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Field assessment of formulations of *Bacillus mycoides* MW 27 for control of *Aphanomyces euteiches* root rot of pea, and assessment of selected spore-forming bacteria for control of *Pythium ultimum* damping-off of lettuce

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1. Summary

Oomycete plant pathogens cause diseases on a wide range of vegetable crops in New Zealand. They are traditionally more difficult to control than non-Oomycetous fungi, as they are not susceptible to most conventional fungicides. Recently, a number of reports have emerged in the scientific literature pertaining to biological control of these fungi using microbial antagonists. We are investigating the potential for biological control of two Oomycete plant pathogens which are important in New Zealand; Aphanomyces root rot of pea, caused by *Aphanomyces euteiches*, and damping-off of lettuce, caused by *Pythium ultimum*.

Previous work funded by FAR and VegFed has demonstrated the potential of a strain of *Bacillus mycoides* (MW 27) for the control of Aphanomyces root rot of pea. In conjunction with AgResearch, we developed several formulations of the bacterium for testing in a field trial. Key findings were:

two distinct diseases were evident: damping-off and root-rot
damping-off reduced the plot stand while root-rot reduced plant yield
a seed coat formulation of *B. mycoides* significantly increased plot stand from 77.22% to 79.44%
a prill formulation of *B. mycoides* significantly increased yield from 14.37 g plant $^{-1}$ to 16.86 g plant^{-1}
Bacillus mycoides MW 27 was the only treatment to significantly increase yield on a plant-by-plant basis
the fungicide control, Apron, increased plot stand (damping-off control), but was ineffective at increasing yield (root rot control)
all formulations of *B. mycoides* MW 27 were stable in storage under ambient conditions for at least 70 days after manufacture

Seed coat preparations of several spore forming bacteria were assessed for control of *Pythium ultimum* damping-off of lettuce. Conditions in the glasshouse and growth cabinet trials were too conducive for disease and variation between replicate treatments too great to detect any beneficial effects. Suggestions are made for improving the trial protocols.

2. Introduction

Plant pathogenic Oomycete fungi occur in agricultural soils worldwide, but are more common in countries such as New Zealand which have a temperate climate (Martin, 1992). They cause disease on a wide range of host plant species (Domsch et al., 1980; Martin, 1992) including many high value crops. Diseases caused by Oomycete fungi are particularly severe in cool, moist, heavy soils with high levels of organic material.

The control of Oomycete fungi is much more difficult than with the 'true' fungi. For the most part, this is due to their susceptibility to only a narrow range of fungicides (Bruin and Edgington, 1983), such as the acylalinine-type of chemicals. However, because these chemicals are expensive, their use is often uneconomic for low-value or broad acre crops, and the use of soil fungicides is currently causing concern because of toxicity to non-target organisms and disruption to soil ecosystems. Furthermore, certain Oomycete pathogens, such as *Aphanomyces euteiches*, are not susceptible to any registered fungicides.

Recently, there have been several examples of biological control of Oomycete plant pathogens reported in the scientific literature. For example, Hwang *et al.* (1996) demonstrated the control of *Pythium ultimum* and *P. irregulare* Buisman damping-off of pea in field trials using *Bacillus subtilis* and *Paenibacillus polymyxa* Paul *et al.* (1995) demonstrated control of *P. mamillatum* Meurs damping-off of cucumber with a strain of *B. mycoides* in the glasshouse, and *B. cereus* has been shown to control *P. aphanidermatum* (Edson) Fitz. on cucumber (Smith *et al.*, 1993).

As this class of bacteria have clear potential for control of these types of disease, we are investigating their potential for biological control of two Oomycete diseases of economic importance in New Zealand: Aphanomyces root rot on pea, caused by *Aphanomyces euteiches*, and damping-off of lettuce, caused by *Pythium ultimum*. This report describes a field trial assessing the effect of different formulations of *Bacillus mycoides* MW27 on Aphanomyces root rot disease of peas; the result of over 3 years of research into the biological control of the disease using spore forming bacteria. In addition, preliminary glasshouse and growth chamber assays are described, investigating the potential for control of damping-off of lettuce.

3. Materials and Methods

3.1 Field trial assessment of *Bacillus mycoides* for control of pea root rot disease

3.1.1 Trial location and date

The field trial was carried out on a high *A. euteiches* indexed field on a farm at Punawai, mid-Canterbury (S 43°46'40", E 171°35'06") on an Eyre stony silt loam soil. The trial was planted on the 4th of October 2000 and recovered on the 27th of December 2000.

