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BIOLOGICAL CONTROL OF *APHANOMYCES EUTEICHES* ROOT ROT OF PEA WITH SPORE-FORMING BACTERIA

A thesis
submitted in fulfilment
of the requirements for the Degree of
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By

S. A. Wakelin

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Abstract of a thesis submitted in fulfilment of the
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BIOLOGICAL CONTROL OF *APHANOMYCES EUTEICHES* ROOT ROT OF PEA WITH SPORE-FORMING BACTERIA

By S. A. Wakelin

Spore-forming bacteria were investigated as potential biological control agents of the pea root rot fungus, *Aphanomyces euteiches* (Oomycete). Isolations of the bacteria were made both directly from field soils (558) and from the rhizosphere of peas grown in soil (146). When the 704 isolates were screened for inhibition of *A. euteiches* mycelial expansion *in vitro*, a low frequency of suppressiveness was observed in isolates taken directly from field-soil (0.05%), whilst 19% of the bacterial isolates taken from the rhizosphere were suppressive.

Mycelial suppressive isolates were subsequently screened for inhibition of zoospore germination and germ tube growth. The seven isolates *Bacillus cereus* 15·80, *Paenibacillus polymyxa* 18·25, *P. polymyxa* 18·94, unidentified bacterium PB 45, *Paenibacillus macerans* PT 1, *Bacillus pumilus* PT 10 and *Bacillus subtilis* PT 69, inhibited growth by 50% or more. Bacterial isolates were identified using carbohydrate fermentation profiles and, for *Bacillus mycoides* MW 27 and *P. polymyxa* 18·25, on their 16S rRNA gene sequences.

The seven suppressive isolates identified, and an additional five isolates which had appeared promising in an earlier study (unidentified bacterium MW 9b, *B. cereus* MW 10, *B. pumilus* MW 12, *B. pumilus* MW 18, *B. mycoides* MW 27), were tested for disease control in glasshouse trials. *Bacillus cereus* 15·80 and Apron C70SD significantly

(α=0.05) reduced visual root rot disease in the initial trial. However, the presence of other root rot pathogens, causing disease with symptoms similar to Aphanomyces root rot, confounded the results. In a subsequent trial, counts were made of oospore numbers per gram of root tissue which allowed specific distinction of *A. euteiches* disease. Several species of bacteria were able to reduce oospores and root rot symptoms. *Paenibacillus polymyxa* 18·25 was the most effective, reducing oospores from 450 to 160 $gram^{-1}$ of root. Apron C70SD reduced overall root disease but had no effect on oospore counts.

On the basis of both *in vitro* and glasshouse trial results, *B. subtilis* PT 69, *B. pumilus* MW 18 and PT 10, *B. mycoides* MW 27, *B. cereus* 15·80, *P. polymyxa* 18·25 and 18·94 were selected for field testing. In the first trial, no significant differences (α=0.05) were found between nil and each of the other treatments. In part, this was due to low overall disease pressure (disease score of 1.5 in untreated) and inadequate separation / resolution of treatment means (all bacterial treatment means were 1.0). *Bacillus mycoides* MW 27 and *P. polymyxa* 18·25 were selected for further field trials. *Bacillus mycoides* MW 27 significantly (α=0.05) increased the mean pod weight per plant from 2.39 g (nil) to 2.71 g, and the average plant root weight from 0.81 g to 0.95 g. No benefits were found by applying the two isolates together as opposed to individually, nor was there a benefit by combining them with Apron C70SD.

Seed coat, prill and granule formulations of *B. mycoides* MW 27 were evaluated in a field trial. As a seed coat formulation, the isolate significantly (α=0.05) increased plot stand by about 10%. In a prill formulation, *B. mycoides* MW 27 had no effect on plot stand (early damping-off disease), but significantly (α=0.05) increased the number of pods per plant and average pod weight per plant. Apron C70SD controlled damping-off disease but had no effect on root rot disease.

Bacillus pumilus PT 10, *B. mycoides* MW 27, *P. polymyxa* 18·25 and fluquinconazole were tested as seed treatments for control of another root disease, take-all of wheat. *Bacillus pumilus* PT 10 significantly (α=0.05) increased plant yield by 77%. *Bacillus mycoides* MW 27 had similar efficacy as fluquinconazole, increasing yield by approximately 10% (not significant at α=0.05). As a seed coat treatment, *B. mycoides* MW 27 also effectively controlled damping-off disease.

