IN VITRO TESTING FOR BIOLOGICAL CONTROL OF *APHANOMYCES EUTEICHES*

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ABSTRACT

Soil samples were collected from 18 pea cropping sites in Canterbury and tested for natural suppressiveness towards Aphanomyces root rot of pea. Highly significant differences were found between soil types and *Aphanomyces* inoculum concentration on root rot development. Three soils gave good suppression of disease. Bacterial isolations were made from soil samples and assayed for inhibition of mycelial growth and zoospore germination of *A. euteiches* using *in vitro* assays. Twenty seven isolates significantly inhibited mycelial growth and, of these, a total of nine also inhibited zoospore germination in two separate assays.
Keywords: bacteria, biological control, pea, suppressive soil.

INTRODUCTION

Common root rot of peas, caused by *Aphanomyces euteiches* Drechsler, has been reported in most pea growing areas of North America, northern Europe, Australia, Japan and New Zealand and is one of the most destructive diseases of peas worldwide (Pfender 1984). It was first detected in New Zealand in the 1977-78 growing season near Nelson (Manning and Menzies 1980). Subsequently, the disease has been found in Canterbury, Hawke's Bay, Horowhenua and Marlborough. Annual yield losses are estimated at 10% worldwide (Pfender 1984); however, under disease conducive conditions complete crop losses are not uncommon.

Despite extensive efforts to develop suitable control methods for Aphanomyces root rot, to date the only effective control is to avoid planting pea crops in moderately to heavily infested soils. Inoculum (oospores) of the pathogen can persist in infected plant debris in the soil for as long as 10 years (Pfender 1984) and can build up rapidly in soil where peas are planted. This persistence in the soil excludes control through crop rotation and a lack of both economic fungicide control and resistant pea cultivars has resulted in unavoidable crop losses and economic disadvantage to the grower and industry. In the Canterbury region, a soil indexing service is provided to farmers so that they may avoid planting peas in moderately to heavily infested areas.

Following the publication of several overseas reports showing effective control of Aphanomyces root rot with the use of microbial antagonists (Bowers and Parke 1993; Parke *et al.* 1991), and considering the lack of chemical and cultural control methods, there appears to be merit in investigating the potential for biological control of *A. euteiches* under New Zealand conditions. The objectives of this study were to identify local soils suppressive to Aphanomyces root rot, to isolate bacteria from local soils and to evaluate their ability to suppress the mycelial growth and zoospore germination of the pathogen.

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MATERIALS AND METHODS

Screening soils for suppressiveness to root rot

Eighteen soils from the Canterbury region (Lincoln, Prebbleton, Leeston, Aylesbury and Southbridge districts), which had recently been cropped with pea, were evaluated for suppressiveness of Aphanomyces root rot. For each site, 15 polythene bags (PB3/4) were filled with soil and four infected pea seedlings per bag were planted (Walter *et al*. 1995). Roots and epicotyl regions of the seedlings were dipped for 3 seconds into a zoospore solution of *A. euteiches* 1143 (isolated from the roots of infected pea seedlings grown in disease indexing soil), produced using the method of Mitchell and Yang (1966). Four infected pea seedlings were planted in each of 15 polythene bags (PB3/4) filled with soil from each of the sample sites. Control treatments consisted of washed river sand. Three rates of zoospore application were evaluated for each soil (0, 500 and 10000 spores/ml). Pea root rot was assessed using a Disease Severity Index (Sherwood and Hagedorn 1958) based on a 0-4 disease rating (0 = healthy plant and epicotyl firm and white; 4 = epicotyl rotted through or plant dead). Analysis of variance (ANOVA) was used to determine effects between treatments.