3.1.2 Treatments

The following treatments were assessed for control of pea root rot disease:

1 Nil control – surface disinfected seed only
2 Apron C 70 SD - fungicide
3 Carrier control – carrier in the Lincoln University seed coat formulation
4 *B. mycoides* MW 27 seed coat formulation: Lincoln University
5 *B. mycoides* MW 27 seed coat formulation: AgResearch
6 *B. mycoides* MW 27 prill formulation: AgResearch
7 *B. mycoides* MW 27 granule formulation: AgResearch

Prior to treatment, all pea seeds (c.v. Dwarf Massey) were surface-disinfected by immersing in 0.5% NaOCl for 5 min followed by three washes with sterile distilled water.

Apron C 70 SD (Ciba-Geigy; 350 g Kg^{-1} metalaxyl and 350 g Kg^{-1} captan) was applied at 2 g Kg^{-1} seed.

The Lincoln seed coat formulation was made using procedures developed during previous field trial tests (Stewart *et al.*, 2000). The bacterium was cultured onto 30 Nutrient agar (NA) +Mn Petri dishes for 14 d at 27°C (Priest, 1989) and the resultant growth recovered into phosphate-buffered saline (PPBS; pH 7.0; 6% NaCl) and freeze dried. The bacterial powder was mixed with 20% (w/w) of a carrier consisting of 8:1:1 of $CaCO_3$:methyl-cellulose:glucose. Chalk talc, $CaCO_3$, was included based on its apparent stimulatory effect in a previous field trial (Stewart *et al.*, 2000). Methyl-cellulose was used as a sticking agent and glucose as an initial carbon source for the bacterium. In addition, a treatment consisting of the carrier only was included as a control.

Three formulations of the bacterium were generated by AgResearch New Zealand Ltd. (AgResearch), Lincoln. The bacterium was grown on five NA Petri plates (as before) for 7 d at 27°C. The resultant growth was recovered into PPBS and used to inoculate six, 2 L flasks, each containing 500 mL of Nutrient broth. The flasks were incubated in the dark at 27°C for 2 weeks and the resultant bacterial growth concentrated into 200 mL by centrifugation (4 500 × g for 45 min). The concentrated solution, containing 2×10^8 colony forming units (c.f.u.) mL^{-1} was supplied to Dr Von Johnson, AgResearch, for formulation into prills (Figure 1), granules and as a seed coat using propriety technology (New Zealand Patent Nos. NZ506484, NZ506485,

NZ506486, NZ506487, and NZ506488). Prills and granules (0.1 g) were placed in the planting hole immediately below surface-disinfected pea seeds.

3.1.3 Determination of bacterial colony forming units

The numbers of c.f.u. per seed or gram of prill or granule formulations were determined. In each case, either five pea seeds or 1 g of prill or granule formulation, were placed into a Universal bottle containing 10 mL of PPBS amended with Tween 80 (polyoxyethylene (20) sorbitan mono-oleate; BDH Limited) at 2 drops L^{-1}. Samples were mixed thoroughly by vortexing for 1 min. A series of dilutions was made from each treatment (to 10^{-6}) and 0.1 mL aliquots of each dilution spread onto three half-strength NA Petri plates. The plates were incubated in the dark at 30°C and the number of bacterial colonies on each plate was counted the following day.

The numbers of c.f.u. of bacteria in the prill and granule formulations of *B. mycoides* MW 27 were repeatedly determined over a 70 day period to determine their shelf life / longevity.

3.1.4 Experiment design

The field trial was set-up using a complete randomised blocking design. Each treatment was replicated once in each of five blocks. Each treatment replicate consisted of 36 pea seeds planted in a 6 × 6 grid at a depth of 5 cm. A 10 cm spacing was left both above and below between each seed. The distance between seedlings in each treatment was at least 20 cm.

3.1.5 Preparation, cultivation and maintenance

The soil at the trial site was cultivated to a depth of approximately 20 cm using a rotary-hoe (Figure 2). Up to six passes over each area was necessary to till the soil to a suitable texture. A rake was used to level the planting bed and remove remaining large soil particles immediately prior to planting.

Bird netting was erected on scaffolding over the trial site (Figure 2) to protect the plants from bird and rabbit damage. Slugs and snails were controlled using Mesurol (20 g Kg^{-1} methiocarb; Bayer NZ Ltd.). Galant (100 g L^{-1} haloxyfop; DowElanco) was sprayed over the trial site 3 weeks after planting to control grass weeds and MCPB (phenoxy butyric 385 g L^{-1}; DowElanco) was sprayed 6 weeks after planting to control broad-leaf weeds.