Paenibacillus polymyxa 18·25 suppressed a wide range of plant pathogenic fungi *in vitro*, possibly through production of the cell wall-degrading enzymes chitinase or cellulase, or an antibiotic compound which the bacterium was found to produce. *Bacillus mycoides* MW 27 was slightly suppressive to only two pathogenic fungi *in vitro*, and did not degrade chitin or cellulose.

The soil bacterium *Bacillus mycoides* MW 27 has excellent potential for development as a biological control agent of Aphanomyces root rot of pea. A formulation of the bacterium could be marketed on both its ability to increase yields under Aphanomyces disease pressure, and to limit oospore development, thereby limiting disease in continuous cropping situations.

Keywords: *Aphanomyces euteiches*, *Pisum sativum*, biological control, endospore-forming bacteria, *Bacillus mycoides*, *Bacillus pumilus*, *Paenibacillus polymyxa*, *Gaeumannomyces graminis,* glasshouse trial, field trial.

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Chapter 1 General introduction

1.1 The pea

The pea, *Pisum sativum* L., is a member of the agriculturally-important family of plants the Leguminosae (occasionally referred to as pulses, grain legumes or food legumes). Like other members of the Leguminosae, such as green / navy beans (*Phaseolus vulgaris* L.), broad / field bean (*Vicia faba* L.), soya bean (*Glycine max* L.), and lentils (*Lens culinaris* Medik.), peas are valued for their edible seeds which have high food value and good storage characteristics (Davies *et al.*, 1985).

The modern pea plant is largely the result of hybridisations made by Thomas Knight in the late 1700's and bears little resemblance to the original wild plant. Currently, there is still such a large degree of morphological and physiological variation within *P. sativum* that an all-inclusive description of the species is impossible (Hagedorn, 1984). An extensive description of typical cultivated forms (Figure 1.1) may be found in Hagedorn (1984). Briefly, the plant may be simply described as a cool-season, herbaceous, annual legume of a bushy climbing nature. The edible seed consists of two fleshy cotyledons which remain below ground following germination. The root system consists of a taproot with secondary branches capable of bearing nitrogen-fixing nodules. The leaves are pinnately compound, consisting of two large, leaf-like stipules and terminal tendrils are arranged along the stem. Inflorescences occur in the leaf axis and are self-pollinating. The flowers have five petals and are bilaterally symmetrical. Physiological maturity of the pod is reached 24-30 days after self-fertilisation, followed by transition to the dry seed stage. Most cultivars used fresh or for processing have wrinkled seed and are harvested when the green, enlarged ovules are sweet. Cultivars used for dried seed are harvested at the end of the growth cycle.

