CP 205 AHDB Horticulture Efficacy Trials 2022
Final Trial Report

Work package:	WP 10
Title:	Seed treatment for spinach damping-off (*Pythium* spp.)
Crop	Spinach
Target	Pythium damping-off
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ORETO Number: (certificate should be attached)	409

We the undersigned, hereby declare that the work was performed according to the procedures herein described and that this report is an accurate and faithful record of the results obtained

Date 28.02.2023 Author's signature E. F. Wedgwood

Date 28.06.2023 Authorised by

Trial Summary

Introduction

Pythium ultimum and *Pythium* species belonging to a group producing hyphal swellings were shown to mainly be the cause of damping-off in spinach samples sent in by UK growers for testing as part of AHDB project FV367 (Green & Gladders, 2012). Variety testing by Green and Gladders suggested susceptibility differences, however, it was concluded that, where germination was rapid, damping-off was less severe, therefore seed stock condition as well as varietal susceptibility should be considered.

Fungicide actives able to control *Pythium* spp. and other oomycetes such as downy mildews are limited in number and there have been reductions in the products available in the UK for foliar spraying. For example, Aliette (fosetyl-aluminium), Fenomenal (fenamidone + fosetyl-aluminium) and Paraat (dimethomorph) have all been withdrawn, and mancozeb, in the products Invader (dimethomorph + mancozeb) and Fubol Gold (mancozeb), is not secure in remaining available for use. Metalaxyl-M has lost its effectiveness in some situations. Cultural management measures, such as rotation, should be employed ahead of any selection of plant protection products, but seed treatments, whether conventional chemistry or biological control, offer a more targeted approach to damping-off management than sprays.

Increasing issues of damping-off diseases have been identified by the leafy salads industry since the loss of thiram as a seed treatment, with a high incidence of damping off in spinach fields in 2020. Pathogens included in the damping-off complex for spinach include *Pythium*, *Fusarium* and *Rhizoctonia* species. AHDB funded grower-led demonstration trials conducted in 2021 (FV 464b) tested seven different seed treatment options on spinach sown into fields with a history of damping-off (Kaye, *et al*., 2021). Chemical and biological fungicides were tested alongside biostimulant products and physical priming. The cause of damping-off (fungal or oomycete) was not determined, but the chemical fungicide coded AHDB 9848 performed well.

Efficacy testing was also carried out within AHDB CP165 using the same seed treatments as in the demonstration trials but sowing in growing medium standing on substrate inoculated with *Pythium ultimum*. AHDB 9848 was again highlighted, with the best emergence, and seedling survival, but losses still occurred. Further efficacy experiments were carried out in the current project using *Pythium ultimum* inoculum to include additional products and preceded by a bioassay to determine an inoculum concentration that might be a less-severe test of protectant ability than arose in CP165.

Methods

Seed treatment and germination testing

Spinach seed was treated at the dose rates provided with the trial products, with two chemical and four microbial experimental products and Integral Pro.

Untreated and treated seed were germination tested in filter-paper pleats directly after treatment (0 days) and again after 10 weeks.

Assessments of the numbers of seedlings emerged and healthy or emerged and then damped-off in the seed trays were made weekly for up to 28 days, with a final count of the number surviving to true-leaf. Vigour of apparently healthy seedlings, principally related to leaf size, and any phytotoxicity were recorded per seed tray was assessed.

Inoculum preparation

Pythium ultimum (isolate C761 sourced from Tim Pettitt) mycelium was grown for two weeks at 20°C in lidded “starter punnets” on a substrate of sterilised moist coir and oatmeal. This inoculated substrate was mixed with further coir and oatmeal plus some sand to produce the desired experimental concentrations of Pythium for efficacy trials.

Inoculum concentration optimisation

Bioassays to evaluate the level of damping-off at particular Pythium dilutions were carried out with untreated seed. These were used to determine the optimal inoculum level to use in the efficacy trials.

Efficacy experiments

Efficacy experiments with Pythium inoculum were conducted with seven seed treatments tested alongside an untreated spinach seed control in seed trays held in controlled environment cabinets. Integral Pro (*Bacillus amyloliquefaciens*) was the standard seed treatment product with experimental chemical products AHDB 9848 and AHDB 9715 and experimental microbial products AHDB 9734, AHDB 9725, AHDB 9955 and AHDB 9716.

Experiment 1 tested a high and low Pythium concentration in the substrate. Experiment 2 used a single, low, concentration, but the seed trays were placed on the inoculated substrate immediately after sowing, or with an 88-hour delay to give seedlings a chance to grow before the pathogen made contact.

12-cell seed trays filled with peat-based growing media were sown with fungicide treated or untreated spinach seeds cv. Montego. Trays were placed on the inoculated substrate in controlled environment cabinets at 20°C for the Pythium mycelium to grow from the infested coir punnets up into the seed trays.

Both experiments included a replicated uninoculated treatment of untreated seed only. Unreplicated observation trays kept without Pythium contact by treated and untreated seeds (unreplicated) were set up alongside Experiment 2.

Weekly assessments of the numbers of emerged healthy seedlings and emerged and then damped-off were conducted up to 28 days post sowing. A final count of the number surviving to true-leaf was made. Vigour of apparently healthy seedlings, principally related to leaf size, and any phytotoxicity were recorded. Weekly post-emergence counts of seedlings that were healthy or collapsed from damping-off recorded those visible at that time rather than being cumulative.

Results

1) Seed treatment and germination testing

- 0-day tests on untreated seeds gave 93% germination, not dissimilar to that of treated seeds which ranged from 89% to 96% germination of normal seedlings, with a range from 4% to 9% abnormal seedlings by 21 days, and the remaining few seeds being dead.
- 10-weeks post-treatment there had been no adverse effect on germination, ranging from a mean 90% to 94% of normal seedlings growing in the treated compared with 95% in the untreated, with similar numbers of abnormal seedlings and dead seeds as in the seeds tested directly after treatment.
- Germination was good at both assessments, with no indications of germination delay nor any increase in abnormal or dead seeds as a result of the seed treatments.

2) Treatment Efficacy experiments

No phytotoxicity, such as yellowing or stunting of foliage, was observed in the efficacy experiments, neither were any vigour differences in healthy plants noted between the seed treatments and those grown from untreated seed.

Experiment 1 – high and low Pythium concentrations

After 21 days the uninoculated untreated had a mean of 75% emergence which was significantly ($P<0.001$) better than the untreated or treated inoculated seeds at both concentrations.

- A maximum of 5% of seedlings emerged in trays stood on inoculated coir. There was no significant difference between the seed treatments or between the treatments and the untreated inoculated control, at both high and low Pythium concentrations.
- Pre-emergence damping-off was likely to have contributed to the similar low proportions of emerged seedlings in both the inoculated untreated and treated seeds at both Pythium concentrations, as emergence was better in the uninoculated untreated. There was little post-emergence damping-off, with seedlings growing to true-leaf while remaining in contact with infested substrate.

Experiment 2 - Immediate Pythium contact or 88 hours delayed contact

- Emergence of the uninoculated untreated seeds was poorer than in Experiment 1 trials, with a mean 33% of seeds sown emerging across the two trials of Experiment 2.
- In Experiment 2 the significant difference seen in Experiment 1 between the greater emergence of uninoculated untreated and the lower of inoculated untreated seeds was lost. A greater proportion of inoculated untreated seedlings emerged in both of the Pythium contact times of Experiment 2 than in the trials of Experiment 1.
- *Immediate contact with Pythium* - after 7 days there was no emergence difference between product treatments. Significantly fewer ($P=0.024$) seedlings had emerged in both the inoculated untreated and treated seed trays (mean 5.6%) than in the untreated uninoculated (20.2%). After 28 days emergence ranged from 7.1% to 20.6% (mean 13.1%) in the treated seeds with no significant difference between treatments.

Untreated inoculated seed emergence was 21.0% and also not significantly different to treated seeds. Emergence in the untreated uninoculated had risen to 33.3% by Day 28, but this was not statistically greater (P=0.110) than any of the inoculated treatments. Of the seedling that emerged, at Day 28 the untreated inoculated had 5.6% visible with damping-off, not statistically different (P=0.052) from either the 0.79% in the untreated uninoculated or the mean 2.6% overall for the inoculated treated treatments.

- *Delayed contact (88h) with Pythium* - there was no significant emergence difference between the seed treatments. Significantly fewer seeds had emerged (P=0.009) in all inoculated treated seed treatments (mean 18.2% by Day 28) than the 42.1% in the untreated inoculated. The emergence of 32.5% seedlings in the untreated uninoculated was, however, similar to the 42.1% in the untreated inoculated rather than being better. Of these seedlings that emerged, at Day 28 the untreated inoculated had 2.4% visible with damping-off, not statistically different from either the 0% in the untreated uninoculated or the mean 1.1% overall for the inoculated treated treatments.

Take home message:

None of the seed treatments, including the standard Integral Pro, was able to significantly reduce the loss of spinach seedlings following contact with Pythium inoculated substrate at the concentrations and moisture levels used.

For the development of a standard inoculation procedure for efficacy testing, the creation of Pythium infestation underneath rather than directly in seed trays did not sufficiently delay the movement of mycelium into the seed or seedling. A delay in contact of 88 hours was insufficient to allow seedlings to “get away” from the pathogen. Where, as in this case, the inoculum used has to be mycelium rather than spores there can be difficulty in creating known inoculum strengths (colony forming units) as mycelial growth is variable and hyphal fragments mixed into the substrate vary in size.

Once seedlings that escaped infection developed true leaves they survived even with Pythium still in contact with their growing-medium.

In field conditions, encouraging a fast germination is likely to be able to reduce the impact of Pythium infested soil. Pythium colonisation of the soil will be lower if the soil can be kept less wet.

SCIENCE SECTION

Objectives

To carry out a laboratory bioassay to determine the levels of Pythium infestation of a culture substrate required to give a moderate and a lighter infection of spinach seedlings growing from sowings in trays placed on the inoculum.

To carry out laboratory efficacy experiments with spinach seed treated with plant protection products and exposed to two levels of Pythium inoculum challenge as guided by the bioassay.

Methods

Trial conduct

The following EPPO guidelines were followed:

Relevant EPPO guideline(s)		Variation from EPPO
EPPO PP1/135(4)	Phytotoxicity assessment	none
EPPO PP1/152(4)	Guideline on design and analysis of efficacy evaluation trials	none
EPPO PP1/225 (2)	Minimum effective dose	none
EPPO PP1/181 (4)	Conduct and reporting of efficacy evaluation trials including good experimental practice	none
EPPO PP 1/214(3)	Principles of acceptable efficacy	none
EPPO PP 1/224(2)	Principles of efficacy evaluation for minor uses	none
EPPO PP 1/125(4)	Seed treatments against seedling diseases (trials under controlled conditions)	Inoculum was not added directly to the seed trays but placed under them. The inoculum was grown on a different formulation of substrate. Three, not four, replicates were used & placed in a controlled environment cabinet.

Common name	Scientific Name	EPPO Code	Infestation levels of Pythium		
			Before application of seed treatment	In growing media at sowing time	In growing media at assessment period end
Damping-off	*Pythium ultimum*	PYTHUL	Products applied to uninoculated spinach seed	Infestation level within peat in the seed trays initially zero. Level following growth up from infested substrate not determined. Inoculum levels in substrate under seed trays differed between experiments.	Not determined

Experiments were carried out to investigate damping-off of spinach seed and seedlings by Pythium. Preliminary and main bioassays (using only untreated spinach seed) were conducted to evaluate the effect of different inoculum concentrations in order to determine the concentrations to use in subsequent efficacy testing. Two runs of efficacy experiments were then conducted in which a higher and lower exposure to Pythium mycelial fragments was given to both treated and untreated seed.