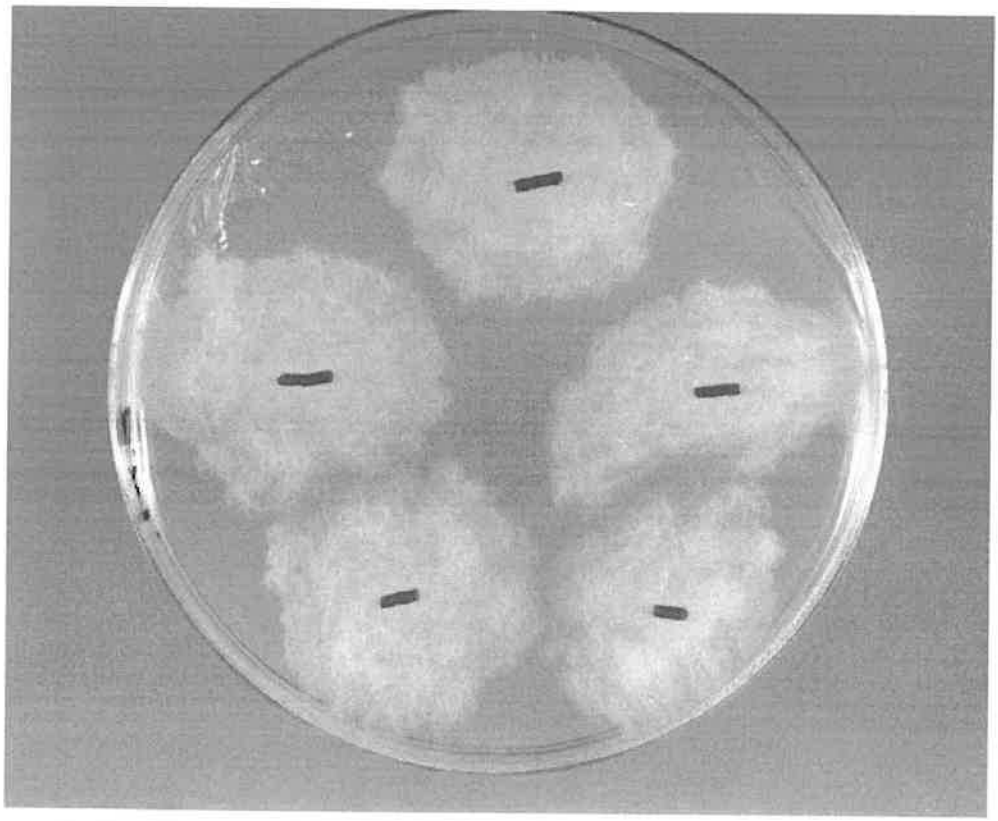

Figure 1: *Bacillus mycoides* MW 27 growing from prills produced by Dr Von Johnson, AgResearch.

Figure 2: Field trial at Punawai. Bird netting was erected over the trial site as protection against bird and rabbit damage. Beds were prepared for planting by rotary-hoeing.

3.1.6 Data collection and analysis

Assessments were made of the percentage plot stand, plant length, numbers of pods per plant, yield of pods per plant, and the total yield per plant for each treatment replicate.

The percentage plot stand, i.e. the number of plants which had emerged as a percentage of those that were originally planted (36), was determined 21 days after planting.

The plant length (cm), at maturity, was measured for the above ground portion of the plant only.

The number of pods per plant and the pod weights were determined at maturity (i.e. when the pods were green and ripe for most plants).

The total plot yield was calculated as follows: percentage germination per plot was multiplied by the number of seeds planted per plot (36) which was multiplied by the average yield per plant. The resultant value, therefore, takes into account both the effect of plant yield and plant stand on overall potential yield.

Analysis of variance (ANOVA) was used to determine if pea seed treatment significantly affected the above factors. Duncans' multiple range test was used as the *post hoc* method to separate treatment means.

Pearsons correlation co-efficients were determined between factors.

3.2 Use of spore-forming bacteria for the biological control of *Pythium ultimum* damping-off of lettuce

Two trials were conducted to determine if spore-forming bacteria could reduce *Pythium ultimum* damping-off disease of lettuce. In the first trial, six bacterial isolates were assayed together under glasshouse conditions. The two best isolates were then tested more thoroughly in a subsequent growth cabinet trial.

3.2.1. Preparation of pathogen inoculum

Inoculum of the damping-off pathogen *P. ultimum* was produced by Dr N. Rabeendran, Lincoln University as part of a broader study. An isolate of the fungus *P. ultimum* taken from the Lincoln University fungal culture collection was grown onto corn-meal agar at 20°C in the dark for 5 d. Small, 2 mm square sections were taken from the growing edge of the culture, and 5-8 pieces placed into 26 deep Petri dishes each containing approximately 40 mL of V8-Juice media amended with $CaCO_3$ at 3 g L^{-1} and cholesterol at 30 mg L^{-1}. The dishes were incubated at 20°C with diurnal lighting for 20 d, after which the mycelial mats were recovered and macerated in a Waring Blender for 2 min to release the oospores into the medium. The resultant slurry was filtered through a double-layer of cheesecloth and the number of oospores in the liquid counted under a haemocytometer.

