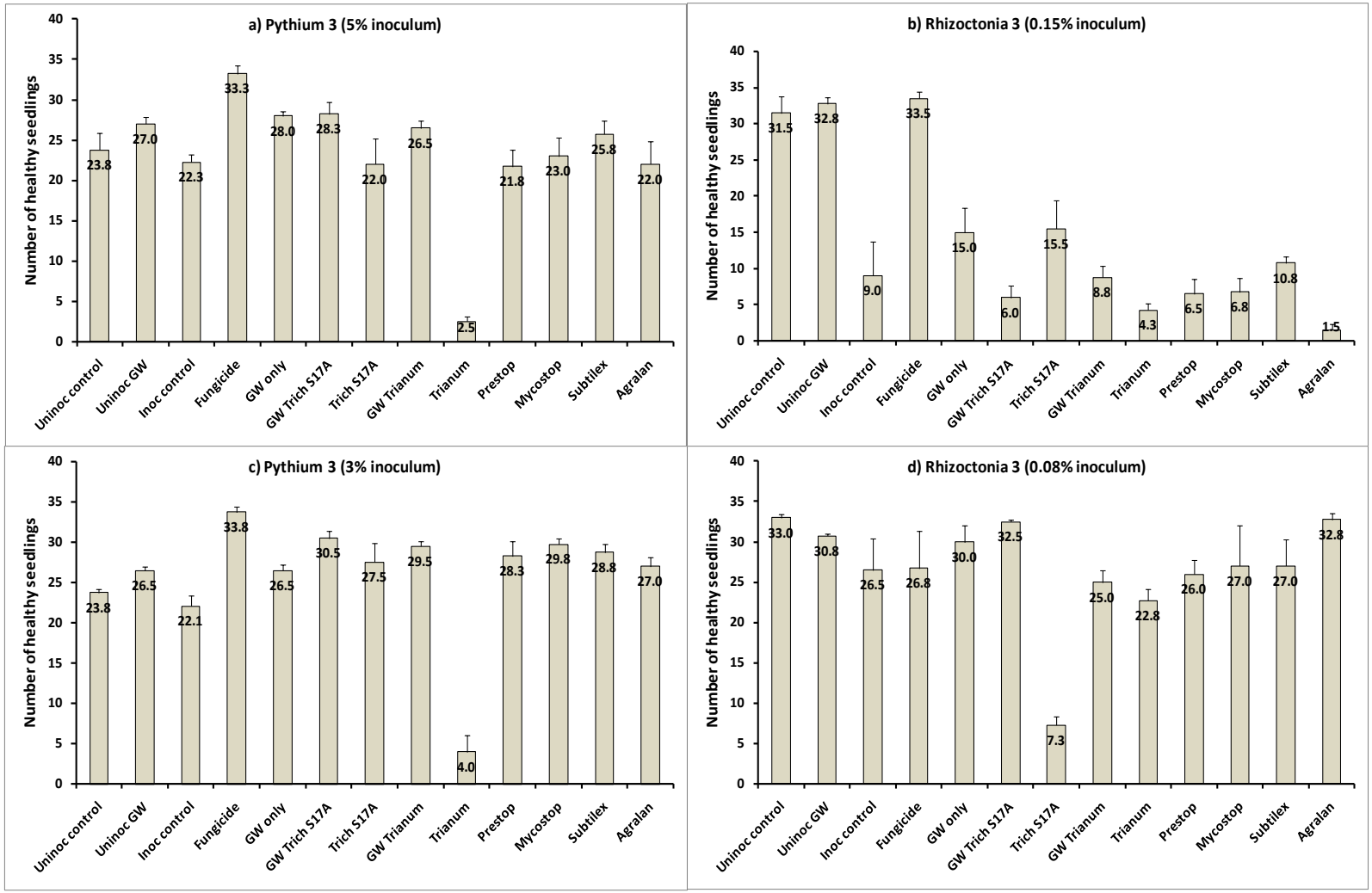

Figure 2. Effect of microbial and green waste treatments on damping-off of cauliflower seedlings due to *P. ultimum* (a, c) and *R. solani* (b, d) for different pathogen inoculum levels for third round of experiments. Numbers of healthy seedlings are means of four replicates of 35. Error bar = standard error of the mean. Uninoc control = no pathogen, Inoc control = pathogen only, Fungicide = thiram treated seed.