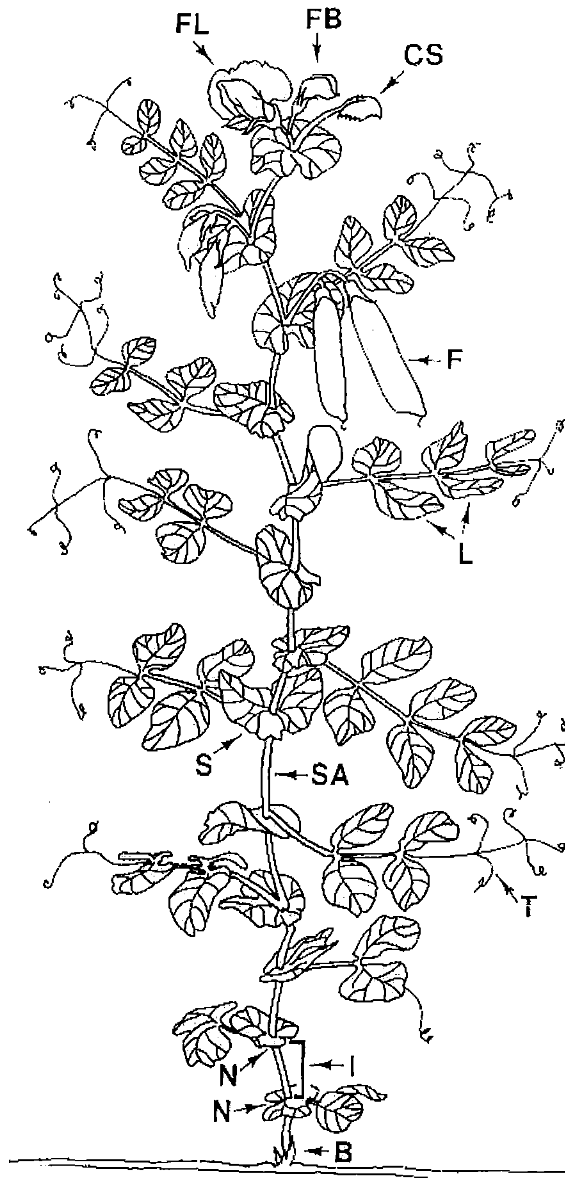


Figure 1.1: The ‘typical’ pea plant. FL = open flower, FB = flower bud, CS = clam shell, F = fruit, L = leaflet, S = stipule, SA = stem axis, T = tendril, N = node, I = internode, B = trifid bracts (from Hagedorn, 1984).

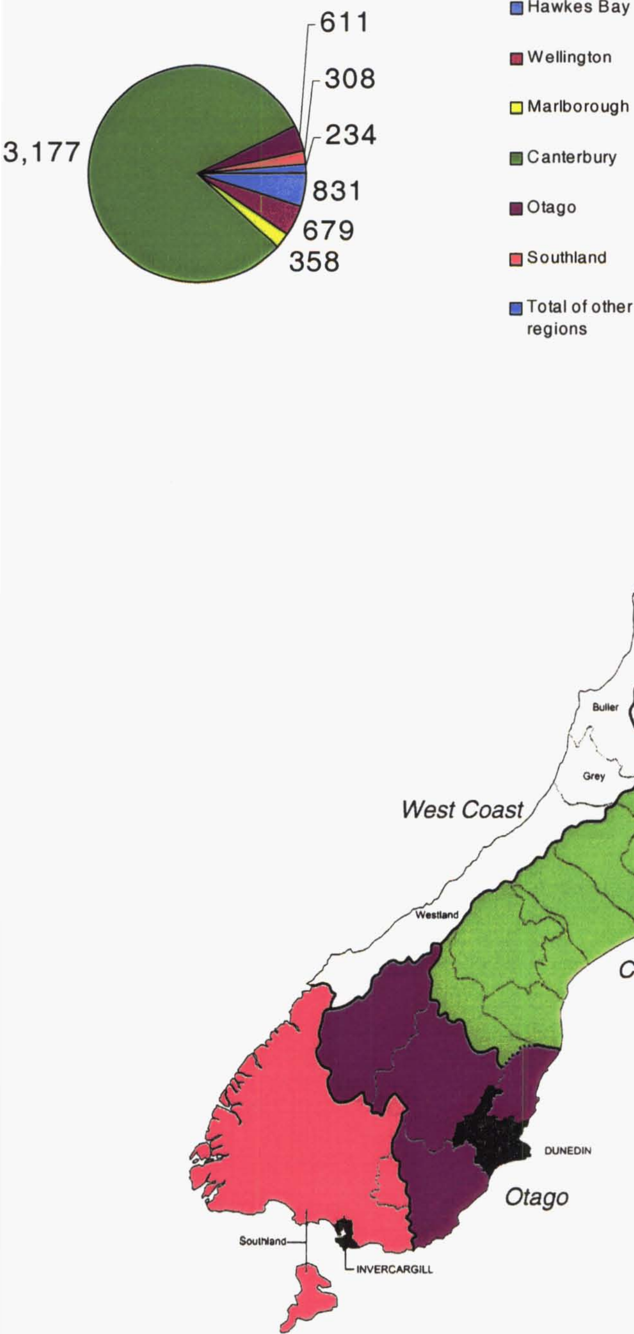
Peas may be grown for a number of different uses. The main harvest worldwide consists of the dry, ripe pea used for human or animal consumption. In developed countries, processing peas (vining peas) are harvested when immature for canning and freezing. Picking peas may also be harvested when immature and sold fresh in markets.