For the first trial, carried out in the glasshouse, the oospore concentration was adjusted to 1.17×10^5 spores mL^{-1} of solution with the addition of distilled water. The oospore suspension was then used to inoculate seedling germination mixture to give a final concentration of 1.17×10^4 spores g^{-1}.

As the disease pressure in the first trial was both too high and too variable within the trial, the preparation of inoculum for the second trial was slightly modified. The level of pathogen inoculum was reduced to 4×10^3 spores g^{-1} of potting mixture, and was mixed more thoroughly than before by tumbling in a concrete-mixer for 5 min followed by turning by hand.

3.2.2. Preparation of biological control inoculum

After recovery from storage at –80°C, the bacterial isolates *B. mycoides* MW 27, *B. pumilus* PT 10, *B. pumilus* PT 1, and *B. subtilis* PT 69 were each cultured onto ten NA plates, and *P. polymyxa* 18·25 was cultured onto ten PDA plates. After 14 d at 23°C, the bacterial growth for each isolate was scraped from the Petri dishes into sterile PPBS and the bacterial spores collected by centrifugation (10 000 × g for 30 min). The pellets of spores were freeze-dried and ground into a powder using a mortar and pestle. Pelleted lettuce seeds (c.v. Casino; Yates, commercially pelleted with clay to 3.25–4 mm diam.; Wrightson Ltd., Christchurch) were moistened with water and coated with the bacterial powder by shaking in a plastic bag. The seeds were then dried overnight in a laminar flow cabinet and planted the following day.

3.2.3. Control treatments

As well as the bacterial treatments, two control treatments were included in each experiment: a pathogen control, in which non-treated lettuce seeds were planted into pathogen-infested potting mixture, and a nil-pathogen control, in which non-treated seeds were planted into potting mixture in which no pathogen had been added.

A fungicide seed treatment was included in the growth cabinet trial. Captan (Orthocide® WDG; Chevron Chemical Co.) was applied to pelleted lettuce seeds at the recommended rate (100 g 500 mL^{-1} water 100 Kg^{-1} seed).

3.2.4. Determination of colony forming units per seed

The c.f.u. $seed^{-1}$ was determined for all treatments in the growth cabinet trial (only). Ten seeds from each treatment were placed into a Universal bottle containing 10 mL PPBS amended with Tween 20 (2 drops L^{-1}). After standing for at least 30 min to soften the clay pellet surrounding the seeds, the Universal bottles were twice vortexed for 2 min with a 10 min interval. A series of dilutions was made from the bottle by spreading 0.1 ml aliquots across the surface of NA plates. The Petri dishes were incubated overnight at 25°C, and the number of bacterial colonies counted.

3.2.5. Experiment design

Both the glasshouse and growth cabinet trials were set up using similar experimental designs, the number of blocks being the only difference: five were used in the glasshouse trial and six in the growth cabinet trial. Each treatment was pseudo-

replicated twice in each block. Each pseudo-replicate consisted of a germination tray filled with 0.5 L of pathogen-infested potting mixture (except for the nil-pathogen control) into which 25 lettuce seeds were planted (Figure 3).

Each germination tray was placed in a 2 L plastic 'ice-cream' container (Figure 3). Water was maintained to an approximate depth of 3 cm in the container to promote pathogenic activity.

The growth chamber was maintained at 20°C with a relative humidity of 65–70%. Lighting was diurnal (12 h : 12 h) at an intensity of 720 $\mu m\ m^{-2}\ sec^{-1}$.

Conditions in the glasshouse were not controlled, other than by opening the door during the day to reduce the temperature.

3.2.6. Data collection and analysis

The number of lettuce seedlings present in each tray was counted at 7 and 14 d after planting. The average germination score was calculated for each treatment per block (i.e. between the two pseudo-replicates per block). Statistical analysis was carried out using the ANOVA procedure. Fishers LSD was used as the *post hoc* test.

4. Results

4.1. Field trial assessment of formulations of *B. mycoides* MW 27

Three formulations of *B. mycoides* MW 27 were produced by AgResearch: a prill which contained 1.5×10^6 c.f.u. $gram^{-1}$, a granule containing 4.3×10^5 c.f.u. $gram^{-1}$ and a seed coat formulation which had 4×10^4 c.f.u. $seed^{-1}$. The seed coat formulation generated at Lincoln University was found to have 6.7×10^7 c.f.u. $seed^{-1}$. The number of viable bacterial cells (c.f.u. counts) in each of these formulations was found to be stable for at least 70 days after the initial manufacture (Figure 4). As the Lincoln University seed coat formulation had a high component of carrier (consisting of $CaCO_3$, methyl-cellulose and glucose), a carrier only control was included in the trial and was found to have 4.9×10^4 c.f.u. $seed^{-1}$ of unidentified bacteria. The use of methyl-cellulose in previous trials did not result in any bacterial contamination, therefore, the contaminating bacteria probably originated from the glucose or $CaCO_3$ carrier components. The fungicide Apron and a nil-control were also included as experimental controls.