Seed Treatments

Materials and Methods

Seed

Samples of Brassica seed lots, suspected or known to be infested with either *Alternaria* sp. or *Phoma lingam,* were requested from a number of seed companies. These were tested for the presence and levels of the respective pathogens. A sample of a seed lot the companion plant species bird's-foot trefoil (BFT, *Lotus corniculatus*) was obtained from the grower co-ordinator.

Seed Health Testing

Seed health testing for was done by one of two standard methods: either the '2,4-D'' method or the 'freeze-blotter' (FB) method. The 2,4-D method is described in the *ISTA Rules for Seed Testing: Annexe to Chapter 7* for detection of *Phoma lingam* in Brassica seed (ISTA-PDC Method Validation Sub-committee 2002). There is no formally recommended or international standard method for the detection of *Alternaria* spp. in Brassica seed. However, the freeze-blotter method is described in the *ISTA Rules* for detection of *Alternaria* spp. in carrot seed (Sheppard *et al.* 2003) and is widely used for detection of fungi in a number of crop species (Mathur and Kongsdal 2003).

The 2,4-D method was performed with the following modifications: seeds were not rinsed in distilled water prior to placing in the Petri dishes; 25 seeds were used per Petri dish instead of 50 (experience suggests that using too many seeds in each Petri dishes leads to difficulties in interpretation of results and inaccuracy). The FB method was also performed with 25 seeds per Petri dish, with a freezing time of approx. 6-8 hrs. In both cases plates were incubated at 20°C with alternating 12 h periods of light and darkness.

For initial tests on Brassica seed half the seed was tested using the 2,4-D method and half using the FB method. Subsequently all Brassica seed was tested using the 2,4-D method. The BFT seed was tested using the FB method.

Seed Treatment

Seed treatments were applied to the seed at the concentrations and using the methods described in Table 5.

Table 5. Seed treatment details.

Code	Treatment	Details or Concentration	mL or g per g	Application method
U	Untreated	n/a	n/a	n/a
H	Hot water	50°C 30 min	n/a	Immersed water bath in stainless steel mesh basket, then re-dried in air-flow at RT.
F	Thiram	5 mL/kg	0.01	Added to wall of bag/bottle containing seeds and shaken to distribute.
T	Thyme oil	1% water emulsion	0.5	Added to seeds in flask or bottle, then shaken and left to stand for 30 min, before tipping out onto paper towel/filter paper and drying in air flow at RT
C	Clove oil	1% water emulsion	0.5	As thyme oil
P	HDC B0003[1]	1g per 100g	0.01	Added to wall of glass flask or bottle above seed then shaken to coat
A	HDC B0002[1]	0.9 mL/kg	0.0036	Diluted 1+3 and added as 4x vol, then shaken.
B	HDC B0001[1]	7.2 mL/kg	0.007	Added to wall of bag/bottle and shaken
S	Serenade ASO (*Bacillus subtilis*)	20 mg/g	0.02	Added to wall of bag/bottle and shaken
M	Mycostop (*Streptomyces griseoviridis*)	0.8 g per 100 g	0.008	Weighed directly into bag/bottle then shaken

[1] Experimental biological product

Emergence/Transmission Tests

Standard seed trays (approx. 35 x 22 x 5.5 cm) were loosely filled with a standard volume (2.5 x 5" pots) of Bulrush modular organic compost. The surface of the compost was levelled and lightly compressed to ca. 1.5 cm below the rim of the tray. Seeds were placed on the surface of the compost in 4 blocks of 60 seeds (240 total per tray) with a slight gap between each block, using a specially-made vacuum seeder. The vacuum head was disinfected with 70% iso-propanol between each seed lot/treatment. Seed was then covered with a standard volume of compost (1 x 5" pot) and the surface levelled. Trays were then placed in randomised blocks on a bench in the glasshouse. Glasshouse temperature was set to a minimum of 18/14°C (day/night) with venting at 20/16°C (day/night). Supplementary lighting was provided to give a minimum daylength of 12 h. Watering was via an overhead sprinkler system controlled by a timer. Trays were initially given 10 minutes watering, then subsequently 5 min daily at 08:00 (with occasional manual cancelling). Emergence and the presence of disease symptoms was recorded approx. 14 d after sowing, with an additional records of disease made approx. 21-28 d after sowing.

Isolations

Seedlings or tissue pieces were surface sterilised by briefly dipping in 70% iso-propanol and then rinsed in sterile de-ionised water. Small (2-4 mm) sections of tissue were then aseptically excised and placed on the surface of potato dextrose agar (PDA) plates and incubated at 20°C.