Project timeline

Task description	Date completed
Preliminary bioassay: Untreated seed tested for damping-off with Pythium to determine colony concentrations in substrate and disease levels. Seeds sown 10 August 2022 & damping-off recorded over 16 d.	26.08.2022
Main bioassay: Untreated seed used with a range of Pythium concentrations to determine extents of damping-off. Seeds sown 31 August 2022 & damping-off & emergence recorded over 12 days.	16.09.2022
Product list finalised by AHDB & last product received.	14.09.2022
Seed treated with seven products by Elsoms Ltd., near Spalding.	14.09.2022
0-day post-treatment germination tests of untreated / treated seed on filter papers at Elsoms started on 14.09.2022 and grown for 21 days with weekly assessments of the number germinated out of 100 seeds.	05.10.2022
1st Efficacy experiments: higher and a lower Pythium cfu in substrate trays sown on 20 September 2022. Assessment of damping-off, vigour & phytotoxicity on trays grown for 21 days.	11.10.2022
10-week post-treatment germination tests at Elsoms started on 23 November on filter papers and grown for 21 days with weekly assessments.	14.12.2022
2nd Efficacy experiments: Seeds sown at 16:30 on 10 November 2022 and trays from one experiment put in contact with a low Pythium level. For second experiment contact with low Pythium level delayed for 88h until 08:30 on 14 November 2022. Assessment of damping-off, vigour & phytotoxicity on trays grown for 28 days.	08.12.2022

Test site for seed tray bioassays and efficacy experiments

Item	Details
Location address	ADAS Boxworth, Battlegate Road, Boxworth, CB23 4NN
Crop	Spinach
Cultivar	Montego (susceptible to Pythium)
Isolate	Pythium isolate C761 ex. Spinach Midlands field damping-off
Substrate type	Levington Advance F1 peat-based seed and modular growing media (low nutrient). Stated pH5.3-6.0, particle size 0-3mm, conductivity 200-294 µ, H2Gro wetting agent. 96 N: 49 P : 159 K
Agronomic practice	Overhead watered module trays (by small can with a fine rose)
Growing environment	Two controlled environment cabinets set at 20°C 60% RH 16h light : 8h dark using overhead white LED strips set at medium intensity. Air ventilation through the perforated back-panel.

The principal methods used across the bioassay and efficacy experiments were the same and so these are described in this section, with specific treatments and any

variations then given under the individual experiment headings. The bioassays and both efficacy experiments used grower-sourced spinach seed of cv. Montego. The seed bulk for the efficacy experiments was divided for treatment by Elsoms Seeds with the untreated seed for the efficacy experiments, whether or not subsequently exposed to Pythium, used from the same sub-sample seed packet.

Experimental set up

All experiments were carried out in new 3 x 4 cell seed trays (37 x 34 mm wide) with a large central hole in the bottom (volume approx. 45 ml). Five seeds were sown per cell in the preliminary bioassay (total 60 seeds) but increased to nine seeds in the main bioassays (total 108 seeds). This was reduced to seven seeds per cell (84 per tray) in the efficacy experiments to aid seeing damped-off seedlings amongst the healthy spinach. Seeds were covered with a uniform depth of 10 mm of sieved peat modular growing media. Watering overhead by watering-can was done to achieve "field capacity" so that the growing media remained moist. As an aid to the judgement of field capacity, during the efficacy experiments samples of trays together with their punnets were check weighed regularly.

Two controlled environment cabinets in the ADAS Boxworth laboratory were set at 20°C 60% relative humidity and used both for inoculated substrate production ("starter punnets") and for growing the spinach seedlings. The controlled conditions allowed some comparison across a series of trials with each trial utilising results from the preceding. For production of the Pythium "starter" coir/oatmeal substrate the punnets were kept in 24 h darkness. Once seed trays were put on top of the open punnets of coir/oat/sand substrate the setting was changed to 16 h light / 8 h dark (with dark during night-time). Temperature and humidity loggers recording every 30 minutes were placed on a shelf in each of the cabinets.

Inoculum preparation

Where Pythium inoculation was required, it was not carried out directly into the seed trays, instead a more-realistic inoculum challenge was given to the spinach by placing Pythium inoculum in substrate under the seed trays. This followed the procedure used by Tim Pettitt in the bioassays for the 2021 AHDB FV 464b spinach damping-off project. In the current project, each seed tray was fitted into a sterilised transparent colourless polyethylene terephthalate (PET) 1 Litre salad punnet measuring 164 mm x 113mm that held a coir / oatmeal / sand substrate with or without inoculum. The punnets initially had hinged transparent lids which were kept on during the incubation of "starter substrate punnets". Further punnets used to contain the diluted incubated substrate, had their lids removed.

Artificial inoculations were made using an isolate of *Pythium ultimum* morphologically identified and provided by Tim Pettitt from an isolation from a Midlands spinach field which had had severe losses. This C761 code was used in previous bioassays (AHDB FV 464b Spinach damping-off seed treatment trials by Kaye, Eyre & Pettitt, 2021). The isolate was cultured on potato dextrose agar (PDA) plates amended with streptomycin

antibiotic which were incubated in the dark for 3 days at 20°C, and then moved to 8°C and allowed to grow for another 3 days to before they reached the plate edge.

The “starter substrate punnets” of Pythium were prepared from 3 parts by dry weight of coir slab (equivalent to 5 parts by volume) and 1 part by dry weight of medium-ground oatmeal (equivalent to 1 part by volume). Autoclave bags batches sufficient for nine punnets each were made up with 300 g of the coir and 100 g of medium ground (food quality) oatmeal and fully wetted with 2 L of distilled water. The substrate was autoclaved for 45 minutes twice on separate days, the last being no more than two days before use. The coir used was from Agrovista fruit Botanicoir Cocopeat Precision Ultra Plus dry strawberry planting slabs, with a water holding capacity of 55%.

The “starter substrate punnets” were filled with 230 g wet weight of the cooled autoclaved coir/oatmeal, the surface flattened, and (following the bioassays) for the efficacy experiments six agar plugs of Pythium were cut from agar plates, using an 8 mm diameter cork-borer, and placed face-down on the surface of the ‘starter’ substrate. The lids were then closed on the punnets, and they were incubated in the dark in a controlled environment cabinet at 20°C for 14 days. The uninoculated punnets contained plugs of clean agar.

To create the punnets that were to be used under the seed trays, after incubating for 7 days, the pythium/coir/oatmeal mixture in the “starter punnets” was sliced up and mixed together. The required wet weight (as given in the tables below for each experiment) was added to uninoculated autoclaved substrate which was also of 1 part oatmeal : 3 parts coir. 23 g of sterile horticultural sand was then sprinkled over the substrate in each punnet and the whole mixed for 15 seconds with a spoon and then flattened down. The final punnets for use under the seed trays contained 253 g wet weight of substrate per punnet. All punnets were then watered with 25 ml of sterile distilled water to achieve field capacity. The make-up of the substrate differed from the 10% oatmeal: 90% sand mix used previously by Tim Pettitt as following further work he had shown a mix using coir to be better for Pythium growth, and the methodology developed for preparation of this inoculum in the current project is detailed below. The substrate in the punnets was kept moistened by drainage of overhead irrigation water down out of the seed tray.

Colony forming unit determination post-inoculation

The inoculum concentration after dilution of the “starter substrate punnets” was measured by colony forming units (cfu) was determined by dilution plating. 10 g wet weight of coir/oatmeal/Pythium/sand mix was taken from across a sample of punnets of the same dilution rate. Each 10 g was placed in a conical flask with 100 ml sterile water and put on a rotary shaker for an hour, before removing aliquots of 1 ml to create a dilution series of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} (Hunter *et al*., 2006). Three replicate PDA plus streptomycin plates were made per concentration using 1 ml per plate. Plates were also made of uninoculated substrate (before starting on the inoculated substrate) to check for any fungal or Pythium contamination. Plates were sealed with Parafilm and incubated at 25°C in the dark for 24 hours. Colonies that had grown from the mycelial fragments were counted through the agar using an inverted microscope (x4 objective). Calculation from plate counts to cfu / g wet weight and conversion to the standard cfu / g dry weight was carried out (a web link to methodology is given in the References section).

Application details for seed treatments used in Germination tests and Efficacy experiments

Seeds were treated by Elsoms Seeds on 14 September 2022 using a bench seed treatment device with STR film coating used as part of the process of product application. Treated seed had a shiny green coating which the seed could not be seen through. The sowing on 20 September 2022 of the 1st efficacy experiment was done within seven days of treatment. The 2nd experiment used more seeds from the same batch on 10 November 2022. Untreated seed did not have any film-coating or other treatments. Seed varied in size and all sizes were sown at random across the trays.

Application schedule for Germination tests and 1st & 2nd Efficacy Experiments

Treatment number	Treatment: product name or AHDB code	Application code
1	Untreated uninoculated	A
2	Untreated inoculated	A
3	AHDB 9848	A
4	AHDB 9715	A
5	Integral Pro	A
6	AHDB 9734	A
7	AHDB 9725	A
8	AHDB 9955	A
9	AHDB 9716	A

Application timing of products for Germination tests and 1st & 2nd Efficacy Experiments

	Application A only - to seed
Application date	14.09.2022
Time of day	Not recorded
Crop growth stage (Max, min average BBCH)	Seed GS00
Crop height (cm)	n/a
Crop coverage (%)	n/a
Application Method	Film coating
Application Placement	Seed surface
Application equipment	Desk-top seed treatment machine
Nozzle pressure	n/a
Nozzle type	n/a
Nozzle size	n/a
Application water volume/ha	n/a
Temperature of air – shade (°C)	Not recorded
Relative humidity (%)	n/a
Wind speed range (m/s)	n/a
Dew presence (Y/N)	n/a
Temperature of soil – 2-5 cm (°C)	n/a
Wetness of soil – 2-5 cm	n/a
Cloud cover (%)	n/a

Seed germination tests on filter paper for seeds used in all experiments

Elsoms Seeds carried out post-treatment germination tests at 0 days (14 September 2022), and 10 weeks post-treatment (23 November 2022). 100 seeds of each batch of treated seeds were used at 0 days. For the 10-week germination test, two batches of 100 seeds were tested. Seed was stored between tests in re-sealed foil pouches within an air-tight box at 4°C ±2°C.

Germination tests were conducted on filter paper pleats (50 pleats, 110 mm x 580 mm strip of filter paper) in a clean clear plastic box. 2 seeds were placed in each pleat to allow space for growth. 40ml of distilled water was sprayed to wet papers, a lid applied and then incubated at 15°C. At each count, at 7, 14 and 21 days, the seedlings together with the seedcoat were removed with forceps and recorded as either normal or in any way abnormal as standard for the testing laboratory, with the number dead recorded at the final count.

Bioassays

A series of experiments were conducted to determine the optimal experimental inoculum concentrations and the best technique to prepare the inoculum for the growth trials. Concentrations of Pythium mycelium were sought that would allow time for the seed treatments to protect emerging seedlings in advance of massed hyphal contact. Information on levels used in glasshouse trials set up in Project AHDB CP165 was used as a starting point for those that might be suitable for the current work in controlled environment cabinets. Details of these trials can be found in Appendix A.