Following lack of significance when measuring visual root rot disease symptoms (Stewart *et al.*, 2000), and the realisation that other factors such as plant length are closely associated with root rot (Hagedorn, 1984) but are both easier to measure and are truly quantitative, this factor was not assessed in the current trial.

Overall, the treatments resulted in significant differences in plant stand ($P<0.01$), the number of pods per plant ($P<0.01$), the yield of pods per plant ($P<0.01$), the total plot yield, ($P<0.01$), and nearly significant differences in plant length ($P=0.0504$).

The *B. mycoides* MW 27 prill formulation was the most effective treatment at increasing the number of pods per plant and increasing the yield of pods per plant, both of which were found to be significantly higher ($P \leq 0.05$) than the nil-control treatment. This treatment, however, did not have a significant effect on plant stand ($\alpha=0.05$) compared with the nil-control. Instead, peas treated with the fungicide treatment, Apron, or the AgResearch seed coat formulation of *B. mycoides* MW 27, were the most effective, increasing the percentage stand from 77.22% (nil-control) to 92.78% and 86.11%, respectively.

When the yield data was combined with the plot stand data to produce a theoretical 'yield of pods per plot' factor, both Apron and the *B. mycoides* MW 27 prill treatments were found to significantly increase yield compared with the nil-control. In the case of the fungicide treatment, the yield increase was predominately the result of an increased plot stand while that of the *B. mycoides* MW 27 prill was through yield per plant-based increases. Compared with the nil-control, therefore, the Apron treatment increased plot yield from 399.62 g $plot^{-1}$ to 503.15 g $plot^{-1}$, and *B. mycoides* MW 27 prill treatment to 482.20 g $plot^{-1}$. Under different disease pressure conditions, for example higher root rot and lower damping off, the relative rankings of these two treatments would be likely to swap.

The increase in plot stand resulting from the AgResearch-based seed coat formulation of *B. mycoides* MW 27 was not great enough to result in significantly higher overall plot yield increases relative to the nil-control, but was significantly better than the Lincoln seed coat formulation ($\alpha=0.05$).

Table 1: Results of Aphanomyces root rot field trial – testing formulations of *B. mycoides* MW 27

Treatment	c.f.u.[1]	Percentage plot stand[2]	Length (cm)	Pods per plant	Pod weight (yield) per plant (g)	Yield of pods per plot (g)[3]
Apron	870	92.78 a	39.21	2.78 b	15.07 ab	503.15 a
B. mycoides prill	1.5×10^6	79.44 bc	37.49	3.26 a	16.86 a	482.20 ab
B. mycoides SC A[4]	4.0×10^4	86.11 ab	37.28	2.77 b	14.13 bc	437.96 abc
B. mycoides granule	4.3×10^5	81.67 bc	39.08	2.76 b	14.30 b	420.28 bcd
Control	4.0×10^1	77.22 c	38.03	2.59 b	14.37 b	399.62 cd
Carrier[5]	4.9×10^4	83.33 bc	37.53	2.65 b	13.41 bc	426.32 cd
B. mycoides SC L[4]	6.7×10^7	83.33 bc	33.62	2.41 b	11.83 c	355.03 d
Duncans critical range		8.629		0.4587	2.529	76.42

[1] – Colony forming units per seed or gram of prill or granule
[2]- Percentage seedlings present per plot after 21 days from an initial planting of 36 seeds.
[3]- Yield of pods per plot: Average yield per plant multiplied by the percentage
[4]- SC L – Lincoln seed coat formulation; SC A – AgResearch seed coat formulation
[5]- Carrier control (for the *B. mycoides* SC L treated seeds only)
Values based on the average of 20 plants per replicate, 5 replicates per treatment.
Means within columns which are followed by the same letter are not significantly different at α=0.05 using Duncan's Multiple Range Test

Table 2: Pearson correlation coefficients for plant length, number of pods per plant and the yield per plant measured in the Aphanomyces field trial at Punawai.

	Length	Pods / plant	Yield / plant	Yield per plot[1]
Stand[1]	r^2=0.184	r^2=-0.028	r^2=-0.028	r^2=0.488
	P=0.291	P=0.426	P=0.871	P=0.003
Length		r^2=0.403	r^2=0.579	r^2=0.612
		P=0.0001	P=0.0001	P=0.0001
Pods / plant			r^2=0.802	r^2=0.668
			P=0.0001	P=0.0001
Yield / plant				r^2=0.856
				P=0.0001

Pearson correlation coefficients / Prob > |R| under Ho: Rho=0.
[1] – Correlations involving stand or yield per plant as factors are based on 35 observations (i.e. resulting from plot averages). All other observations based on 875 observations (plant by plant basis).