Statistical analysis

Seed health test data were analysed by fitting a series of generalised linear models with a binomial error distribution and logistic link function to the data for each plate (i.e. no infected in each plate out of the total of 25 seeds). The means presented in graphs and tables were obtained as predictions after fitting the appropriate model. Dispersion was estimated from the data and if greater than the theoretical minimum value of one was used in the calculation of confidence intervals. Emergence and seedling disease data were analysed in a similar way, except that the models were fitted to the data for each seed tray or block of 60 seedlings within each tray.

Results

Based on the initial seed tests, two Brassica seedlots (one infested with *Phoma* and one infested with *Alternaria brassicicola*) were selected for treatment, together with the supplied BFT seedlot. In initial tests on Brassica seed, the 2,4-D method resulted in greater detection of both *Phoma lingam* and *A. brassicicola* than the FB method; therefore the 2,4-D method was used in all subsequent tests on Brassica seed.

Effect of treatments on seed health

Lot S1098 – Phoma

Ten percent of untreated seed of Brassica seed lot S1099 was infested with *Phoma* in the 2,4-D seed test. All treatments appeared to reduce the level of infestation compared to the untreated control (Figure 3), but only Hot Water, Thyme oil, and HDC B0002 gave statistically significant reductions. These three treatments gave a greater reduction than the chemical standard Thiram and Hot Water in particular reduced infestation to undetectable levels (i.e. <1.5%).

Lot S1099 – Alternaria

Seventy-eight percent of untreated seed of Brassica seed lot S1098 was infested with *A. brassicicola.* All treatments gave a statistically significant reduction in the level of infestation compared to the untreated control (Figure 4). The greatest reductions were achieved with Hot Water, Thyme oil, clove oil, the BCAs Serenade and HDC B0002, and the fungicide Thiram. Again Hot Water reduced infestation to undetectable levels (i.e. < 1.5%).

BFT – All fungi

Seed tests on the untreated BFT did not indicate the presence of any specific pathogenic fungi, therefore results are presented as the percentages of seed infested with any fungi. Twenty-six percent of untreated BFT seed carried some fungal contamination. All

treatments gave a statistically significant reduction (to 3% or less) in the level of contamination compared to the untreated control, with little difference between them (Figure 5).

Effect of treatments on emergence and seedling disease

Symptoms on seedlings were recorded in three different categories:

1. Brown/necrotic lesions on one or both cotyledons, some of which subsequently developed into clear '*Phoma*' symptoms in the *Phoma* infected seedlot.

2. Some of the lesions initially apparent as necrotic lesions usually on one but sometimes on both cotyledons, developed down one side of the hypocotyl (stem) as a brown streak to compost level, often resulting in collapse of the seedling. Isolations from representative symptoms confirmed that these were invariably caused by *Phoma.*

3. 'Damping off' symptoms were apparent as a 'water-soaked' lesions developing initially at compost level. Isolations from representative symptoms confirmed the presence of *Pythium* in some of these lesions.

Emergence

None of the treatments increased emergence compared to the untreated controls. Hot water significantly reduced emergence in the Phoma infected Brassica seedlot S1098, and in BFT but not in the *Alternaria* seedlot S1099, clove oil treatment also significantly reduced emergence in the *Phoma* infected seed lot. None of the other treatments had a detrimental effect on emergence. (Figure 6)

Damping-off

Treatment with hot water led to a small but significant increase in damping off in all three seed lots. None of the treatments gave a significant reduction in damping-off compared to the untreated control, but no damping off was apparent in the thiram treated seed (Figure 8)

Lot 1098 - Phoma

The proportion of seedlings affected by Phoma was significantly reduced by treatment with hot water, thiram, thyme oil and Serenade, with hot water having the greatest effect.

Lot S1099 – Alternaria

Excluding damping-off symptoms, none of the treatments had any effect on the proportion of seedlings with necrotic lesions, and there were no obvious symptoms which could be specifically attributed to *Alternaria*

BFT

Apart from damping-off, there were no other disease symptoms on the bird's foot trefoil seedlings.