Efficacy Experiments

Two runs of efficacy experiments were carried out. Experiment 1 tested high and low inoculum concentrations. Experiment 2 tested immediate contact between inoculum and seed trays and a delayed contact of 88 hours. Layouts of the treatment plots are given in Appendix B.

Spinach seed cv. Montego fungicide coated on 14 September 2022 was used for Experiment 1 sown 20 September and Experiment 2 sown 10 November 2022.

Trial design Efficacy Experiments 1 & 2

Efficacy experiment design	Details for each of two experiments per run
Trial design:	Randomised block with 9 treatments
Number of replicates:	3
Row spacing:	7 seeds within a 37 x 34 mm seed tray cell
Plot size: (w x l)	112 mmm x 165 mm
Plot size: (m^2)	0.012 m^2
Number of plants per plot:	84

In both experiments, all plots of treated seed received Pythium inoculation, together with the same number of replicates of untreated seed. A further three replicates of untreated seed were left uninoculated. Treatment details are given in the table below. Integral Pro (*Bacillus amyloliquefaciens*) was used as the standard.

In Experiment 2, unreplicated uninoculated 'observation trays' were sown for each of the nine treatments, to give information on emergence in the absence of pathogen.

Treatment details for Germination tests and Efficacy Experiments 1 & 2

AHDB Code	Active substance	Product name/ manufacturer code	Formulation batch number	Content of active substance in product	Formula-tion type	Adju-vant
AHDB 9848	Conventional fungicide	-	Not given	confidential	Liquid	-
AHDB 9715	Conventional fungicide	-	Not given	confidential	Solid	-
Not needed	*Bacillus amylo-liquefaciens* strain MBI600	Integral Pro	Not given	2.2×10^{10} cfu/ml (6.12% w/w)	Flowable concentrate	-
AHDB 9734	microbial	-	Not given	confidential	Liquid	-
AHDB 9725	microbial	-	SAL2566093	confidential	Solid	-
AHDB 9955	microbial	-	02202202	confidential	Solid	-
AHDB 9716	microbial	-	2503	confidential	Solid	-

Further details of the application of these products to the seed are given below in the section on germination tests.

Biological plant protection products were stored at 4°C and shipped with cool blocks to Elsoms for seed treatment.

Efficacy Experiment 1 – high and low inoculum concentration

High and low concentrations of Pythium in the substrate were created by the dilutions of infested coir/oatmeal from "starter punnets" with uninfested coir/oatmeal (see Inoculation Treatments table below). Concentrations obtained were 2.0×10^4 cfu / g dry substrate in the high inoculum and 1.0×10^3 cfu /g dry substrate in the low. The untreated uninoculated had 230 g of only the uninfested substrate. 23 g of dry sand per punnet was subsequently added to each punnet.

Seed trays were placed onto inoculum punnets immediately after sowing (20 September 2022). High and low concentration experiments were in separate controlled environment cabinets but under the same conditions and practices. Each experiment was statistically analysed separately.

Efficacy Experiment 2 – immediate and delayed inoculum contact

The dilution rate that produced the lower concentration (7.7 g of Pythium infested "starter punnet" in 222 g) used in Experiment 1 was used in Experiment 2 for immediate (directly after sowing, 10 November 2022) and delayed contact (88 h post-sowing, 14 November 2022) (see Inoculation Treatments table below). The concentration used was 4.6×10^3 cfu /g dry substrate for both immediate and delayed contact. Seed trays for both conditions were set up at the same time and watered in parallel.

The delayed contact treatment was designed to give the seeds a chance to grow before being challenged by the Pythium mycelium growing up from the substrate into the seed trays.

The experiments ran in separate controlled environment cabinets but were subject to the same practices. Each experiment was statistically analysed separately.

Inoculation Treatments per punnet for Efficacy Experiments

Seed Treatment	Dilution ratios of wet weights (g) of Pythium infested "starter" : uninfested coir + oatmeal substrate			
	1st Efficacy experiments		2nd Efficacy experiments	
	Low Pythium 0 h delay	High Pythium 0 h delay	88 h Pythium contact delay	0 h Pythium contact delay
T1 untreated	0.0 : 230	0.00 : 230	0.0 : 230	0.0 : 230
T2 untreated	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T3 AHDB 9848	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T4 AHDB 9715	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T5 Integral Pro	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T6 AHDB 9734	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T7 AHDB 9725	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T8 AHDB 9955	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222
T9 AHDB 9716	7.7 : 222	23.0 : 207	7.7 : 222	7.7 : 222

Assessments of spinach seedlings

Seedlings were assessed on the following metrics. The timing of the records taken during the efficacy experiments is given within those sections of this report.

- *Growth stage*: BBCH identification key from GS 09 to 11 for leaf vegetables not forming heads. Mode (most common), maximum and minimum stage recorded.
    - *Principal growth stage*
        - 00 Dry seed
        - 01 Beginning of seed imbibition
        - 03 Seed imbibition complete
        - 05 Radicle emerged from seed
        - 07 Hypocotyl with cotyledons breaking through seed coat
    - *Leaf development (main shoot)*
        - 10 Cotyledons completely unfolded: growing point or true leaf visible
        - 11 First true leaf unfolded
        - 12 Second true leaf unfolded (up to 19 9 or more true leafs unfolded)

- *Emergence, healthy* – count of number of seedlings emerged in whole tray
- *Emergence, healthy, cotyledons down* - count of seedlings where the cotyledons were still facing downwards, as an indication of continuing emergence**.**
- *Damping off* - The number of seedlings at the time of the emergence count.

    - damped seedlings were not always still visible at future counts and more seedlings were emerging, so a total tally of all the emerged was not possible at the end.

- *Survival to first true leaf* - number of seedlings of those emerged.
- *Vigour* - 0-10 scale across each tray, where 5 was average vigour and 10 was excellent for those present. Where there were seedlings of a more-advanced growth stage these increased the index scored. Vigour differed within trays and the score was an average. Vigour also reflected plant height and any paler leaf colour within the first three weeks.
- *Phytotoxicity* **-** distortion, yellowing or stunting scored by an index, or an assessment of the proportion affected depending on what was seen or marked as absent.
- *Plant size* – record of tallest height seedling per tray. Measured to top of stem at cotyledon stage. At true leaf stage the height from the soil up along the longest leaf tip was recorded.
- Photographs were taken of whole trays and any individual seedling symptoms.

Statistical analysis

Results from seed germination tests performed immediately and 10 weeks after treatment were not statistically analysed.

Statistical analysis was not carried out in either of the bioassay experiments, with comparisons made by examination of the raw data.

Treatment comparison for the efficacy experiments was made within each inoculum concentration trial of Experiment 1 or initial contact time trial of Experiment 2 using Analysis of Variance. Each trial was also analysed using Duncan's Multiple Range Test and the rank position of the products were compared as this picked up differences with a little less statistical probability then the ANOVA and could indicate some benefits.

Observation plots of one tray of each treatment without inoculum (set up in the same cabinet as each of the two inoculum-contact times of Experiment 2) were not analysed statistically. They were examined to see any potential differences between plant survival in the peat-based growing-media and the filter-paper germination tests.

Efficacy Experiments Assessments

Evaluation timings were calculated from seed treatment day on 14 September 2022 to days after (DA) seed treatment.

Counts were made separately of healthy seedlings as they came through the growing medium and still had their heads down (GS09) and of healthy seedlings that had cotyledons completely unfolded (GS10) or in later scores had true leaves (GS11-14). This was because GS09 seedlings could be disintegrated by the next score if attacked by Pythium and so any further GS09 counted indicated additional emergence. GS10 seedlings when attacked by Pythium could remain visible for a week but could be lost from the total present by the next count.

First efficacy experiments sown 20 September 2022 with assessment on the same day for High and Low inoculum cabinets The second efficacy experiments were sown on 10 November 2022 with assessments on the same day for both the immediate and delayed inoculum challenge cabinets. Assessment dates are given in the two tables below together with the number of days after seed application (DA)**.**

Assessment Timings Efficacy Experiment 1: low and high Pythium inoculum

Evaluation date	**Evaluation Timing (DA) after conventional & biofungicide application on 14 September**	**Modal Crop Growth Stage (BBCH)**	**Evalua-tion type**	**Assessment following sowing on 20 September 2023**
27.09.2022	13	10	Efficacy	GS09 Healthy emerged count 7 d
27.09.2022			Efficacy	≥GS10 Healthy emerged count 7 d
27.09.2022			Efficacy	Damped-off emerged count 7 d
27.09.2022			Efficacy	Vigour 0-10 index whole tray 7d
27.09.2022			Phytotox.	Count of affected emerged 7d
04.10.2022	20	10/12	Efficacy	GS09 Healthy emerged count 14 d
04.10.2022			Efficacy	≥GS10 Healthy emerged count 14 d
04.10.2022			Efficacy	Damped-off emerged count 14 d
04.10.2022			Efficacy	Vigour 0-10 index whole tray 14 d
04.10.2022			Phytotox.	Count of affected emerged 14 d
11.10.2022	27	12	Efficacy	GS09 Healthy emerged count 21 d
11.10.2022			Efficacy	≥GS10 Healthy emerged count 21 d
11.10.2022			Efficacy	Damped-off emerged count 21 d
11.10.2022			Efficacy	Survival to true leaf count 21 d
11.10.2022			Efficacy	Vigour 0-10 index whole tray 21 d
11.10.2022			Phytotox.	Count of affected emerged 21 d

Assessment Timings Efficacy Experiment 2: immediate & delayed inoculum contact

Evaluation date	Evaluation Timing (DA) after conventional & biofungicide application on 14 September	Modal Crop Growth Stage (BBCH)	Evaluation type	Assessment following sowing on 10 November 2022
17.11.2022	64	10	Efficacy	GS09 Healthy emerged count 7 d
17.11.2022			Efficacy	≥GS10 Healthy emerged count 7 d
17.11.2022			Efficacy	Damped-off emerged count 7 d
17.11.2022			Efficacy	Vigour 0-10 index whole tray 7d
17.11.2022			Phytotox.	Count of affected emerged 7d
24.11.2022	71	10	Efficacy	GS09 Healthy emerged count 14 d
24.11.2022				≥GS10 Healthy count 14 d
24.11.2022			Efficacy	Damped-off emerged count 14 d
24.11.2022			Efficacy	Survival to true leaf ≥GS11 count 14 d
24.11.2022			Efficacy	Vigour 0-10 index whole tray 14d
24.11.2022			Phytotox.	Count of affected emerged 14d
01.12.2022	79	12	Efficacy	GS09 Healthy emerged count 21 d
01.12.2022				≥GS10 Healthy count 21 d
01.12.2022			Efficacy	Damped-off emerged count 21 d
01.12.2022			Efficacy	Survival to true leaf ≥GS11 count 21 d
01.12.2022			Efficacy	Vigour 0-10 index whole tray 21 d
01.12.2022			Phytotox.	Count of affected emerged 21 d
08.12.2022	86	12	Efficacy	GS09 Healthy emerged count 28 d
08.12.2022				≥GS10+ Healthy count 28 d
08.12.2022			Efficacy	Damped-off emerged count 28 d
08.12.2022			Efficacy	Survival to true leaf ≥GS11+ count 28 d
08.12.2022			Efficacy	Vigour 0-10 index whole tray 28 d
08.12.2022			Phytotox.	Count of affected emerged 28 d

Results

Seed germination tests on filter paper

At the germination test carried out straight after treatment (Table 1), there were no obvious differences in the proportion of healthy normal seedlings having germinated after three weeks, with a range from 89% to 95%. Abnormal seedlings were 7% of the untreated and the range of 4% to 9% in the treated was not notably different. There was, however, quite a wide range in the proportion of seeds that had germinated after a week, being a relatively low 84% in the untreated, whereas AHDB 9848, Integral Pro, AHDB 9734 and AHDB 9725 had 90% or more germinated.