Correlation analysis (Table 2) showed that there was no significant association between the plot stand and any of the other parameters measured except for the yield per plot. In this case, the association related to the use of the plot stand in the determination of the yield value. All other factors, however, were strongly correlated. Increase in plant length was associated with an increase in the number of pods per plant and, not surprisingly, increases in either of these factors were associated with increasing yield values.

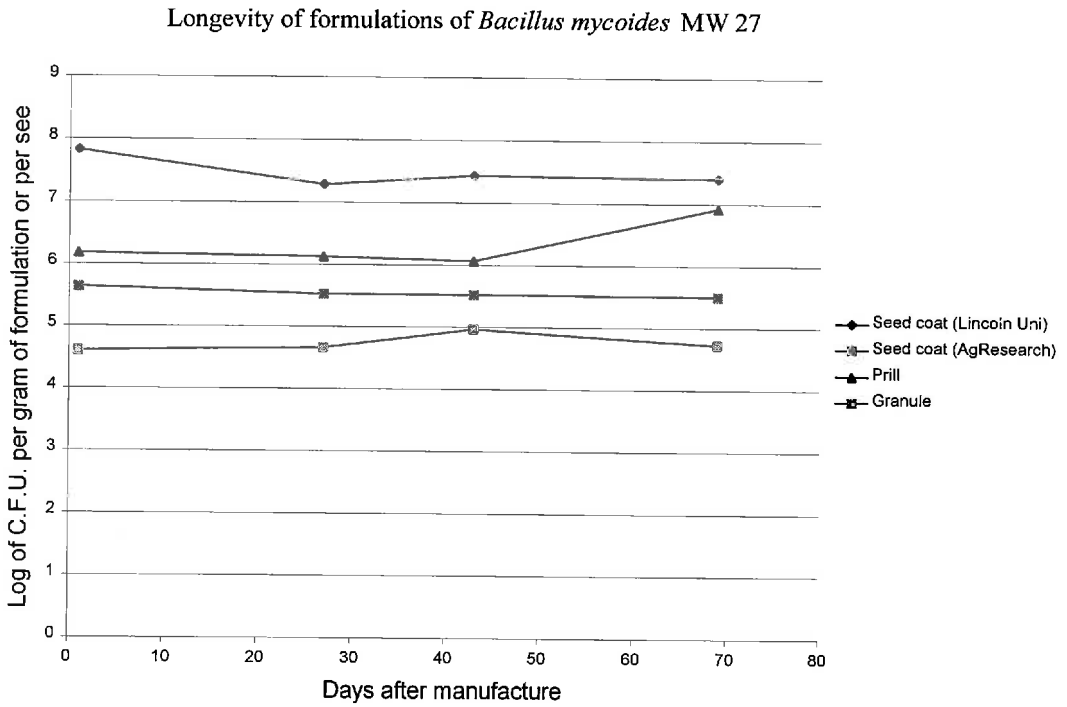

Figure 4.: Longevity of different formulations of the bacterium *B. mycoides* MW 27 used in the Aphanomyces root-rot field trial.

4.2. Pythium ultimum damping-off of lettuce

Differences existed between treatments at both the week 1 and week 2 assessment dates (P=0.0001). At both assessment dates, lettuce seeds planted in potting mixture containing no added *P. ultimum* had significantly higher ($P \leq 0.05$) seedling counts than all other treatments (Table 3). Seed treatment with spore-forming bacteria did not increase germination compared to the pathogen-only control ($\alpha=0.05$). Treatment with *B. subtilis* PT 69 and *B. pumilus* PT 10 reduced germination in lettuce relative to all other treatments at the week 2 assessment ($\alpha=0.05$). The only bacterial treatment to increase the germination relative to the nil-control was *P. polymyxa* 18·25. However, the increase was too small (only 3.6 % at week 2), and variability within the experiment too high, to result in statistical levels of significance (Table 3).

Table 3: Effect of treating lettuce seeds with different spore-forming bacteria on germination rates when planted into potting mixture infested with the damping-off fungus, *Pythium ultimum*.

Treatment	Seedling count[1]			
	Week 1		Week 2	
No pathogen	21.0	a	21.0	a
Pathogen only	8.4	bc	12.8	b
P. polymyxa 18·25	11.1	b	13.7	b
B. mycoides MW 27	8.4	bc	11.6	b
P. macerans PT 1	6.7	cd	10.1	b
B. subtilis PT 69	3.1	d	5.9	c
B. pumilus PT 10	2.8	d	5.1	c
LSD (α=0.05)	4.118		4.084	

[1] – average for five blocks, each block containing two pseudo-replicated trays of 25 seeds each.
Treatment means followed by the same letter are not significantly different at α=0.05.