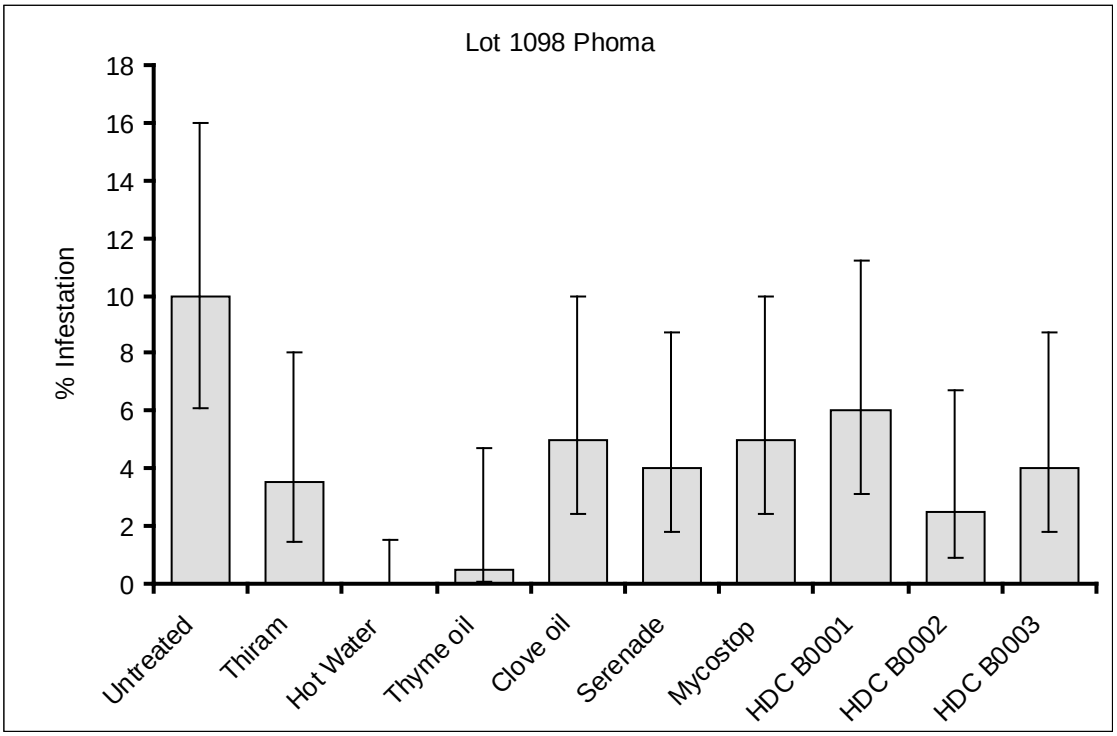

Figure 3. Effect of seed treatments on levels of brassica seed infestation by *Phoma lingam* in a 2,4-D blotter seed test. Error bars represent the 95% confidence limits.