At the germination test carried out after 10 weeks of cold storage following seed treatment (Table 2), emergence after a week was 93% in the untreated and this was only exceeded (marginally) by Integral Pro although only in one replicate. Germination by three weeks was similar to that seen in the 0 days germination test, with a range from 87% to 96% without any obvious benefit or detriment from any treatment and means of 4% to 7.5% of seedlings being abnormal.

Therefore, no indication of phytotoxicity was seen from any treatment either immediately or 10 weeks after treatment, and the proportion germinating was acceptable for both the untreated and treated seeds.

Table 1. Germination tests commenced on 14 September 2022 in filter-paper boxes on sub-samples of 100 seeds directly following seed treatment

Treatment	Number germinated out of 100 by each count			Final number	
	7 days	14 days	21 days	Abnormal seedlings	Dead seeds
Untreated	84	92	93	7	0
Untreated	84	91	92	7	1
AHDB 9848	91	95	95	5	0
AHDB 9715	89	92	92	8	0
Integral Pro	93	92	94	4	2
AHDB 9734	90	96	96	4	0
AHDB 9725	91	93	93	5	2
AHDB 9955	85	87	89	9	2
AHDB 9716	88	90	91	9	0

Table 2. Germination tests commenced on 23 November 2022 in filter-paper boxes on sub-samples of 100 seeds 10 weeks after seed treatment

Treatment	Number germinated out of 100 by each count (two replicates per treatment)			Final numbers	
	7 days	14 days	21 days	Abnormal seedlings	Dead seeds
Untreated	93	96	96	4	0
Untreated	93	95	95	4	1
AHDB 9848	92	98	98	2	0
AHDB 9848	84	86	90	8	2
AHDB 9715	88	94	94	5	1
AHDB 9715	80	84	87	10	3
Integral Pro	84	89	90	8	2
Integral Pro	96	91	91	7	2
AHDB 9734	87	96	96	4	0
AHDB 9734	85	91	91	7	2
AHDB 9725	86	94	94	5	1
AHDB 9725	89	92	92	8	0
AHDB 9955	86	93	94	4	2
AHDB 9955	86	94	94	5	1
AHDB 9716	93	94	95	3	2
AHDB 9716	87	90	90	7	3

Bioassays

Detailed results are presented in Appendix A.

In the first bioassay using untreated seed, lower (52 to 348 cfu/ g dry substrate) and higher (5.2×10^3 to 1.2×10^4 cfu/ g dry substrate) Pythium ranges were obtained for the substrate placed under the seed trays. A mean 62% of seedlings emerged and survived in the uninoculated trays, compared with inoculated trays of commonly only 3%, and at most 22% emergence. The numbers damped-off did not alter in line with the range of Pythium dilutions.

In the second bioassay using untreated seed with five increasing concentrations of Pythium in the substrate, a mean 89% of seedlings emerged and survived in the uninoculated trays. There was a mean 68% survival at the highest concentration tested (6.5×10^4 cfu / g dry substrate) and this dilution rate was subsequently selected to produce the high concentration for Efficacy Experiment 1. An interim dilution rate from the bioassay was used as the low concentration in both Efficacy Experiments 1 and 2.

Efficacy Experiment 1: Low and high inoculum

Non-statistical comparisons between the trials in Experiment 1.

Very few seedlings emerged from either the treated or untreated seed in trays stood on either the lower or the higher concentration of Pythium mycelium. This was probably due to pre-emergence damping-off as trays not in contact with inoculum grew well (Figures 1 and 2). Excavation in the peat of samples of trays at the end of the experiment showed a large proportion of un-emerged seedlings with no signs of germination having started, the seed coating film containing the product sometimes still apparently intact.

The poor emergence of seedlings subject to both the lower and higher levels of Pythium inoculum meant that true plant vigour was not able to be assessed. Similarly, few plants emerged in the untreated inoculated plots as in the treated of both experiments, so it was unlikely that poor emergence in the treated plots was due to any phytotoxicity, but instead resulted from Pythium infection.

Efficacy Experiment 1 - lower inoculum level

The concentration for the lower Pythium inoculation as determined by dilution plating was 1.0 x 10^3 cfu /g dry substrate (oven dry substrate weight 85% of the wet weight).

Figure 1. 1st Efficacy Experiment lower inoculum 13 October 2022. Trays of spinach seedlings sitting on punnets of coir / oatmeal / sand substrate. All the yellow-labelled punnets contained the lower colony concentration of Pythium. The purple-labelled trays with abundant seedlings growing were uninoculated. Seeds sown 20 September.

At Day 21, 75.4% of seeds were emerged and apparently healthy in the untreated uninoculated. However, significantly fewer ($P<0.001$) were present (at most 1.98% of those sown) in the treated, with no differences between the untreated inoculated and the various treatments (Table 3).

Post-emergence damping-off was little evident in the inoculated because of the few seedlings grown (Table 4). Totalling both healthy emerged and those that damped-off after emergence confirmed equally poor emergence across the inoculated plots by Day 21 (Table 5), although some seedlings had been able to survive to true leaf (Table 6).

Table 3. Efficacy Experiment 1 - low Pythium: The number and calculated % of healthy seedlings present at weekly assessments.

1st Efficacy expt. With lower Pythium	Number healthy seedlings emerged & present at each assessment			% of 84 seeds sown that were present & healthy at each assessment		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	25.67b	58.33b	63.33b	30.56b	69.44b	75.40b
Untreated inoculated	2.00a	0.67a	1.00a	2.38a	0.79a	1.19a
AHDB 9848	2.00a	0.67a	0.67a	2.38a	0.79a	0.79a
AHDB 9715	3.67a	1.00a	1.33a	4.37a	1.19a	1.59a
Integral Pro	3.33a	1.67a	1.33a	3.97a	1.98a	1.59a
AHDB 9734	4.67a	1.33a	1.67a	5.56a	1.59a	1.98a
AHDB 9725	4.00a	1.33a	1.67a	4.76a	1.59a	1.98a
AHDB 9955	1.67a	0.33a	0.67a	1.98a	0.40a	0.79a
AHDB 9716	2.33a	1.00a	1.00a	2.78a	1.19a	1.19a
Grand mean	5.48	7.37	8.07	6.53	8.77	9.61
P.Fr	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L.S.D.	7.377	7.251	6.988	8.782	8.633	8.32
D.F.	16	16	16	16	16	16

Table 4. Efficacy Experiment 1 - low Pythium: The number of emerged seedlings present that were damped off and visible at each weekly assessment and calculated % of post-emergence damped-off seedlings out of the total seeds sown.

1st Efficacy expt. With lower Pythium	Number emerged that were damped-off on each assessment			% of 84 seeds sown that emerged then damped off		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	0.00	0.33	1.33	0.00	0.40	1.59
Untreated inoculated	0.33	1.00	0.67	0.40	1.19	0.79
AHDB 9848	0.00	0.33	0.33	0.00	0.40	0.40
AHDB 9715	0.67	2.00	0.67	0.79	2.38	0.79
Integral Pro	0.67	1.00	0.33	0.79	1.19	0.40
AHDB 9734	0.00	2.00	0.67	0.00	2.38	0.79
AHDB 9725	0.33	2.00	2.67	0.40	2.38	3.17
AHDB 9955	0.00	1.33	0.67	0.00	1.59	0.79
AHDB 9716	0.00	0.33	0.00	0.00	0.40	0.00
Grand mean	0.22	1.15	0.81	0.26	1.37	0.97
P.Fr	0.713	0.456	0.326	0.713	0.456	0.326
L.S.D.	1.06	2.156	2.087	1.262	2.566	2.484
D.F.	16	16	16	16	16	16

Table 5. Efficacy Experiment 1 - low Pythium: The total seedlings visible at each weekly assessment whether healthy or damped-off and as a % of seeds sown.

1st Efficacy expt. With lower Pythium	Total emerged, both healthy and damped-off			% of 84 seeds sown that emerged (healthy or damped-off)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	25.67b	58.67b	64.67b	30.6b	69.8b	76.98b
Untreated inoculated	2.33a	1.67a	1.67a	2.80a	2.00a	1.98a
AHDB 9848	2.00a	1.00a	1.00a	2.40a	1.20a	1.19a
AHDB 9715	4.33a	3.00a	2.00a	5.20a	3.60a	2.38a
Integral Pro	4.00a	2.67a	1.67a	4.80a	3.20a	1.98a
AHDB 9734	4.67a	3.33a	2.33a	5.60a	4.00a	2.78a
AHDB 9725	4.33a	3.33a	4.33a	5.20a	4.00a	5.16a
AHDB 9955	1.67a	1.67a	1.33a	2.00a	2.00a	1.59a
AHDB 9716	2.33a	1.33a	1.00a	2.90a	1.60a	1.19a
Grand mean	5.70	8.52	8.89	6.80	10.10	10.58
P.Fr	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L.S.D.	7.629	7.876	7.116	9.08	9.38	8.472
D.F.	16	16	16	16	16	16

Table 6. Efficacy Experiment 1 - low Pythium: Survival to 21 days showing the number of healthy seedlings visible that were at cotyledon stage or true-leafed.

1st Efficacy expt. With lower Pythium	Number at cotyledon stage Day 21	Number at true leaf Day 21
Untreated uninoculated	6.67b	56.67b
Untreated inoculated	0.67a	0.33a
AHDB 9848	0.00a	0.67a
AHDB 9715	0.33a	1.00a
Integral Pro	0.00a	1.33a
AHDB 9734	0.67a	1.00a
AHDB 9725	1.00a	0.67a
AHDB 9955	0.33a	0.33a
AHDB 9716	0.33a	0.67a
Grand mean	1.11	6.96
P.Fr	<0.001	<0.001
L.S.D.	1.999	7.978
D.F.	16	16

Efficacy Experiment 1 – higher inoculum level

The concentration for the higher Pythium inoculation as determined by dilution plating was 2.0 x 10^4 cfu / g dry substrate (oven dry substrate weight 85% of the wet weight).

At Day 21 71.4% of the seeds were emerged and apparently healthy in the untreated uninoculated. However, significantly fewer ($P<0.001$) were present (at most 1.19% of those sown), in the treated (Figure 2), with no differences between the untreated inoculated and the various treatments (Table 7).

Post-emergence damping-off was little evident in the inoculated because of the few seedlings grown (Table 8). Totalling both healthy emerged and those that damped-off after emergence confirmed equally poor emergence across all the inoculated plots by Day 21 (Table 9), although some seedlings had survived to true leaf (Table 10).

Figure 2. Efficacy Experiment 1 - high Pythium on 13 October 2022. Trays of spinach seedlings sitting on punnets of coir / oatmeal / sand substrate. All orange-labelled punnets with Pythium. Uninoculated trays with many seedlings. Sown 20 September.