Figure 3: Set-up of the lettuce damping-off trial. Each container holds a seedling-germination tray in which 25 treated lettuce seeds were planted.

4.3. *Pythium ultimum* trial 2: growth cabinet

Despite using lower rates of pathogen inoculum, the overall disease pressure was still very high (approximately 96% disease). Furthermore, conditions were not favourable overall for the germination of the seeds: in the pathogen-free potting mix, germination rates were approximately 50%. Despite mixing the pathogen inoculum into the potting mixture with a concrete mixer, there was still a large variation in germination within replicates of the same treatment.

As in the previous trial, significant differences between treatments were found at all assessment dates (P=0.0001) and lettuce seeds planted in potting mixture containing no added *P. ultimum* had significantly higher levels of germination than all other treatments (Table 4). Treatment of lettuce seeds with Captan, *B. mycoides* MW 27 or *P. polymyxa* 18·25 did not significantly increase (α=0.05) seedling counts over the pathogen-only control (Table 4). Pea seeds treated with *B. mycoides* MW 27 had a significantly lower level of germination than those treated with *P. polymyxa* 18·25.

Table 4: Effect of various biological and fungicidal treatments on germination of pelleted lettuce seeds planted into potting mixture infested with the damping-off fungus, *Pythium ultimum*.

Treatment	C.F.U.[1]	Seedling count (out of 25)[2]		
		Week 1	Week 2	Week 3
No pathogen	30	12.58 a	16.17 a	16.42 a
Pathogen only	30	3.25 bc	4.33 bc	3.92 bc
Captan	126	2.00 c	3.08 bc	3.08 bc
B. mycoides MW 27	7.3×10^6	4.92 b	6.75 b	6.50 b
P. polymyxa 18·25	5.1×10^5	2.08 c	2.83 c	2.83 c
LSD(α=0.05)		2.821	3.695	3.623

[1] – colony forming units per seed

[2] – Average for six blocks, each block containing two pseudo-replicated trays of 25 seeds each.

Treatment means followed by the same letter are not significantly different at α=0.05.

5. Discussion

Different formulations of the bacterium *B. mycoides* MW 27 were tested for control of Aphanomyces root rot disease on peas in a field trial. The environmental conditions during the trial were more typical of average Canterbury conditions than those of previous trials (Stewart *et al.*, 2000). Planting of the trial occurred in early spring into damp soil conditions. Although overall rainfall for the duration of the trial was light, some early wet weather induced both damping-off and root-rot disease pressures. Subsequently, significant effects were observed between the various formulations, with the prill formulation of *B. mycoides* MW 27, produced by AgResearch, performing particularly well. Prills placed in the planting hole directly beneath the pea seed significantly increased the number of pods set per plant and the yield per plant. The yield of pods per plot was increased from 399.62 g for the nil-control, to 482.20 for the prill treatment. Only the fungicide treatment, Apron, had a higher yield of pods per plot than the prill treatment. However, this resulted from a greater pea survival rate (plot stand), due to damping-off disease control, than increased yield per plant. In conditions where damping-off was not as severe, the results indicate that the prill treatment would have been more effective. The lack of damping-off disease control by the prill treatment, may be explained by its spatial displacement from the actual pea seed. The plot stand was increased from 77.22% (nil-control) to 86.11% when the bacterium was applied as a seed coat formulation. This was comparable to the fungicide control (92.78% plot stand).

These results clearly demonstrate the potential for biological control of pea root rot with *B. mycoides* MW 27. The bacterium was the only treatment which could significantly increase yield per plant; a parameter affected by Aphanomyces disease. Furthermore, these results highlight the effect that formulation and placement have on biological control efficacy. The prill formulation of the bacterium was more effective, at a significant level, than other treatments at reducing root rot disease. However, the seed coat formulation was effective at reducing damping-off disease. A combination of both treatments would, presumably, lead to increased yields overall following control of damping-off and root rot diseases. Further development of the prill formulation by AgResearch may also lead to significant improvements in its efficacy (Pers. Comm., Dr Von Johnson, AgResearch).

Spore-forming bacteria were also tested for biological control of damping-off disease on lettuce. In the first glasshouse-based trial, no bacterial treatments had a significant effect on germination, except for *P. macerans* PT 1, *B. subtilis* PT 69 and *B. pumilus* PT 10 which significantly increased damping-off. Given the large amount of variation both between and within the treatments, the trial was repeated using a slightly modified protocol. In this trial, the level of pathogen inoculum, oospores, was decreased from 1.17×10^4 spores g^{-1} of seedling germination mixture to 4×10^3 spores g^{-1} in the second trial, and a greater emphasis was placed on thorough mixing of the germination mixture. In addition, the second trial was carried out in a growth cabinet, providing greater uniformity in environmental conditions. Despite these measures, the variation in the second trial was still high and disease pressure was greater. In the first trial, the

germination rate for lettuce plants in the pathogen control treatments was 40% and in the second 15%.