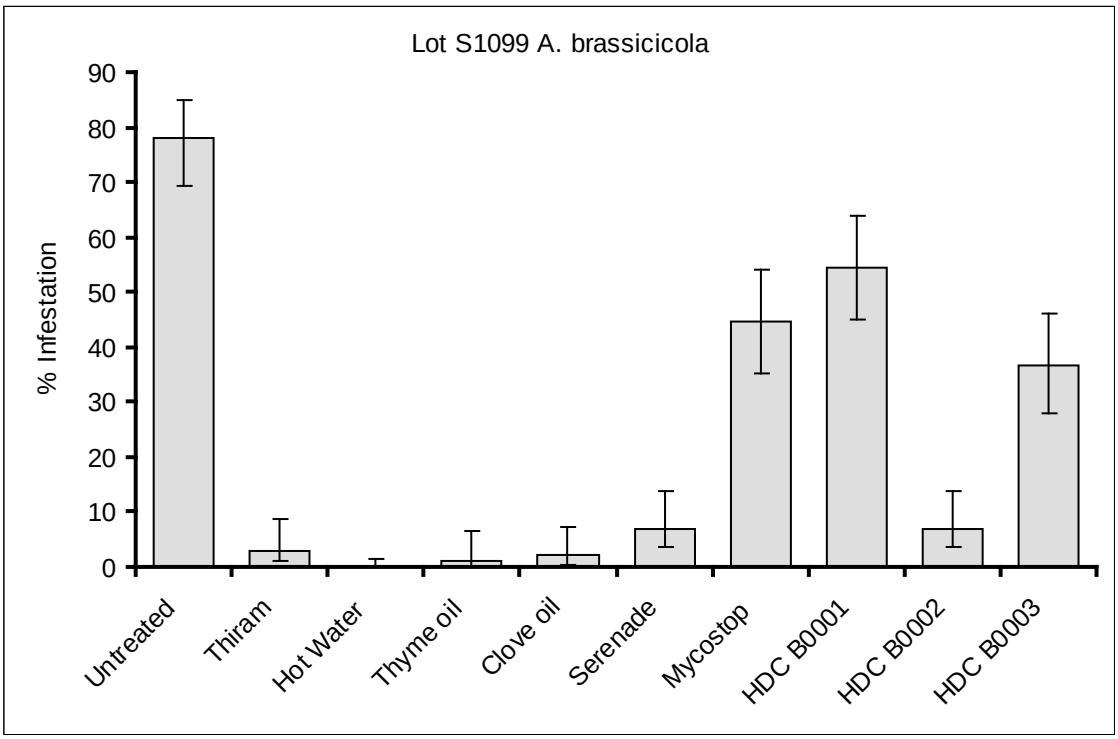

Figure 4. Effect of seed treatments on levels of *Alternaria brassicicola* infestation, as determined by a 2,4-D blotter test. Error bars represent 95% confidence limits.

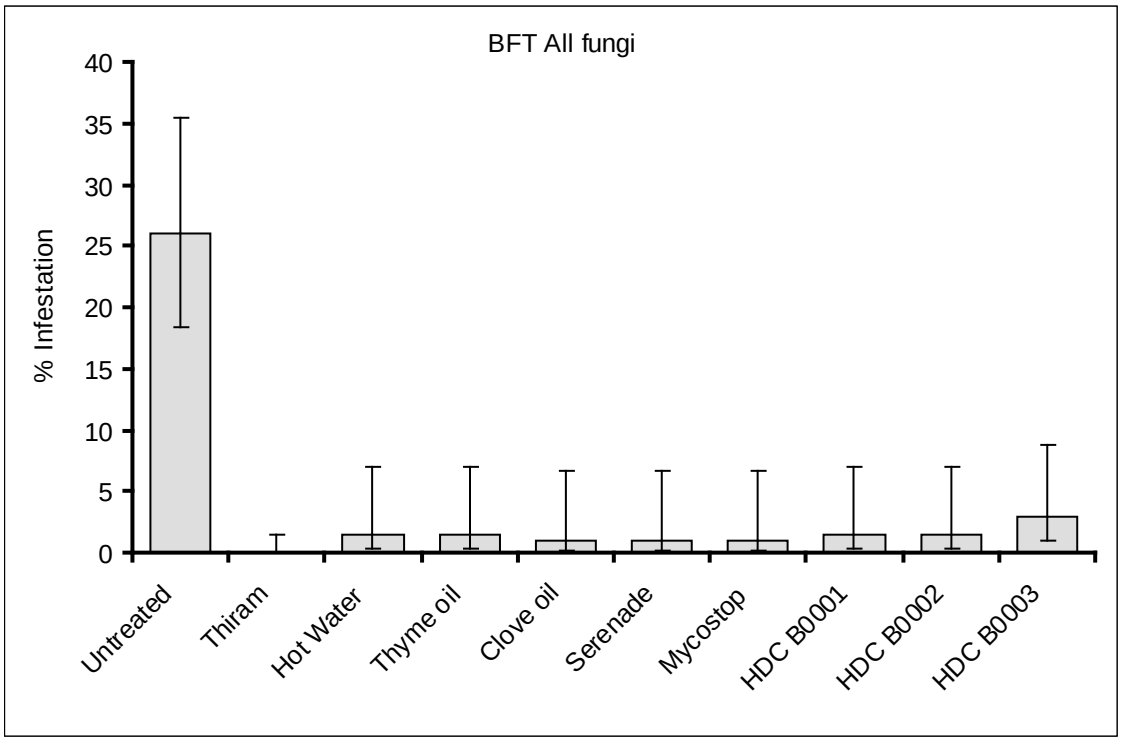

Figure 5. Effect of seed treatments on levels of fungal contamination in a bird's foot trefoil seed lot. Error bars represent 95% confidence limits.