1.1.1 New Zealand pea production

In New Zealand, approximately 75% of the total pea production occurs in the Canterbury region (Statistics New Zealand, 1996); the Nelson / Marlborough, Southland, Hawke's Bay, Wellington and Otago regions crop peas to a lesser extent (Figure 1.2). The total farmed area for peas in New Zealand is currently 28,500 ha and yields are approximately 115,000 tonnes per annum (FAO, 2000). Farmers grow peas for two main reasons: for a cash return and as a break crop for disease control and soil fertility maintenance (White, 1987).

Processing peas, grown under contract to one of the three major processing companies (Heinz-Watties, McCain Foods, Talley Frozen Foods) have a high export value. For the year ending 30 June 1999, frozen peas were the third largest vegetable export (onions $101.7M, squash $59.9M) earning about $45M from 35,000 tonnes (VegFed, 2000a). Furthermore, these figures do not include peas exported as a component of frozen mixed vegetables, as dried (dehydrated) or canned pea products. The major export markets are Australia, Japan, United Kingdom and Taiwan (VegFed, 2000b). In the past 8 years, the value of process pea exports has increased by over 40%.

Peas are commonly grown in rotation with cereals, such as barley and wheat, on arable cropping farms, or before winter feeds, such as grasses and brassicas, on livestock / mixed livestock and cropping farms. As such, their role is to provide a break to assist in control of cereal diseases (e.g. wheat take-all disease) and for maintaining soil fertility, particularly nitrogen (White, 1987).



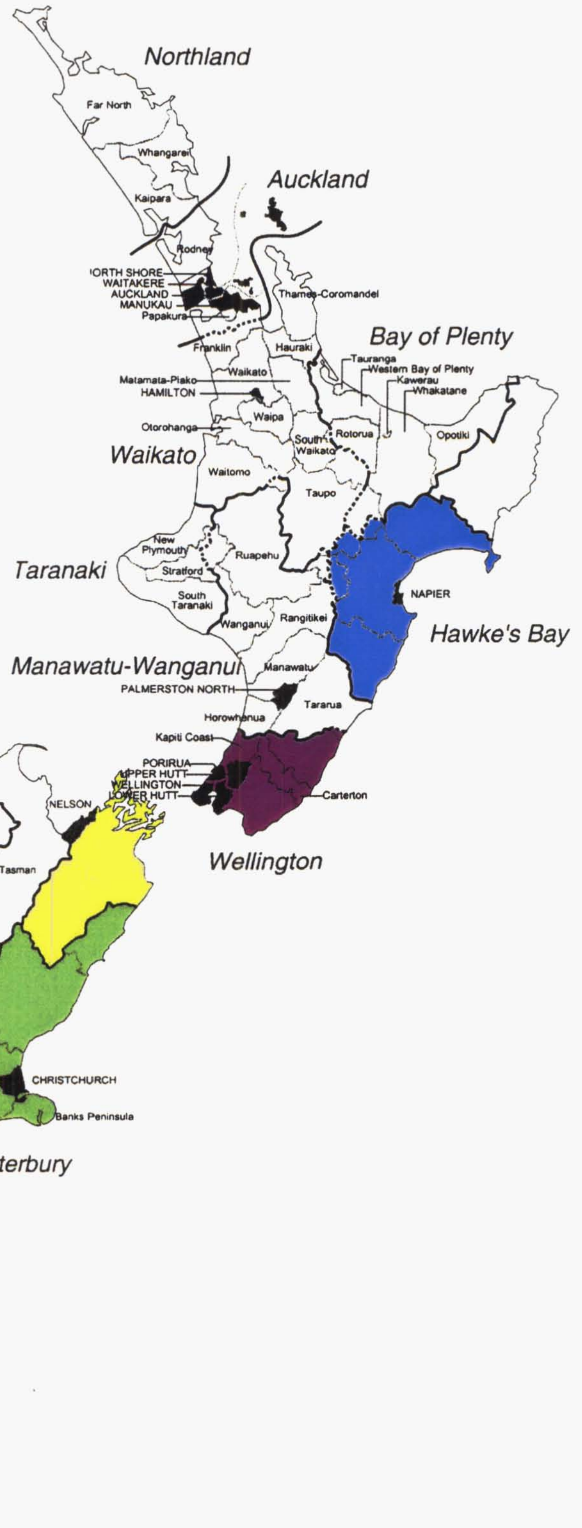


Figure 1.2: Regional pea production in New Zealand for the year ended 30th June, 1999 (Agricultural Production Series, Statistics New Zealand).