In the second trial, the level of disease in the treatment in which no pathogenic inoculum had been added was approximately 55%. Therefore, a large proportion of the variation and disease expression in the trial was generated independently of the *P. ultimum* inoculum and, as such, was difficult to control. Due to either the large level of non-*P. ultimum* disease or unfavourable plant growth conditions, definitive conclusions regarding the ability of the spore-forming bacteria to control damping-off disease are difficult to make. In addition, the relative performance of *B. mycoides* MW 27 and *P. polymyxa* 18·25, was inconsistent between trials. The lack of performance of the other bacterial isolates (*B. pumilus* PT 10, *B. subtilis* PT 69 and *P. macerans* PT 1) in the first glasshouse trial should not therefore exclude them from further testing. The bacterium *B. mycoides* MW 27 should, in particular, be tested further as it significantly controlled damping-off on pea under field conditions in the field trial described before.

Examples in the scientific literature show that spore-forming bacteria have potential for control of this type of disease (Hwang *et al.*, 1996; Paul *et al.*, 1995; Smith *et al.*, 1993), and given the difficulties described in the trials, these bacteria should be re-evaluated under conditions that are more appropriate.

A significant modification to the protocol used in the glasshouse or growth cabinet trials, would be an alteration of the watering regime. In the trials described, the germination mixture was maintained at near-saturation for the entire duration of the trial; conditions which are both unfavourable for the seed and seedling *per se*, and particularly favourable towards Oomycetous fungi. As, in moist soil conditions, the spores of *Pythium* spp. can germinate and infect host tissue within a few hours of detecting the host (Martin and Loper, 1999), it is possible that the fungus was able to infect the seed prior to the germination of the bacterial spores. Furthermore, as most vegetable seeds are rarely, if ever, planted directly into soils that are saturated, the type and level of disease pressure encountered was both artificial and high. A more realistic protocol would have the soil conditions damp for the first few days after planting followed by a period of saturation. This should not only promote initial germination of the seeds, but also allow time for the biological control agents to germinate and establish prior to initiation of disease pressure.

6. References

Bruin, G.C.A.; Edgington, L.V. 1983: Chemical control of diseases caused by zoosporic fungi: a many sided problem. In *Zoosporic plant pathogens: A modern perspective* ed.. Buczacki, S. T. pp 193-223. London: Academic Press.

Domsch, K.H.; Gams, W.; Anderson, T.H. 1980: Pythium. In *Compendium of soil fungi* ed. Domsch, K.H.; Gams, W.; Anderson, T.H. pp. 678-697. New York: Academic Press.

Hagedorn, D. J. 1984: *Compendium of Pea Diseases*. Minnesota: The American Phytopathological Society. 57pp.

Hwang, S.F.; Chang, K.F.; Howard, R.J.; Deneka, B.A. and Turnball, G.D. 1996: Decrease in incidence of *Pythium* damping-off of field peas by seed treatments with *Bacillus* spp. and metalaxyl. *Journal of Plant Disease and Protection* **103**: 31-41.

Martin, F.N. 199: Pythium. In *Methods for Research on Phytopathogenic Fungi* ed. Singleton, L.L.; Mihail, J.D.; Rush, C.M.. pp 39-49. St Paul: The American Phytopathological Press.

Martin, F.N.; Loper, J.E. 1999: Soilborne plant diseases caused by *Pythium* spp.: Ecology, epidemiology, and prospects for biological control. *Critical Reviews in Plant Sciences* **12**: 111-181.

Paul, B.; Charles, R.; Bhatnagar, T. 1995: Biological control of *Pythium mamillatum* causing damping-off of cucumber seedlings by a soil bacterium, *Bacillus mycoides*. *Microbiological Research* **150**: 71-75.

Priest, F.C. 1989: Isolation and identification of aerobic endospore-forming bacteria. In *Bacillus* ed. Harwood, C.R. pp. 27-56. New York, Plenum Press.

Smith, K.P.; Harvey, M.J.; Handelsman, J. 1993: Suppression of cottony leak of cucumber with *Bacillus cereus* strain UW85. *Plant Disease* **77**: 139-142.

Stewart, A.; Jaspers, M; Wakelin, S.A.; Walter, M. 2000: Field assessment of formulations of biological control agents against *Aphanomyces euteiches*. *Industry Report to The Foundation for Arable Research*: 24pp.

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