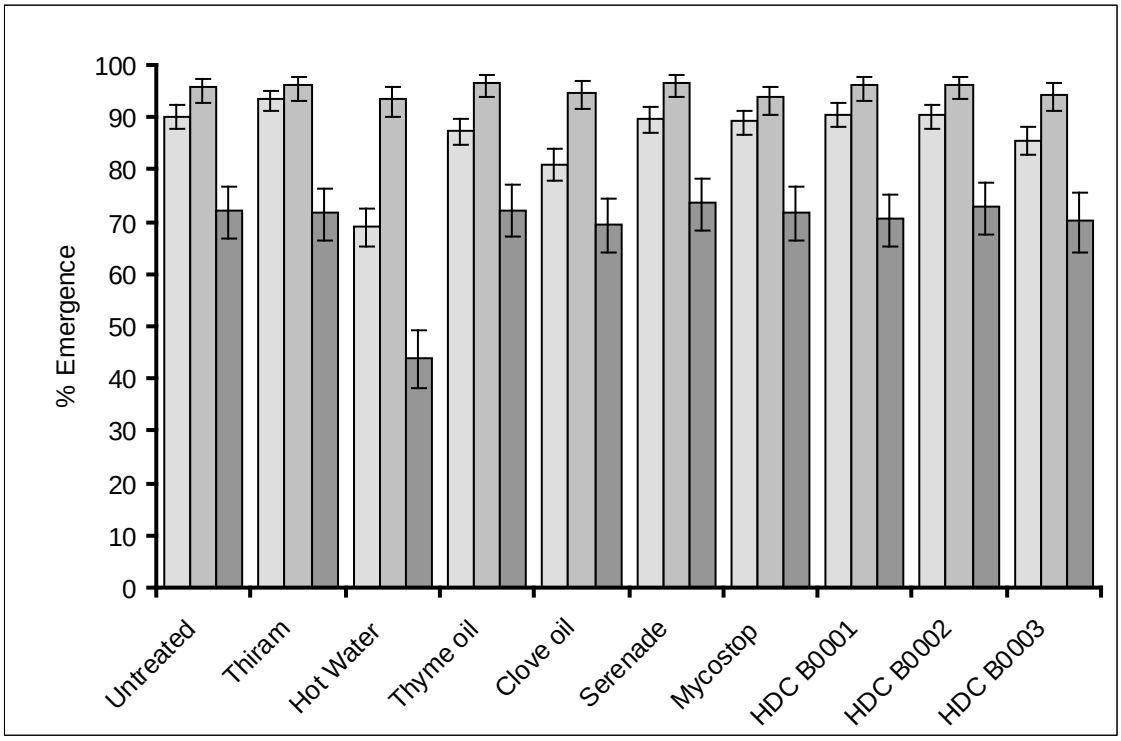

Figure 6. Effect of seed treatments on emergence in Brassica seed lot 1098 (left bar), lot 1099 (middle bar) and bird's foot trefoil (right bar). Error bars represent 95% confidence limits.