Table 7. Efficacy Experiment 1 - high Pythium: The number and calculated % of healthy seedlings of those sown that were present at weekly assessments.

1st Efficacy expt. With higher Pythium	Number healthy seedlings emerged & present at each assessment			% of 84 seeds sown that were present & healthy at each assessment		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	23.67b	57.67b	60.00b	28.17b	68.65b	71.43b
Untreated inoculated	2.33a	0.00a	0.00a	2.78a	0.00a	0.00a
AHDB 9848	1.33a	0.00a	0.00a	1.59a	0.00a	0.00a
AHDB 9715	4.33a	0.67a	0.67a	5.16a	0.79a	0.79a
Integral Pro	3.00a	0.67a	0.67a	3.57a	0.79a	0.79a
AHDB 9734	4.33a	0.33a	0.33a	5.16a	0.40a	0.40a
AHDB 9725	3.00a	0.67a	0.67a	3.57a	0.79a	0.79a
AHDB 9955	2.00a	0.67a	1.00a	2.38a	0.79a	1.19a
AHDB 9716	1.33a	0.33a	0.33a	1.59a	0.40a	0.40a
Grand mean	5.04	6.78	7.07	6.00	8.07	8.42
P.Fr	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L.S.D.	6.823	2.11	2.026	8.122	2.512	2.412
D.F.	16	16	16	16	16	16

Table 8. Efficacy Experiment 1 - high Pythium : The number of emerged seedlings present that were damped off and visible at each weekly assessment and calculated % of post-emergence damped-off seedlings out of the total seeds sown.

1st Efficacy expt. With higher Pythium	Number emerged that then were damped-off on each assessment			% of 84 seeds sown that emerged then damped off		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	0.33	0.67	1.00	0.40	0.79	1.19
Untreated inoculated	0.33	1.67	1.33	0.40	1.98	1.59
AHDB 9848	0.00	0.00	0.33	0.00	0.00	0.40
AHDB 9715	1.67	2.33	1.67	1.98	2.78	1.98
Integral Pro	1.00	2.00	2.00	1.19	2.38	2.38
AHDB 9734	1.33	2.33	1.67	1.59	2.78	1.98
AHDB 9725	0.00	1.33	0.67	0.00	1.59	0.79
AHDB 9955	0.00	1.33	1.00	0.00	1.59	1.19
AHDB 9716	0.67	1.33	0.67	0.79	1.59	0.79
Grand mean	0.59	1.44	1.15	0.71	1.72	1.37
P.Fr	0.066	0.656	0.904	0.066	0.656	0.904
L.S.D.	1.201	2.66	2.633	1.43	3.166	3.135
D.F.	16	16	16	16	16	16

Table 9. Efficacy Experiment 1 - high Pythium: The total seedlings visible at each weekly assessment whether healthy or damped-off and as a % of seeds sown.

1st Efficacy expt. With higher Pythium	Total emerged, both healthy and damped-off			% of 84 seeds sown that emerged (healthy or damped-off)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Untreated uninoculated	24.00b	58.33b	61.00b	28.57b	69.44b	72.62b
Untreated inoculated	2.67a	1.67a	1.33a	3.17a	1.98a	1.59a
AHDB 9848	1.33a	0.00a	0.33a	1.59a	0.00a	0.40a
AHDB 9715	6.00a	3.00a	2.33a	7.14a	3.57a	2.78a
Integral Pro	4.00a	2.67a	2.67a	4.76a	3.17a	3.17a
AHDB 9734	5.67a	2.67a	2.00a	6.75a	3.17a	2.38a
AHDB 9725	3.00a	2.00a	1.33a	3.57a	2.38a	1.59a
AHDB 9955	2.00a	2.00a	2.00a	2.38a	2.38a	2.38a
AHDB 9716	2.00a	1.67a	1.00a	2.38a	1.98a	1.19a
Grand mean	5.63	8.22	8.22	6.7	9.79	9.79
P.Fr	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
L.S.D.	6.817	3.586	3.649	8.115	4.269	4.344
D.F.	16	16	16	16	16	16

Table 10. Efficacy Experiment 1 - with high Pythium: Survival to 21 days showing the number of healthy seedlings visible that were at cotyledon stage or true-leafed.

1st Efficacy expt. With higher Pythium	Number at cotyledon Day 21	Number at true leaf Day 21
Untreated uninoculated	3.67b	56.33b
Untreated inoculated	0.00a	0.00a
AHDB 9848	0.00a	0.00a
AHDB 9715	0.33a	0.33a
Integral Pro	0.33a	0.33a
AHDB 9734	0.33a	0.00a
AHDB 9725	0.00a	0.67a
AHDB 9955	0.33a	0.67a
AHDB 9716	0.00a	0.33a
Grand mean	0.56	6.52
P.Fr	0.012	<0.001
L.S.D.	1.825	2.665
D.F.	16	16

Pythium mycelium was confirmed to still be growing in the coir / oatmeal / sand mix in the punnets under the seed trays when the seed trays were taken off at the end of the experiment (Figure 3).

Figure 3. Pythium mycelium clearly visible growing upwards on the substrate in a punnet in between the seed tray cells after the tray was removed following efficacy testing. Directly under tray cells mycelium was able to grow up through the central drainage hole into the growing-media containing the seedlings. 19 October 2022.

Efficacy Experiments 2 – immediate and delayed inoculum contact

Non-statistical comparisons between the trials in Experiment 2 and with Experiment 1.

For both experiments the inoculum concentration used was 4.6 x 10^3 cfu /g dry substrate (oven dry weight of the substrate 77% of the wet weight). This was similar to the low inoculum concentration experiment in Efficacy Experiment 1.

In the uninoculated untreated only 33% of seedlings emerged for both the immediate and delayed contact experiments (Tables 13 &17 and Figures 4 & 5). This was low compared with a mean 75% observed in the two trials of efficacy Experiment 1 (Tables 5 & 9). An extra recording week in Experiment 2 did not show further emergence.

More seedlings emerged in the Pythium inoculated trays in Experiment 2 (Figures 4, 5, 6 & 7) than in Experiment 1 (Figure 1). In Experiment 1, very few untreated inoculated seedlings emerged at either inoculum concentration (Tables 5 & 9). However, in Experiment 2, 21% of seeds emerged following immediate inoculation (Table 17) and 42% when inoculation was delayed (Table 13).

The number of healthy emerged untreated seedlings was similar both with delayed Pythium contact and no contact (Table 11), but fewer (P=0.07) healthy untreated seedlings were present in trays following immediate contact than if left uninoculated (Table 15). As in Experiment 1, post-emergence damping-off in Experiment 2 was a small proportion of those sown. Therefore, any comparisons between treatments of the numbers lost post-emergence should consider the seedling numbers actually available in each to damp-off.

Efficacy Experiment 2 – delayed contact with inoculum

Figure 4. Top shelf of cabinet CE01 Experiment 2. Trays sown 10 November delayed contact with Pythium inoculum until 08:30 h 14 November. Day 28; plants starting to run out of nutrients from the growing media and so yellowing. Purple labels mark uninoculated untreated plots. 8 December 2022.

Figure 5. Bottom shelf of cabinet CE01 Experiment 2. Trays sown 10 November delayed contact with Pythium inoculum until 08:30 h Monday 14 November 2022. Day 28; plants were starting to run out of nutrients from the growing media and so yellowing. 8 December 2022. Right-hand nine trays were all un-inoculated observation plots.

Healthy plant vigour did not differ significantly between the treatments at any of the assessment dates; with indices each week ranging 4.3 - 6.0 at Day 7, 5.3 - 6.7 at Day 14, 4.7 - 7.3 at Day 21 and 6.0 to 7.0 at Day 28 (where index 10 would be excellent). There were no foliar phytotoxicity symptoms.

At all four assessments, the numbers of healthy seedlings and total emergence in the untreated inoculated were significantly greater ($P>0.01$) than for any of the treated inoculated (Tables 11 and 13).

At seven days post-sowing, chemical AHDB 9848, with 9.3 healthy seedlings, was the only treatment not significantly different from the untreated uninoculated (15.7 seedlings, $P<0.01$). The other products had fewer healthy seedlings. At Days 14 and 28 emergence was increased and both AHDB 9848 and AHDB 9715 had similar numbers of healthy seedlings compared with the uninoculated. However, there was no statistical difference between any products in totals emerged and numbers healthy (Tables 11 and 13).

The greatest increase in emergence was between Days 7 and 14 (Table 13). After this, except in the uninoculated, one or two seedlings per tray were lost to damping-off, with no significant differences between treatments (Table 12).

Healthy seedling growth to true-leaf was not complete by Day 28. At this timepoint, all products conversely had significantly fewer ($P<0.01$) true-leaved spinach plants than the untreated inoculated (20.7 plants). There was no significant difference between the untreated uninoculated (14.7 plants) at true-leaf and treatments AHDB 9848 and AHDB 9715. However, these products did not have significantly better survival than any other product (Table 14).

Table 11. Efficacy Experiment 2 - delayed Pythium contact: Weekly counts of healthy seedlings and calculated % of the total sown.

2nd Efficacy expt. With delayed Pythium	Number healthy seedlings emerged & present at each assessment				% of 84 seeds sown that were present & healthy at each assessment			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Untreated uninoculated	15.67bc	27.33bc	29.00bc	27.33bc	18.65bc	32.54bc	34.52bc	32.54bc
Untreated inoculated	20.33c	31.00c	34.33c	33.33c	24.21c	36.90c	40.87c	39.68c
AHDB 9848	9.33ab	18.67ab	19.67ab	19.67ab	11.11ab	22.22ab	23.41ab	23.41ab
AHDB 9715	6.33a	16.33ab	16.33a	16.70ab	7.54a	19.44ab	19.44a	19.84ab
Integral Pro	7.33ab	10.00a	12.67a	15.00ab	8.73a	11.90a	15.08a	17.86ab
AHDB 9734	7.00ab	13.67a	14.00a	12.67a	8.33a	16.27a	16.67a	15.08a
AHDB 9725	4.67a	12.33a	13.67a	14.33a	5.56a	14.68a	16.27a	17.06a
AHDB 9955	2.33a	11.33a	11.33a	10.67a	2.78a	13.49a	13.49a	12.70a
AHDB 9716	4.67a	10.33a	11.33a	11.67a	5.56a	12.30a	13.49a	13.89a
Grand mean	8.63	16.80	18.00	17.90	10.30	20.00	21.50	21.30
P.Fr	0.005	0.008	0.001	0.011	0.005	0.008	0.001	0.011
L.S.D.	8.16	11.22	10.25	11.8	9.71	13.35	12.20	14.05
D.F.	16	16	16	16	16	16	16	16

Table 12. Efficacy Experiment 2 - delayed Pythium contact: The number of emerged seedlings present that were damped off and visible (including ones counted before) at each weekly assessment and calculated % of post-emergence damped-off seedlings out of the total seeds sown.