Bacterial isolations

Bacteria were isolated from each soil site using a dilution plating technique. From each site, two measures of soil, each weighing 1 g, were transferred into Universal bottles and 10 ml of sterile Tween water (0.05% Tween 80 in sterile distilled water) added. Universals were shaken for 10 min on an orbital shaker (100 rpm) to fully disperse soil particles. Following shaking, samples were diluted six times in a ten-fold series (ie. dilutions of 10^0, 10^{-1}, 10^{-2} - 10^{-6} were made). At each dilution, isolations of bacteria were made onto Kings medium (Difco) and nutrient agar (NA; Gibco). Plates were incubated at 20°C for 3 days and all visibly different bacteria were streaked onto individual NA plates. Isolates (total of 225) were stored at 4°C.

Mycelial inhibition assay

Each bacterial isolate was looped from the NA plate and streaked across the surface of a potato dextrose agar (PDA; Difco) plate. Four replicate plugs of *A. euteiches* (7 mm diameter PDA culture, grown for 5 d at 25°C in the dark) were placed onto each plate. Plates were incubated at 20ºC for 3 days and colony radii measured (mm). Controls consisted of plates inoculated with *A. euteiches* alone. The experiment was duplicated to determine reproducibility of results.

Zoospore germination inhibition test

Twenty seven bacterial isolates were cultured overnight in Universal bottles containing potato dextrose broth (Gibco), shaken at 100 rpm on an orbital shaker. Bacterial colony forming units (CFU) in each stock solution were determined by dilution plating onto NA plates and enumerating following overnight incubation at 30°C. In Experiment 1, for each isolate, three 30 µl droplets of the stock solution were pipetted onto PDA plates for each pre-colonisation time. The bacterial plates were allowed to pre-colonise the agar for 0, 4 or 8 h prior to inoculation of each bacterial droplet with a 20 µl droplet of *A. euteiches* zoospore solution (5 x 10^4 spores/ml). Control plates consisted of sterile water droplets inoculated with *A. euteiches* zoospores at each concentration. Plates were incubated at 20ºC for 3 d and assessed for germination of *A. euteiches*. Each bacterial droplet was scored for the presence (1) or absence (0) of *A. euteiches* and the scores tallied for each isolate. A potential score of 9 was possible provided all *A. euteiches* droplets germinated (ie. no germination inhibition occurred). In a repeat experiment, Experiment 2, each isolate was cultured overnight, the CFU/ml of stock solution determined, and then diluted six times in a ten-fold manner. For each isolate, three 30 µl droplets of the stock solution and each of the six dilutions were pipetted onto PDA plates for each pre-colonisation time. Plates were inoculated with zoospores, grown and assessed (total scores recorded for each dilution at each pre-colonisation time) as in Experiment 1. An example of how each isolate was scored is given in Table 1. A potential score of 63 was possible provided all *A. euteiches* droplets germinated.

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TABLE 1: Sample layout for scoring bacterial isolates for zoospore germination inhibition.

Isolate #	Dilution	Bacterial pre-colonisation time: 0 Hours 1[1]	0 Hours 2	0 Hours 3	4 Hours 1[1]	4 Hours 2	4 Hours 3	8 Hours 1[1]	8 Hours 2	8 Hours 3	Total
18	Stock	0	0	0	0	0	0	0	0	0	
	10^{-1}	0	0	0	0	0	0	0	0	0	
	10^{-2}	1	1	1	1	1	1	0	0	0	
	10^{-3}	1	1	1	1	1	1	1	1	1	
	10^{-4}	1	1	1	1	1	1	1	1	1	
	10^{-5}	1	1	1	1	1	1	1	1	1	
	10^{-6}	1	1	1	1	1	1	1	1	1	
Subtotal			15			15			12		42

[1]Replicate 1, 2 or 3

RESULTS

Screening soils for suppressiveness to root rot

Highly significant differences were found between soil types and between inoculum concentration on pea root rot (Figure 1). Three soils were found to be suppressive to Aphanomyces root rot: Soil 1, from D. Lemon's farm at Leeston; soil 4 from the Kimihia research station (G block) at Lincoln; and soil 12 from S. Lemon's farm at Southbridge. Soils 1 and 4 suppressed disease (as measured by the disease severity index) by more than 50% at the highest zoospore application concentration (10000 spores/ml). Soil 12 suppressed disease by 35% at the highest zoospore application rate and by 82.5% at 500 spores/ml. Disease occurred in several soils (soils 13-18) when no inoculum was added, indicating natural infestation by *A. euteiches*.