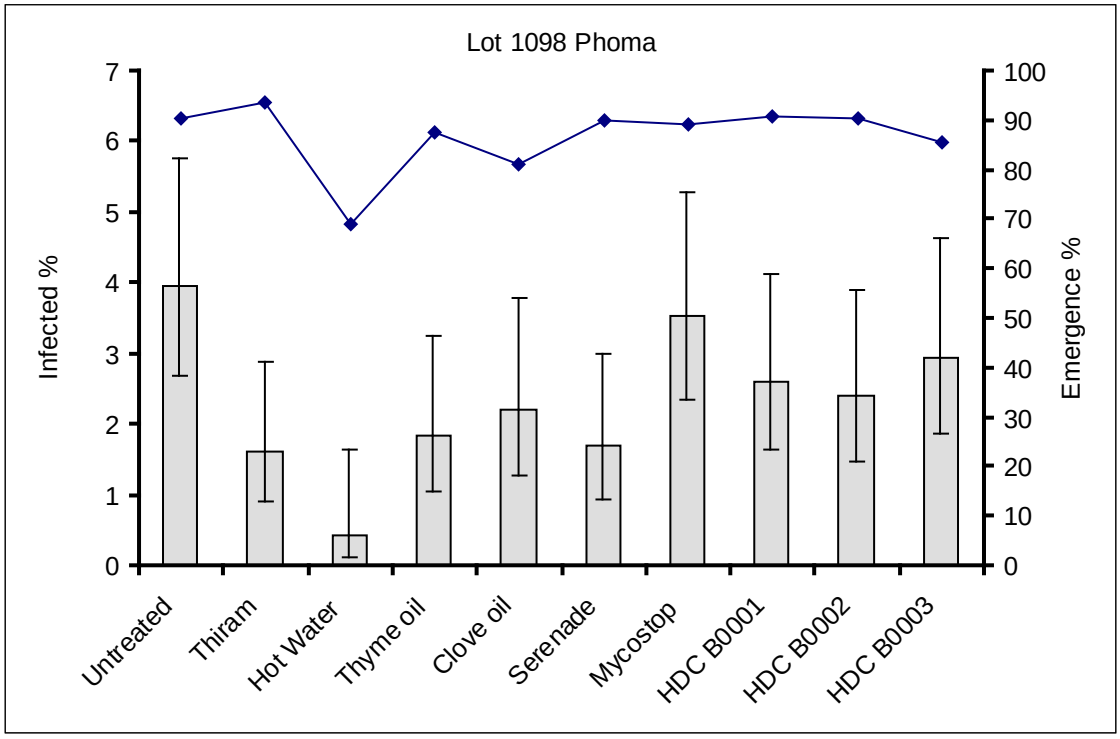

Figure 3. Effect of seed treatments on seedling infection by *Phoma lingam* (bars) and emergence (line). Error bars represent 95% confidence limits.

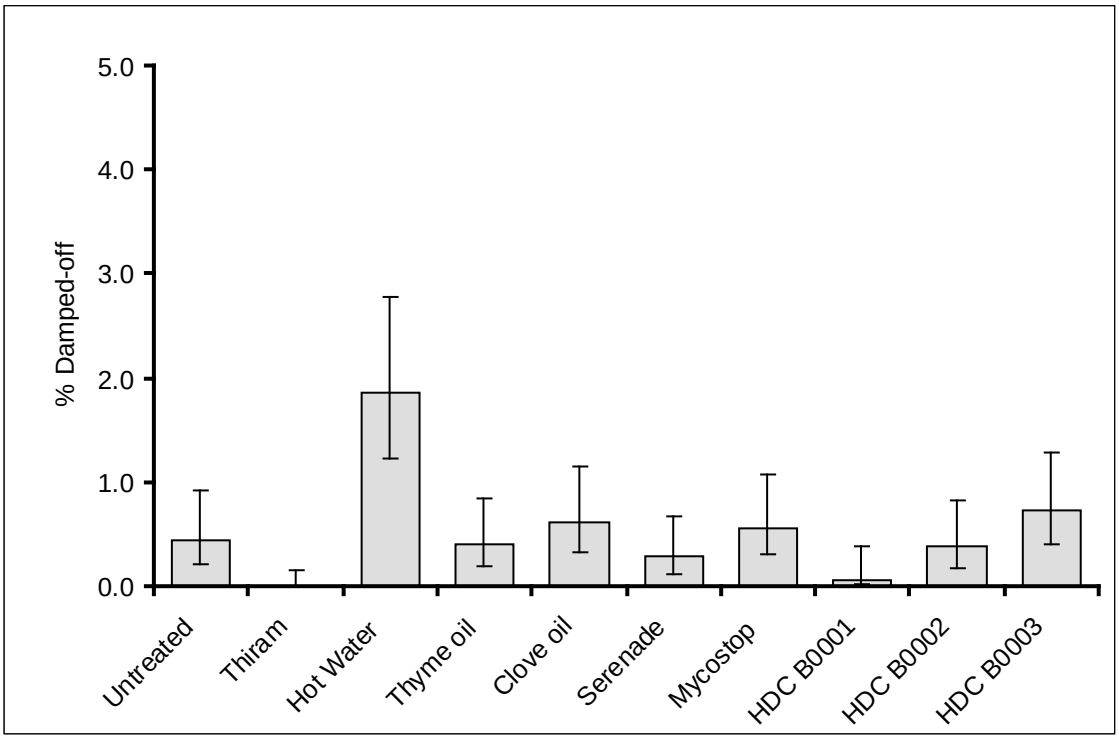

Figure 4. Effect of seed treatments on levels of damping-off (combined results of two brassica seedlots and one bird's foot trefoil seedlot). Error bars represent 95% confidence limits.

Discussion

Both the seed health test results and the emergence/transmission experiments showed a significant effect of hot water and thyme oil on *Phoma* infection. Unfortunately hot water treatment also caused a significant reduction in emergence in the *Phoma*-infected Brassica seed lot, but not in the *Alternaria*-infected brassica seed lot. Thus it is possible that *Phoma*-infected seeds are more sensitive to hot water, and at least some of the seeds which failed to emerge may have been infected.

There were also indications of reductions in *Phoma* by two of the microbial treatments. HDC B002 gave a significant reduction in the seed health test, and also gave a slight reduction in the seedling transmission experiments (although not statistically significant). Serenade gave a significant reduction in the seedling transmission experiment and also gave a reduction in the seed health test (but not statistically significant).