1.2 Pea diseases

As with other cultivated plant species, peas are susceptible to diseases caused by a large number of infectious pathogens (Hagedorn, 1984). Duke (1981) lists over 150 fungal, 12 bacterial, and 30 viral species which have been recorded as pathogenic to peas worldwide. In New Zealand, 21 fungi, four bacteria, and 10 viral species have been found on peas (Pennycook, 1989). Overall, pathogenic fungi cause the most common and economically significant biotic diseases of peas.

1.2.1 Fungal diseases of peas

Fungal diseases of peas may be arbitrarily divided into three categories: seed and seedling diseases, foliar diseases and root diseases.

Seed and seedling diseases occur pre-germination or in the early stages of the plants growth. A number of non-specialised (*sensu* Garrett, 1970) pathogens may be involved, particularly *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani* Kühn. Fungicidal seed treatments, seed testing (e.g. for exudate leakage) and cultivar resistance are commonly used to control these diseases (Hagedorn, 1984).

Foliar-type diseases occur on the above-ground portions of the plant such as leaves, stems, pods and tendrils. In New Zealand, the most serious foliar diseases are caused by *Ascochyta pisi* Lib. (blight), *Erysiphe pisi* Syd. (syn. *E. polygoni* DC.; powdery mildew) and *Peronospora pisi* (Berk.) Caspary (downy mildew) (New Zealand Institute for Crop & Food Research, 1995). In each case, the disease may be visually identified, assessed and treated with appropriate fungicides (Hagedorn, 1984).

Diseases of roots are less obvious than foliar diseases; their only visible effect is often reduced yield which can be mistakenly credited to agronomic factors. They are also more difficult to quantify and to control. Fungicides are difficult to apply and target to the root zone and may cause problems due to persistence and toxicity to non-target organisms. Developing plants resistant to root diseases has also proved difficult. In part this is due to the difficulties in quantifying the diseases *per se*, and also because many of the pathogens are unspecialised in their host range (Cook, 1980).

Historically, the two most serious root diseases of peas in New Zealand were caused by the pathogens *Fusarium oxysporum* Schlecht. Emend. Snyd. & Hans. f. sp. *pisi* (van Hall) Snyd. & Hans. Race 2 and *Aphanomyces euteiches* Drechsler. However, resistance to Fusarium wilt (*F. oxysporum* f. sp. *pisi* Race 2) has been bred into modern pea cultivars meaning the disease is no longer a serious problem to pea growers (New Zealand Institute for Crop & Food Research, 1995). Subsequently, the major disease occurring on pea roots is Aphanomyces root-rot (alternatively referred to as common root-rot or foot-rot). This disease is gaining notoriety as being 'particularly difficult to study and control' due to its apparent absence during non-favourable conditions, its aggressive impact on pea yields in disease-favourable seasons, and the lack of disease control options (Section 1.6). Farmers may see no visible sign of the disease in a pea crop during one season, only to lose a crop the following year.

1.3 Aphanomyces root-rot of peas

Aphanomyces root-rot disease has a world-wide distribution, occurring in most pea growing regions (Pfender, 1984). In New Zealand, it was first detected during the 1977-1978 growing season near Nelson (Manning and Menzies, 1980). Subsequently, the disease has been found to be present throughout the major pea growing districts.

1.3.1 Symptoms

Peas infected with *A. euteiches* may express a variety of symptoms, similar in many respects to those caused by *Pythium* spp. (Burke *et al.*, 1969; Haenseler, 1925; Jones and Drechsler, 1925; Manning and Menzies, 1980). The most diagnostic feature of the disease, however, is the formation of water-soaked, honey-coloured lesions on the root and epicotyl (Figure 1.3). Often, as the disease progresses, these lesions may darken due to invasion by secondary organisms. Ultimately, there may be a total collapse of the root tissue. Root damage causes a loss in turgor of the entire plant, causing wilting and yellowing of the lower leaves (Figure 1.4). Infected plants are often stunted and set fewer pods per plant with a reduced number of seeds per pod.