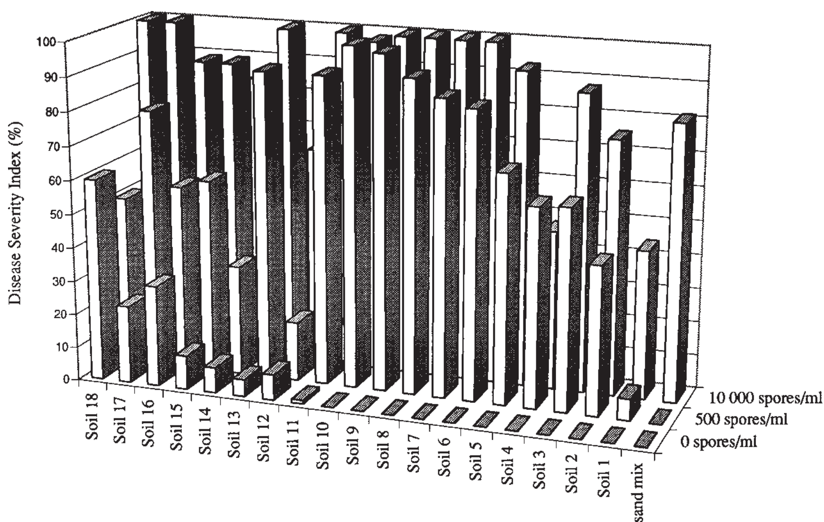

FIGURE 1: Effect of soil type and *Aphanomyces euteiches* inoculum (zoospore) concentration on pea root rot. Disease severity index as described by Sherwood and Hagedorn (1958).

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Mycelial inhibition assay

Data from the mycelial inhibition assay are summarised in Table 2. Of the 58 isolates expressing high inhibition intensity, 27 isolates were found to reproducibly inhibit fungal growth in duplicate assays. These isolates were selected for zoospore germination inhibition testing.

TABLE 2: Inhibition of mycelial growth of *Aphanomyces euteiches* 1143 by soil bacteria.

Degree of inhibition[1]		Number of bacterial isolates
Nil-low	(0-10%)	115
Medium	(10-50%)	52
High	(>50%)	58

[1] Inhibition relative to mycelial growth on control plate.

TABLE 3: Scores for test bacteria against *A. euteiches* zoospore germination. Lower scores correlate to greater suppression of *A. euteiches* zoospore germination and growth.

	Experiment 1		Experiment 2	
Isolate number	CFU[1]/ml	Total Score[2]	CFU[3]/ml	Total Score[2]
1	10^7	9	10^7	63
2	10^7	9	10^3	63
3	10^7	0	10^6	63
4	10^7	9	10^5	63
5	10^6	9	10^8	63
6	10^8	9	10^5	57
7	10^8	9	10^7	56
8	10^7	1	10^5	62
9	10^7	0	10^8	24*
10	10^8	0	10^5	28*
11	10^7	1	10^8	27*
12	10^8	9	10^8	49
13	10^8	9	10^5	63
14	10^7	9	10^8	42
16	10^7	9	10^7	63
17	10^8	9	10^6	63
18	10^7	9	10^6	42
19	10^7	9	10^7	57
21	10^8	9	10^6	63
22	10^7	9	10^4	63
23	10^7	9	10^7	60
24	10^8	9	10^7	63
25	10^6	9	10^6	63
27	10^7	3	10^6	50*

[1] Bacterial colony forming units.
[2] Number of droplets from which hyphae of *A. euteiches* grew. Maximum score (no suppression) for Exp 1 is 9 and for Exp 2 is 63.
[3] Bacterial colony forming units in the stock solution only (from which serial dilutions were made.
* Selected for further evaluation.