Despite the apparently high level of infestation with *Alternaria* in Brassica seed lot S1099, this did not translate into any detrimental effects in emergence or apparent transmission to seedlings. Thus none of the treatments gave any improvement in emergence of seedlings and the efficacy of the treatments can only be judged on the basis of the seed health testing. All of the treatments that were effective against *Phoma* (i.e. hot water, thyme oil, Serenade and HDC B0002) were also effective in reducing the *Alternaria* loading on the seed, plus clove oil and the fungicide thiram were also effective.

The BFT seedlot had relatively poor emergence. No specific fungal pathogens were detected/identified in the BFT seedlot and no seed-borne diseases were apparent in the seedlings. All of the treatments gave a marked reduction in fungal loading on the seeds. On the other hand none of the treatments gave an improvement in emergence, although this is perhaps not surprising given that no specific pathogens were detected in the untreated seed. Hot water was extremely detrimental to BFT emergence. BFT has relatively small seed compared to Brassicas, and is clearly much more sensitive to hot water. Thus if hot water treatment were to be considered for BFT, further experiments would be needed to define appropriate temperature-time regimes.

It was apparent that there was a low level of *Pythium* in the compost and this resulted in classic damping-off symptoms in a proportion of the seedlings. Most notably the levels of damping-off were slightly (but significantly) increased in the hot-water treated seeds. It is possible that the hot water treatment caused some sub-lethal damage to some seeds, increasing their susceptibility to or providing entry points for the pathogen. Alternatively it is possible that the non-specific impact of hot water on the natural seed micro-flora, may reduce the natural bio-control exerted by some of the organisms normally present. Optimisation of the hot-water treatment on a per seed-lot basis might overcome some of this issues and of course the problem would not be apparent if the compost was free from damping-off pathogens.

Overall there would appear to be four leading treatments for control of seed-borne disease in Brassicas: hot water, thyme oil, Serenade and HDC B0002. The first three of these treatments have also been tested and shown promise in previous work on both

seedborne fungi and bacteria (Green and Roberts 2009; Koch *et al.* 2010; Roberts *et al.* 2006; Roberts 2009; Schmitt *et al.* 2008). Currently, hot water is the only treatment that can be legally used, as it does not require approval. Unfortunately its routine application is not without problems and the temperature-time regimes need to optimised on a per seed-lot basis to ensure maximum efficacy and minimum seed damage. Thyme oil is a natural plant product with broad spectrum disinfectant activity but would require formal approval as a pesticide before it could be used as a commercial seed treatment. Serenade ASO is a microbial product based on a strain of *Bacillus subtilis*. It is currently approved for foliar application to all crops (via a SOLA), but does not have approval as a seed treatment. The manufacturer should be encouraged to seek approval for seed treatment. Experimental product HDC B0002 is also a microbial product and the manufacturer should be encouraged to seek approval as a seed treatment.

Conclusions

- Hot water (50°C, 30 min) gave the greatest control of *Phoma* and reduced *Alternaria* Brassica seed infestation to undetectable levels, but is not without problems and precise temperature-time conditions should be determined on a per seed lot basis.
- Thyme oil (1%) reduced both *Phoma* and *Alternaria* in Brassica seed, without any detrimental effects on emergence, but its use is not currently approved.
- Two microbial treatments (Serenade ASO and an experimental product) gave promising results against both *Phoma* and *Alternaria.* Their use as seed treatments is not currently approved.
- Emergence in the bird's foot trefoil was relatively poor, but this could not be attributed to any specific fungal pathogens and none of the seed treatments gave any improvement in emergence compared to the untreated control.

Approval status of products

Table 6. Pesticide approval status of the various treatment products used in this study

Treatment/Product	Status
Compost treatments	
Trianum	Not approved in the UK, listed on Annexe 1 of 91/414.
Prestop	Not approved in the UK, listed on Annexe 1 of 91/414.
Mycostop	Not approved in the UK. Approved in several EU countries.
Subtilex	Not approved.
Revive P	Not approved, but marketed as a 'Microbial Soil Treatment'
Green Waste	Approval not required.
Seed treatments	
Hot water	Approval not required
Thiram	Approved as a seed treatment for Brassicas.
Thyme oil	Not approved, Annexe 1 listing in progress?
Clove oil	Not approved, listed on Annexe 1 of 91/414
Serenade ASO	Not approved for application to seeds. Approved for foliar application to all crops (SOLA).
Mycostop	Not approved in the UK. Approved in several EU countries.
HDC B0001	Experimental product. Not approved.
HDC B0002	Experimental product. Not approved.
HDC B0003	Experimental product. Not approved.

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