2nd Efficacy expt. With delayed Pythium	Number emerged that then damped-off				% of 84 seeds sown that emerged then damped-off			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Untreated uninoculated	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Untreated inoculated	0.00	1.67	2.33	2.00	0.00	1.98	2.78	2.38
AHDB 9848	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.19
AHDB 9715	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.40
Integral Pro	0.00	1.67	1.67	1.67	0.00	1.98	1.98	1.98
AHDB 9734	0.33	0.67	0.33	1.33	0.40	0.79	0.40	1.59
AHDB 9725	0.00	0.33	0.33	0.67	0.00	0.40	0.40	0.79
AHDB 9955	0.00	0.00	0.33	1.00	0.00	0.00	0.40	1.19
AHDB 9716	0.00	0.33	0.67	0.33	0.00	0.40	0.79	0.40
Grand mean	0.04	0.52	0.63	0.93	0.04	0.62	0.75	1.10
P.Fr	0.473	0.408	0.477	0.651	0.473	0.408	0.477	0.651
L.S.D.	0.33	1.96	2.48	2.30	0.37	2.34	2.95	2.73
D.F.	16	16	16	16	16	16	16	16

Table 13. Efficacy Experiment 2 - delayed Pythium contact: The total seedlings visible at each weekly assessment whether healthy or damped-off and as a % of sown.

2nd Efficacy expt. With delayed Pythium	Total emerged, both healthy and damped-off				% of 84 seeds sown that emerged (healthy or damped-off)			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Untreated uninoculated	15.67bc	27.33bc	30.33bc	27.33bc	18.65bc	32.54bc	36.11bc	32.54bc
Untreated inoculated	20.33c	32.67c	36.67c	35.33c	24.21c	38.89c	43.65c	42.06c
AHDB 9848	9.33ab	18.67ab	20.33ab	20.67ab	11.11ab	22.22ab	24.21ab	24.60ab
AHDB 9715	6.33a	16.33ab	17.00a	17.00ab	7.54a	19.44ab	20.24a	20.24ab
Integral Pro	7.33ab	11.67a	17.00a	16.67ab	8.73ab	13.89a	20.24a	19.84ab
AHDB 9734	7.33ab	14.33a	14.33a	14.00a	8.73ab	17.06a	17.06a	16.67a
AHDB 9725	4.67a	12.67a	14.67a	15.00ab	5.56a	15.08a	17.46a	17.86ab
AHDB 9955	2.33a	11.33a	11.67a	11.67a	2.78a	13.49a	13.89a	13.89a
AHDB 9716	4.67a	10.67a	12.00a	12.00a	5.56a	12.70a	14.29a	14.29a
Grand mean	8.67	17.30	19.30	18.90	10.30	20.60	23.00	22.40
P.Fr	0.005	0.004	0.002	0.009	0.005	0.004	0.002	0.009
L.S.D.	8.108	10.78	10.84	11.79	9.65	12.83	12.90	14.04
D.F.	16	16	16	16	16	16	16	16

Table 14. Efficacy Experiment 2 - delayed Pythium contact : The number of healthy seedlings that had grown to true leaf at 14, 21 and 28 days from sowing.

2nd Efficacy expt. With delayed Pythium	Survival to true leaf	Survival to true leaf	Survival to true leaf
	Day 14	Day 21	Day 28
Untreated uninoculated	5.00	12.33bc	14.67bc
Untreated inoculated	6.67	15.00c	20.67c
AHDB 9848	3.00	8.00abc	10.67ab
AHDB 9715	2.67	7.33abc	9.00ab
Integral Pro	3.00	6.33ab	6.67a
AHDB 9734	2.33	5.67ab	8.00ab
AHDB 9725	1.00	5.67ab	6.67a
AHDB 9955	1.33	2.67a	5.67a
AHDB 9716	0.67	3.67a	5.00a
Grand mean	2.85	7.41	9.67
P.Fr	0.171	0.043	0.005
L.S.D.	4.44	7.23	7.08
D.F.	16	16	16

Efficacy Experiment 2 – immediate contact with inoculum

Figure 6. Experiment 2 – immediate contact with inoculum. Trays sown 10 November. Orange-labelled stood on inoculum same day as sowing. Purple labelled were uninoculated trays Plants on Day 28 starting to run out of nutrients from the growing media and starting to yellow. 8 December 2022.

Figure 7. Experiment 2 – immediate contact with inoculum Bottom shelf of cabinet CE02 2nd run. Trays sown 10 November with Pythium inoculum contact the same day at 16:30 h. Plants were starting to run out of nutrients from the growing media and so were starting to yellow. Day 28, 8 December 2022. Right-hand half of this shelf (nine trays) were all un-inoculated observation plots.

Healthy plant vigour did not differ significantly between the treatments at any of the assessment dates. with the indices each week ranging 3.0 - 5.6 at Day 7, 4.0 - 5.3 at Day 14, 3.7- 6.0 at Day 21 and 4.3 - 6.3 at Day 28 (where index 10 would be excellent). There were no foliar phytotoxicity symptoms.

Seven days from sowing, total emergence and the number of healthy plants in the uninoculated untreated were greater (P<0.05) than in any inoculated treatments and continued to rank best. None of the products gave better emergence counts of healthy seedlings at any assessment date than the untreated inoculated (Tables 15 and 17).

Across the assessment dates a maximum of 3% of seedlings emerged and then damped off. There were never significantly fewer in the treated than the untreated inoculated. At Day 21, post-emergence damping-off in AHDB 9734 of 7.9% was significantly worse (P<0.05) than all other treatments, including the untreated controls (Table 16).

The total number of seedlings that emerged in the untreated uninoculated ranked above the inoculated at each assessment but was only significantly (P<0.05) better at Day 7. By Day 28, Duncan's Test indicated that the untreated inoculated and the biological products AHDB 9734 and AHDB 9955 had higher total emergence, the other products ranking below 14% emergence (Table 17).

Not all healthy seedlings had progressed from cotyledon stage by Day 28. By then no significant treatment differences had arisen in survival to true leaf, with a maximum of 4.4 plants seen in the untreated inoculated, but not significantly more than any other treatment (Table 18). This was notably less plants than the 14.7 in the delayed inoculum contact experiment.

Table 15. Efficacy Experiment 2, - immediate Pythium contact: Weekly counts of healthy seedlings and calculated % of the total sown.

2nd Efficacy expt. With immediate Pythium	**Number healthy seedlings emerged & present at each assessment**				**% of 84 seeds sown that were present & healthy at each assessment**			
	Day 7	**Day 14**	**Day 21**	**Day 28**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Untreated uninoculated	17.00b	27.67b	27.33b	27.33b	20.24b	32.94b	32.54b	32.54b
Untreated inoculated	7.67a	18.00ab	15.33ab	13.00a	9.13a	21.43ab	18.25ab	15.48a
AHDB 9848	2.33a	9.67a	10.00a	9.67a	2.78a	11.51a	11.90a	11.51a
AHDB 9715	3.33a	7.33a	7.33a	7.33a	3.97a	8.73a	8.73a	8.73a
Integral Pro	6.00a	12.00ab	11.67a	9.67a	7.14a	14.29ab	13.89a	11.51a
AHDB 9734	6.67a	14.67ab	11.33a	11.33a	7.94a	17.46ab	13.49a	13.49a
AHDB 9725	5.00a	7.33a	7.33a	7.33a	5.95a	8.73a	8.73a	8.73a
AHDB 9955	3.33a	13.67ab	12.33a	11.67a	3.97a	16.27ab	14.68a	13.89a
AHDB 9716	3.33a	5.67a	5.00a	4.67a	3.97a	6.75a	5.95a	5.56a
Grand mean	6.07	12.90	12.00	11.33a	7.2	15.3	14.20	13.50
P.Fr	0.026	0.127	0.085	0.071	0.026	0.127	0.085	0.071
L.S.D.	7.62	14.79	7.64	12.81	9.07	17.61	15.76	15.25
D.F.	16	16	16	16	16	16	16	16

Table 16. Efficacy Experiment 2 -immediate Pythium contact: The number of emerged seedlings present that were damped off and visible at each weekly assessment and calculated % of post-emergence damped-off seedlings out of the total seeds sown.

2nd Efficacy expt. With immediate Pythium	Number emerged that then had damped-off				% of 84 seeds sown that emerged then damped-off			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Untreated uninoculated	0.00	0.67a	0.33a	0.67a	0.00	0.79a	0.40a	0.79a
Untreated inoculated	0.00	0.67a	2.33a	4.67ab	0.00	0.79a	2.78a	5.56ab
AHDB 9848	0.00	1.00a	1.33a	1.67a	0.00	1.19a	1.59a	1.98a
AHDB 9715	0.00	0.33a	1.00a	0.67a	0.00	0.40a	1.19a	0.79a
Integral Pro	0.00	0.67a	2.00a	1.67a	0.00	0.79a	2.38a	1.98a
AHDB 9734	0.33	4.00b	6.67b	6.00b	0.40	4.76b	7.94b	7.14b
AHDB 9725	0.00	1.00a	1.00a	0.67a	0.00	1.19a	1.19a	0.79a
AHDB 9955	0.00	1.33a	3.33a	3.33ab	0.00	1.59a	3.97a	3.97ab
AHDB 9716	0.00	0.33a	1.00a	1.33a	0.00	0.40a	1.19a	1.59a
Grand mean	0.04	1.11	2.11	2.3	0.04	1.32	2.51	2.73
P.Fr	0.473	0.195	0.015	0.052	0.473	0.195	0.015	0.052
L.S.D.	0.33	2.66	3.07	3.63	0.40	3.17	3.66	4.32
D.F.	16	16	16	16	16	16	16	16

Table 17. Efficacy Experiment 2 -- immediate Pythium contact: The total seedlings visible at each weekly assessment whether healthy or damped-off and as a % of sown.

2nd Efficacy expt. With Immediate Pythium	Total emerged, both healthy and damped-off				% of 84 seeds sown that emerged (healthy or damped-off)			
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28
Untreated uninoculated	17.00b	28.33b	27.67b	28.00b	20.24b	33.73b	32.94b	33.33b
Untreated inoculated	7.67a	18.67ab	17.67ab	17.67ab	9.13a	22.22ab	21.43ab	21.03ab
AHDB 9848	2.33a	10.67a	11.33ab	11.33a	2.78a	12.70a	13.49ab	13.49a
AHDB 9715	3.33a	7.67a	8.33a	8.00a	3.97a	9.13a	9.92a	9.52a
Integral Pro	6.00a	12.67ab	13.67ab	11.33a	7.14a	15.08ab	16.27ab	13.49a
AHDB 9734	7.00a	18.67ab	18.00ab	17.33ab	8.33a	22.22ab	21.43ab	20.63ab
AHDB 9725	5.00a	8.33a	8.33a	8.00a	5.95a	9.92a	9.92a	9.52a
AHDB 9955	3.33a	15.00ab	15.67ab	15.00ab	3.97a	17.86ab	18.65ab	17.86ab
AHDB 9716	3.33a	6.00a	6.00a	6.00a	3.97a	7.14a	7.14a	7.14a
Grand mean	6.11	14.00	14.10	13.60	7.30	16.70	16.80	16.20
P.Fr	0.024	0.113	0.161	0.110	0.024	0.113	0.161	0.110
L.S.D.	7.57	14.97	15.06	14.36	9.01	17.82	17.93	17.10
D.F.	16	16	16	16	16	16	16	16

Table 18. Efficacy Experiment 2 - immediate Pythium contact: The number of healthy seedlings that had grown to true leaf at 14, 21 and 28 days from sowing.