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Zoospore germination inhibition test

Of the 27 bacteria tested at stock concentration (Table 3) in the first experiment, only 6 isolates (strains 3, 8, 9, 10, 11 and 27) suppressed zoospore germination after 3 days incubation. When bacteria were evaluated at six ten-fold dilutions (Experiment 2) from the stock solution, isolates 9, 10, 11, 12, 14, 18 and 27 were suppressive to zoospore germination (Table 3). Bacterial isolates 9, 10, 11 and 27 were able to repeatedly inhibit zoospore germination of *A. euteiches* 1143, and hence were selected for further evaluation. Bacterial isolates 3, 8, 12, 14 and 18 failed to produce consistent inhibition across both assays. This may have been due to different initial bacterial concentrations in the stock solutions between assays. Bacterial isolates 15, 20 and 26 failed to grow in the zoospore germination inhibition assay.

DISCUSSION

Natural soils suppressive to Aphanomyces root rot have been reported and attempts made to determine the nature of suppression (Worku and Gerhardson 1996; Oyarzan *et al*. 1997). In some instances, the suppression was thought to be biologically based as it was removed by heat treatment (Worku and Gerhardson 1996). Of the 18 Canterbury soils tested in this study, three exhibited a natural suppressiveness to Aphanomyces root rot under glasshouse conditions, although the nature of the suppression was not determined. Soils taken from similar localities (eg. different blocks of the same farm) ranged from being disease suppressive to disease conducive, indicating that soil type did not represent a major factor in disease suppression in this study. Suppression in these soils may, therefore, be based on farm management practices (such as previous cropping history or fertiliser applications) or differences in biological composition. Further work needs to be carried out to determine the basis of suppressiveness in these soils.

Twenty seven of the 225 bacteria isolated from the soil samples were found to reproducibly inhibit mycelial growth of *A. euteiches* in dual-culture assays. Inhibition zones on dual culture plates usually result from production of chemicals inhibitory to the growth of the pathogen. Our results are consistent with the antibiosis mode of action proposed for bacteria so far reported to control *A. euteiches* (Heungens and Parke 1997; Carruthers *et al.* 1994). Bacterial biological control agents antagonistic to the mycelial growth of *A. euteiches* may be able to inhibit ectotrophic hyphal growth along the pea root prior to penetration and colonisation of the root and thus reduce disease.

Of those isolates which effectively inhibited mycelial growth, only four isolates (isolates 9, 10, 11, and 27) were able to reproducibly inhibit zoospore germination. It is likely that the failure of several bacterial isolates (3, 8, 12 and 14) to inhibit zoospore germination in one of the two experiments was due to insufficient inoculum (as stock solution CFU). These bacteria need to be re-evaluated for their ability to inhibit zoospore germination. The identity of the bacterial isolates is, at present, unknown.

Bacteria selected for further evaluation (isolates 9, 10, 11 and 27) were obtained from disease conducive soils. For example, soil 11 yielded three isolates (isolates 9, 10 and 11) antagonistic to *A. euteiches*. Soils conducive for disease should, therefore, not be discounted as potential sources for bacterial biological control agents. Populations of suppressive bacteria were possibly not high enough in the disease conducive soils to control disease.

By inhibiting both zoospore germination and mycelial growth along the root, it is hoped that biological control agents may be able to better inhibit Aphanomyces root rot disease in peas. The best isolates from the *in vitro* tests will, therefore, be assessed for their ability to suppress Aphanomyces root rot disease of peas in a glasshouse trial. Isolates exhibiting good disease control will be identified to species level and further studied to determine a biological basis for control.

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