1.3.2 Yield losses

As with many soil-borne plant diseases, quantitative assessment of yield losses due solely to *A. euteiches* is difficult to determine (Pfender, 1984). The difficulties are largely due to the disease occurring with other pathogenic and semi-pathogenic organisms in a disease complex. Although no yield loss data has been collected for New Zealand, growers are regularly confronted with disease losses during wet seasons and the impact of the disease has been recognised by research bodies, such as The Foundation for Arable Research (FAR) and the Vegetable and Potato Growers Federation (VegFed), and other groups within the industry. Pfender (1984) estimated that worldwide, annual losses due to the pathogen are approximately 10% of production. Considering the large environmental influence on the expression of the disease (principally the requirement of free-soil water), it is likely that in dry seasons losses are lower than 10% but, during wet seasons, large or total crop losses may occur.

Figure 1.3: Honey coloured discolouration of pea roots are symptomatic of *A. euteiches* infection. Plants on the right are more severely infected than those on the left.

Figure 1.4: Above-ground symptoms of *A. euteiches*: yellowing and wilting of lower leaves and stunting of plants.

1.4 Taxonomy of *Aphanomyces*

The genus *Aphanomyces* was established by de Bary in 1860 to include a number of fungi which he had observed to be different to those in other genera in the family Saprolegniaceae (Scott, 1961). The generic name *Aphanomyces*, Greek for "obscure fungus", refers to the appearance of a halo of delicate mycelial growth which extends outwards from infected tissue when placed in water.

Taxonomically, *Aphanomyces* and other Oomycetous fungi are no longer classified within the kingdom Myceteae (the true fungi), but rather the newer kingdom Chromista (Barr, 1992). The Chromista includes the brown algae and all protists that have either tubular ciliary (flagella), mastigonemes (fine, hair-like rodlets attached to the flagella shaft laterally in longitudinal rows on a flagellum), or chloroplast endoplasmic reticulum or both (Erwin and Ribeiro, 1996). One of the three phyla within the Chromista kingdom is Heterokonta under which the class Heteromycotina (Pseudofungi) is included. This class contains Oomycetes, which are characterised by the production of zoospores with two cilia

(flagella) with anterior rootlets having a ribbed triplet and doublet, with posterior rootlets having an octet and doublet, and with cytoplasmic and nuclear-associated microtubules.

The genus *Aphanomyces*, and others within the Oomycetes such as *Pythium* Pringsheim and *Phytophthora* de Bary, are, therefore, fundamentally different from the true fungi by a number of key characteristics (Erwin and Ribeiro, 1996): the major part of their life cycle is diploid whereas the true fungi are haploid; their cell walls are composed of cellulose and ß-glucans (not chitin); and mycolaminarin, a ß-1,3-glucan, is the major storage carbohydrate. Due to their unique cell-wall biology and other physiological characteristics, the Oomycetes are unaffected by many anti-fungal compounds to which the true fungi are susceptible.

The full taxonomic ranking for *Aphanomyces* is:

Kingdom: Chromists
Phylum: Heterokonta
Class: Heteromycotina (Pseudofungi)
Subclass: Oomycetes
Order: Saprolegniales
Family: Saprolegniaceae
Genus: *Aphanomyces*

Although the genus *Aphanomyces* has some examples of saprophytic species, many are parasitic. Their host range includes protozoa, rotifers, crustaceans, algae, other phycomycetous fungi, and the roots of seedlings (Scott, 1961). Only seven of the approximately thirty species are recognised as important plant pathogens: *A. brassicae* Drechsler (cauliflower), *A. campostylus* Drechsler (oats), *A. cladogamus* Drechsler (tomato), *A. cochlioides* Drechsler (sugar beet), *A. iridis* Ichitani *et.* Kodama (Dutch iris), *A. raphani* Kendrick. (radish) and *A. euteiches* (legumes) (Parke and Grau, 1992).