2nd Efficacy expt. With immediate Pythium contact	Survival to true leaf Day 14	Survival to true leaf Day 21	Survival to true leaf Day 28
Untreated uninoculated	1.33	4.00	4.33
Untreated inoculated	0.33	1.33	1.00
AHDB 9848	0.00	3.00	3.00
AHDB 9715	0.00	0.00	0.00
Integral Pro	1.00	2.00	2.00
AHDB 9734	0.00	1.00	2.33
AHDB 9725	0.00	1.00	1.33
AHDB 9955	0.67	2.00	2.33
AHDB 9716	0.33	1.33	1.67
Grand mean	0.41	1.74	2.00
P.Fr	0.226	0.363	0.311
L.S.D.	1.201	3.262	3.251
D.F.	16	16	16

Uninoculated Observation trays alongside Efficacy Experiment 2

In cabinet CE01 a few seedlings had damped-off. However, the greatest loss of seedlings was pre-emergence in both cabinets, so that at most 40 out of 84 seeds per tray emerged (Table 19) and at worst zero (Table 20).

Table 19. Uninoculated, unreplicated, Observation Trays in Cabinet CE01 (alongside the delayed Pythium contact trays of Efficacy Experiment 2).
Weekly records of the number of emerged seedlings present that were damped-off and still visible at each weekly assessment and the total seedlings emerged.

2nd Efficacy expt. Cabinet 01. Uninoculated	Number emerged that then were damped-off on assessment				Total emerged, both healthy and damped-off out of 84 sown				True leaved
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28	Day 28
Untreated	0	0	0	0	18	34	40	39	18
Untreated	0	0	0	0	19	28	28	28	10
AHDB 9848	0	0	0	0	5	12	12	13	4
AHDB 9715	0	0	3	1	3	11	13	11	1
Integral Pro	0	2	0	0	3	5	0	3	3
AHDB 9734	0	0	1	1	12	22	23	23	13
AHDB 9725	0	0	0	0	5	21	22	22	10
AHDB 9955	0	1	0	1	1	6	5	6	3
AHDB 9716	0	0	0	0	5	9	9	9	4

Table 20. Uninoculated, unreplicated, Observation Trays in Cabinet CE02 (alongside the immediate Pythium contact trays of Efficacy Experiment 2).
Weekly records of the number of emerged seedlings present that were damped-off and still visible at each weekly assessment and the total seedlings emerged.

2nd Efficacy expt. Cabinet 02 Uninoculated	Number emerged that then were damped-off on assessment				Total emerged, both healthy and damped-off out of 84 sown				True leaved
	Day 7	Day 14	Day 21	Day 28	Day 7	Day 14	Day 21	Day 28	Day 28
Untreated	0	0	0	0	18	33	37	37	16
Untreated	0	0	0	0	8	21	16	16	12
AHDB 9848	0	0	0	0	5	11	11	11	6
AHDB 9715	0	0	0	0	2	9	9	9	7
Integral Pro	0	0	0	0	2	8	7	7	1
AHDB 9734	0	0	0	0	9	17	17	18	8
AHDB 9725	0	0	0	0	3	17	18	19	8
AHDB 9955	0	0	0	0	4	16	17	18	8
AHDB 9716	0	0	0	0	2	18	18	18	11

Although statistical comparisons cannot be made, in both cabinets the tray with the highest emergence had untreated seed, while treated trays had at most around half the number emerged. In both cabinets the standard, Integral Pro, was one of the products with the poorest emergence (Tables 19 and 20).

Discussion

In this study, both the lower concentration and higher inoculum concentrations tested had poor emergence in the first efficacy experiments. Unexpectedly, better emergence followed use of a lower inoculum concentration in the variable timing of contact efficacy experiments. For comparison, the AHDB FV464b study, found most spinach seedlings survived on top of punnets with a similarly low inoculum, but there was excessive damping off in a repeat trial with only a slightly higher concentration. These results show the difficulty obtaining consistent results when the colonies counted are variable sized mycelial fragments mixed in a solid substrate, rather than spores in liquid.

Emergence in the uninoculated treated seed trays of the efficacy experiments was poorer than in the germination tests on filter paper. Too little or too much moisture can be a factor in spinach seed germination (Liz Johnson, pers. comm.). One explanation for the poorer emergence of treated seed in the uninoculated observation plots compared to untreated could be that film coating, only on the treated seeds, could have adversely affected germination.

With the Pythium isolate that was used the main loss of seedlings was pre-emergence, with further being killed at the point of emergence. It was possible for seedlings in the same cells as killed seedlings to survive to true-leaf. Thus, as mentioned in AHDB FV367, getting seedlings to grow quickly to “escape” the mycelium is important and so treatments and growing conditions that facilitate fast seedling growth and do not favour pathogen movement in the soil are required.

Conclusions

- There were no phytotoxic or clear beneficial effects of any treatments on percentage seed germination (on filter paper) tested either immediately or 10 weeks after treatment, compared to untreated seed.
- The method of Pythium inoculum production and dilution developed in the bioassays allowed a range of colony forming unit concentrations to be tested for their damping-off severity.
- In Efficacy Experiment 1 (high vs low inoculum), few seedlings grew in inoculated trays, and it was therefore not possible to compare products.
- In Efficacy Experiment 2 (immediate vs delayed inoculum contact) more seedlings emerged than in Experiment 1. However, no product was better than the untreated inoculated even when tray contact with the inoculum was delayed by a couple of days to give the seeds a 'head-start' on germination before contact with mycelium.
- In the trays, no foliar phytotoxicity was seen from any of the seed treatments.

Acknowledgements

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https://openwetware.org/wiki/BISC209/F13:_Lab2 for instructions on dilution series and cfu /g dry weight calculations

Appendix

Appendix A - Bioassay experiments

Preliminary Bioassay

The preliminary bioassay trial consisted of four inoculation levels plus an uninoculated substrate control. There were no product treatments on the seed. The experiment sought to determine whether four or six agar plugs of Pythium should be used to inoculate “starter punnets”.

After a fortnight’s incubation, the “starter punnets” of the same plug number were mixed together and weighed out into punnets to go under the seed trays. The experiment used only a tenth of the weight of the 4 and 6-plug “starter punnets” to mix with nine tenths of uninoculated substrate for treatments T2, T4 and T7. For the other inoculated treatments, the whole “starter punnet” weight was used undiluted (see Inoculation Treatments table below). One each of the 4 and 6-plug “starter punnets” became contaminated by Penicillium and so was not used, thus T4 and T7 had no replication. On completion, the punnets all had 230 g of coir / oatmeal substrate before the addition of 23 g of sand (10% by weight).

A sown seed tray was placed on each of the punnets on the same day as the punnet mixing was completed. All trays were in the same controlled environment cabinet. The trial layout is shown below.

Trial design for Preliminary Bioassay

Preliminary bioassay, with untreated seeds	**Details**
Trial design:	Randomised block with 5 treatments
Number of replicates:	2 for T1, T2, T3 & T6. 1 for T4 & T7
Row spacing:	5 seeds within a 37 x 34 mm seed tray cell
Plot size: (w x l)	112 mmm x 165 mm
Plot size: (m^2)	0.012 m^2
Number of plants per plot:	60

Inoculation Treatments per punnet for Preliminary Bioassay

Treatment codes & inoculum levels for Prelim. Bioassay. All seed untreated.	**Wet weight of Pythium infested substrate**	**Wet weight of uninfested coir/oat substrate in punnet**
T1	0 g	230 g
T2 (Low 4-plug)	23 g of 4-plug starter	207 g
T3 (High 6-plug)	230 g of 6-plug starter	0 g
T4 (Low 6-plug)	23 g of 6-plug starter	207 g
T5 (High 4-plug)	230 g of 4-plug starter	0 g
T6 (High 6-plug)	230 g of 6-plug starter	0 g
T7 (Low 4-plug)	23 g of 4-plug starter	207 g

Layout of Preliminary Bioassay

The preliminary bioassay trial was originally intended to consist of a randomised block design with five treatments including an untreated control, replicated twice, but as some of the “starter” trays did not produce enough Pythium mycelium for this then it was redesigned. T4 had one replicate and an additional single replicate was set up for

T7. This observation trial was not put before a statistician. All seed was untreated. A range of inoculum levels were created under the seed trays for T2 to T7.

Back of cabinet

1	**T1**	2	**T3**	3	**T5**	4	**T4**	5	**T2**	6	**T6**
7	**T3**	8	**T7**	9	**T1**	10	**T6**	11	**T5**	12	**T2**

Front of cabinet

Preliminary bioassay results

Appendix Table 1. Preliminary Bioassay weekly counts per seed tray of the numbers of healthy or damped-off seedlings visible at that assessment

Preliminary bioassay inoculum treatments ("starter" content)	Number healthy seedlings emerged out of 60 sown & present at each assessment			Number of damping-off seedlings visible at each assessment out of 60 sown		
	Day 7	Day 13	Day 21	Day 7	Day 13	Day 21
T1 Uninoculated	12	29	38	0	0	0
T1 Uninoculated	22	40	36	0	1	1
T2 4 plug L (tenth)	15	2	2	6	23	21
T2 4 plug L (tenth)	4	3	3	0	2	2
T3 6 plug H (whole)	22	13	13	0	7	7
T3 6 plug H (whole)	3	5	3	0	0	2
T4 6 plug L (tenth)	5	2	2	0	0	2
T5 4 plug H (whole)	12	3	6	6	15	11
T5 4 plug H (whole)	6	8	9	0	4	2
T6 6 plug H (whole)	6	2	1	0	2	5
T6 6 plug H (whole)	2	2	2	0	0	0
T7 4 plug L (tenth)	0	40	2	1	1	1

Seedlings emergence in the uninoculated trays was 66% (40 out of 60 seeds), whereas in the inoculated trays at most 22 healthy seedlings per tray were present at any assessment timing (Appendix Figure 1). Post-emergence damping-off was minimal in all but two of the inoculated trays where it reached 35% of those sown. Neither poorer emergence nor greater damping off were consistently associated with the use of undiluted whole trays of "starter" inoculum (Appendix Table 1).

Appendix Figure 2 shows a typical example of a seed tray after 16 days stood on Pythium infested coir. The majority of seedlings did not emerge. Some seedlings that grew swiftly damped-off from Pythium infection (as shown in the central cells).

Colony counts of Pythium mycelial fragments confirmed that different concentrations had been achieved, with the use of one tenth of a "starter punnet" resulting on between 52 to 348 cfu/ g dry substrate, and undiluted ranging from 5220 to 11606 cfu / g dry weight (5.2×10^3 to 1.2×10^4).

Appendix Figure 1. Preliminary bioassay. Trays of spinach seedlings on punnets of coir / oatmeal / sand substrate. The two trays with many seedlings were uninoculated (T1). The other ten trays show pre- and post-emergence damping-off by Pythium introduced at different concentrations. Seeds sown 3 August, photographed on 26 August 2022.

Appendix Figure 2. Preliminary bioassay T2 (4-plug low weight inoculum) tray of plot 5 in the back row of the cabinet on 26 August 2022, 16 days after sowing.

From this preliminary bioassay it was determined that the use of a whole “starter punnet” was unnecessary for further experiments as there was substantial seedling loss when one tenth of a “starter punnet” had been used. The use of six plugs was adopted for future experiments as the resulting faster covering by Pythium mycelium of the substrate in the “starter punnet” compared with four plugs could reduce the opportunity for substrate colonisation by contaminants such as Penicillium during Pythium incubation.

Main Bioassay

The main bioassay utilised the information from the completed preliminary bioassay on the mixing of inoculum and the resulting concentration of colony forming units of Pythium & damping-off of the seedlings.

The main bioassay consisted of five inoculation levels plus an uninoculated substrate control. There were no product treatments on the seed. Punnets to place under the seed trays received the stated weights of Pythium inoculum from combined "starter punnets". On completion, the punnets all had 230 g of coir / oatmeal substrate before the addition of 23 g of sand (10% by weight). A sown seed tray was placed on each of the punnets on the same day as the punnet mixing was completed. All trays were in the same controlled environment cabinet. The layout is shown below.

Trial design for Main Bioassay

Main bioassay, with untreated seeds	Details
Trial design:	Randomised block with 6 treatments
Number of replicates:	3
Row spacing:	9 seeds within a 37 x 34 mm seed tray cell
Plot size: (w x l)	112 mmm x 165 mm
Plot size: (m^2)	0.012 m^2
Number of plants per plot:	108

Inoculation Treatments, substrate ratios per punnet for Main Bioassay

Treatment codes for Main Bioassay	Wet weight of Pythium infested substrate (g)	Wet weight of uninfested coir/oat in punnet (g)
T1 uninoculated untreated	0	230
T2 untreated	2	228
T3 untreated	4	226
T4 untreated	8	222
T5 untreated	16	214
T6 untreated	24	206

23 g dry weight of sand subsequently added to each tray

Layout of Main Bioassay

The main bioassay trial consisted of a randomised block design with six treatments including an untreated control, replicated three times. This observation trial was not put before a statistician. All seed was untreated. A range of increasing inoculum levels were used in punnets under the seed trays for T2 to T6.

Back of cabinet

1	**T2**	2	**T5**	3	**T4**	4	**T3**	5	**T1**	6	**T6**
7	**T4**	8	**T1**	9	**T3**	10	**T5**	11	**T6**	12	**T2**
13	**T3**	14	**T6**	15	**T2**	16	**T1**	17	**T4**	18	**T5**

Front of cabinet

Main bioassay results

Appendix Table 2. Main Bioassay inoculated substrate weights & Pythium mycelial colony counts. Weekly counts of healthy & damped-off seedlings visible in each seed tray

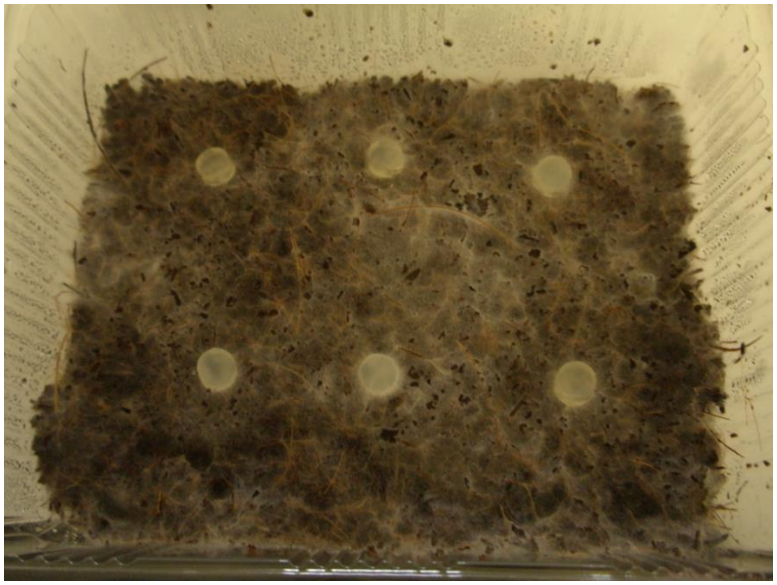
Treatment & weights of starter" added to punnets	Main Bioassay assessments for three replicates of each inoculum concentration used with untreated seeds				
	Pythium colonies in dry coir/oat/ sand	Number of healthy seedlings visible at each assessment out of 108 sown		Number of damping-off seedlings visible at each assessment out of 108 sown	
	Cfu / g	Day 7	Day 14	Day 7	Day 14
T1 0 g	0	86	98	0	0
		77	96	0	1
		77	97	0	0
T2 2 g	3.5×10^3	72	88	0	4
		65	86	0	3
		55	78	0	13
T3 4 g	1.3×10^4	70	87	0	8
		87	79	0	15
		53	85	0	4
T4 8 g	4.9×10^4	79	83	0	11
		53	76	0	10
		72	89	0	1
T5 16 g	8.2×10^4	79	95	0	4
		83	68	0	20
		62	64	0	24
T6 24 g	6.5×10^4	88	88	0	13
		74	64	0	22
		81	69	3	29

Main bioassay experiment results

This bioassay sought to further determine the concentration of inoculum to be used as a higher and lower challenge for the seed treatments in the subsequent efficacy experiments. The one tenth (24 g) "Low" dilution of "starter punnet" used in the preliminary bioassay was compared against dilutions of 16 g, 8 g, 4 g and 2 g of inoculated 6-plug "starter punnet" substrate. The "starter trays" had a good covering of Pythium mycelium when used (Appendix Figure 3).

More seeds were sown, and proportionally many more seedlings emerged across the Main Bioassay experiment (Appendix Figure 4) than in the Preliminary Bioassay, with up to 90% emergence. The cfu counts confirmed that different mycelium concentrations had been achieved, with some counts rising above those in the earlier bioassay. As in the Preliminary Bioassay, there was noticeable variation between replicate trays (see Appendix Table 2). The use of either 16g or 24 g of "starter punnet" gave the most post-emergence damping-off of up to 26.9% of those sown.

As a result of this experiment for the following efficacy experiments 8 g wet weight of Pythium infested coir/oatmeal from the “starter punnet” in 222 g of uninoculated coir/oatmeal was selected as the lower level of inoculum and the 24 g weight for the higher level of inoculum.



Appendix Figure 3. A “starter punnet” on 31 August 2022 14 days after agar plug placement. Showing Pythium mycelium grown to cover the substrate surface. The Pythium mycelium and substrate was broken apart and set weights used in the Main Bioassay.

Appendix Figure 4. Spinach seedlings growing in inoculated and uninoculated treatments in the Main Bioassay on 16 September 2022.

Appendix B - Efficacy Experiments

Layout of First Efficacy Experiments

For the first run of the efficacy experiments, each experiment was a three replicate randomised block design (see below) each in a separate cabinet running concurrently. All but T1 & T2 were treated and only T1 was not exposed to Pythium.

Back of cabinet **CE02** top shelf. Higher inoculum level Replicates 1 & 2

1	**T2**	2	**T5**	3	**T7**	10	**T2**	11	**T4**	12	**T3**
4	**T8**	5	**T3**	6	**T9**	13	**T5**	14	**T1**	15	**T6**
7	**T1**	8	**T4**	9	**T6**	16	**T8**	17	**T9**	18	**T7**

Front of cabinet top shelf

Back of cabinet **CE02** bottom shelf. Higher inoculum level Replicate 3

19	**T3**	20	**T7**	21	**T4**			
22	**T2**	23	**T8**	24	**T5**			
25	**T9**	26	**T6**	27	**T1**			

Front of cabinet bottom shelf

Back of cabinet **CE01** top shelf. Lower inoculum level Replicates 1 & 2

1	**T7**	2	**T8**	3	**T3**	10	**T6**	11	**T2**	12	**T8**
4	**T2**	5	**T1**	6	**T6**	13	**T9**	14	**T5**	15	**T1**
7	**T5**	8	**T4**	9	**T9**	16	**T7**	17	**T3**	18	**T4**

Front of cabinet top shelf

Back of cabinet **CE01** bottom shelf. Lower inoculum level Replicate 3

19	**T8**	20	**T1**	21	**T7**			
22	**T9**	23	**T6**	24	**T4**			
25	**T5**	26	**T2**	27	**T3**			

Front of cabinet bottom shelf

Layout of Second Efficacy Experiments

For the second run of the efficacy experiments, each experiment was a three replicate randomised block design in separate cabinets (CE01 and CE02) with the lower (delayed) and higher (immediate) Pythium exposure trials running concurrently. All but seeds ofT1 & T2 were treated and only T1 was not exposed to Pythium.

Cabinet CE02 top shelf – Replicates 1 & 2 of un-delayed Pythium exposure (Higher challenge)

1 **T3**	2 **T7**	3 **T2**	10 **T5**	11 **T6**	12 **T9**
4 **T5**	5 **T8**	6 **T9**	13 **T3**	14 **T1**	15 **T4**
7 **T1**	8 **T4**	9 **T6**	16 **T2**	17 **T8**	18 **T7**

Front of cabinet top shelf

Cabinet CE02 bottom shelf – Replicate 3 of Higher Pythium challenge. Observation plots 28 - 36 (shaded) with treated seed in T3 to T9 but no inoculum in any of the punnets.

19 **T4**	20 **T8**	21 **T1**	28 **T6**	29 **T1**	30 **T7**
22 **T2**	23 **T6**	24 **T3**	31 **T9**	32 **T5**	33 **T4**
25 **T7**	26 **T9**	27 **T5**	34 **T8**	35 **T2**	36 **T3**

Front of lower shelf of cabinet

Cabinet CE01 top shelf. Replicates 1 & 2 of delayed Pythium (Lower challenge)

1 **T8**	2 **T2**	3 **T6**	10 **T7**	11 **T5**	12 **T1**
4 **T5**	5 **T1**	6 **T3**	13 **T9**	14 **T2**	15 **T4**
7 **T4**	8 **T7**	9 **T9**	16 **T3**	17 **T8**	18 **T6**

Front of cabinet top shelf

Cabinet CE01 bottom shelf. Replicate 3 of Lower Pythium challenge. Observation plots 28-36 (shaded) with treated seed in T3 to T9 but no inoculum in any of the punnets.

19 **T9**	20 **T3**	21 **T4**	28 **T1**	29 **T7**	30 **T6**
22 **T6**	23 **T5**	24 **T7**	31 **T3**	32 **T5**	33 T9
25 **T2**	26 **T1**	27 **T8**	34 **T8**	35 **T4**	36 **T2**

Front of cabinet bottom shelf

Controlled Environment

Data logger readings from within a controlled environment cabinet set at 20°C (blue, lower lines) and 60% humidity (orange upper chart lines) with 16 h light : 8 h dark. Data logger was placed at the same level as the seed trays.

The chart shown was the conditions in a controlled environment cabinet during seed tray growth of the second efficacy experiments, with similar conditions maintained in each of the experiments in this project. The dips in humidity arose when the cabinet was opened to either examine or water the plants, and set conditions returned after the door was shut.

ORETO certificate

Certificate of

Official Recognition of Efficacy Testing Facilities or Organisations in the United Kingdom

This certifies that

RSK ADAS Ltd

complies with the minimum standards laid down in Regulation (EC) 1107/2009 for efficacy testing.

The above Facility/Organisation has been officially recognised as being competent to carry out efficacy trials/tests in the United Kingdom in the following categories:

Agriculture/Horticulture
Stored Crops
Biologicals and Semiochemicals

Date of issue: 1 June 2018
Effective date: 18 March 2018
Expiry date: 17 March 2023

Signature Alison Richardson
Authorised signatory

Certification Number
ORETO 409

HSE
Chemicals Regulation Division

Department of Agriculture and Rural Development