1.5 *Aphanomyces euteiches*

Although first described as a pathogen of peas by Drechsler in Jones and Drechsler (1925), *A. euteiches* has been recovered from the roots of many, mainly leguminous, plant species but also from a range of naturally occurring pasture species in New Zealand (Chan and Close, 1987b) (Table 1.1). Papavizas and Ayers (1974) listed over 90 plant species from a wide range of families to which *A. euteiches* has been reported to exhibit pathogenicity worldwide. However, in many of the experiments described, parasitism studies were performed by pure-culture inoculations and may not occur naturally. It is likely, therefore, that *A. euteiches* exists as a parasite of many different legumes, causing disease of particularly susceptible species under optimal conditions.

Table 1.1: Plant species in New Zealand susceptible to infection by *A. euteiches.*

Common name	Botanical name	Reference
Pea[1]	*Pisum sativum* L	Manning and Menzies, 1980, 1984; Chan and Close, 1987a, b, c.
Alfalfa / Lucerne[1]	*Medicago sativa* L.	Chan and Close, 1987b;
Red clover[1]	*Trifolium pratense* L.	Chan and Close, 1987b.
White clover[1]	*Trifolium repens* L.	Chan and Close, 1987b.
Lentil[1]	*Lens culinaris* Medik	Fletcher *et al.*, 1991; Jermyn, 1986; Russell, 1991.
Shepherd's purse[2]	*Capsella bursa-pastoris* L.	Chan and Close, 1987b.
Chickweed[3]	*Stellaria media* L.	Chan and Close, 1987b.
Field pansy[4]	*Viola arvensis* L.	Chan and Close, 1987b.

[1] Leguminosae

[2] Brassicaceae

[3] Caryophyllaceae

[4] Violaceae

1.5.1 Physiological specialisation

The existence of different strains of *A. euteiches* became apparent soon after attempts to breed pea lines with resistance to the pathogen. Various authors have reported the presence of 'races' of the pathogen with different levels of virulence towards selected lines of pea germplasm (eg. Beute and Lockwood, 1967; Sundheim, 1972). In New Zealand, Manning and Menzies (1984) recovered isolates with pathogenicity corresponding to race 5 of Sundheim (1972), but went on to question the overall concept of races within *A. euteiches* "due to the ambiguous nature of the criteria used for differentiation and wide range of pathogenicity exhibited within races".

Strain differences within the species *A. euteiches* may also occur across alternative host species. Based on pathogenicity tests, morphological differences (oospore sizes) and growth rates in culture, Pfender and Hagedorn (1982) proposed that two *forma speciales* (special forms) of *A. euteiches* be recognised: *A. euteiches* f. sp. *pisi*, which infects peas and beans, and *A. euteiches* f. sp. *phaseoli*, which infects beans but not peas. Holub *et al.* (1991) did detect a wide pathogenic diversity among isolates of *A. euteiches* isolates. However, they found a high proportion of the strains were pathogenic towards alfalfa and red clover, but not necessarily towards pea, and therefore concluded that the formation of a new group of *A. euteiches* was not warranted. Parke and Grau (1992) recognised three strains of *A. euteiches*: *A. euteiches*, which was virulent towards legumes in general, *A. euteiches* f. sp. *pisi* which was virulent towards peas, and *A. euteiches* f. sp. *phaseoli* which was virulent to snapbeans. Recently, attempts have been made to associate pathogenic diversity with genetic diversity of *A. euteiches* isolates using randomly amplified polymorphic DNA (RAPD) analysis (Malvick *et al.*, 1998; Malvick and Percich, 1998). Results from these studies have shown that populations of *A. euteiches* are both pathogenically and genetically variable, and that genetic similarities within groups of isolates can be associated with the host of origin and pathogenicity towards bean and alfalfa.

It may be concluded therefore, that pathogenic variation occurs both within pea and between different leguminous plants, however there is disagreement between researchers as to the validity of differentiating isolates of *A. euteiches* based on these often arbitrary observations.

1.5.2 Life cycle and infection process

The life-cycle of *A. euteiches* on peas is outlined in Figure 1.5.

Oospores, formed as a result of sexual fusion between the antheridium and oogonium, are the means by which the pathogen is maintained in the soil. Oospore dormancy, which may last longer than 10 years (Pfender, 1984), may be broken by a stimulus from a potential substrate (i.e. leguminous roots) (Nelson, 1990; Scharen, 1960) indicating a host-specific chemical trigger. Oospores may germinate to form germ tubes, which can directly infect root tissue (myceliogenic infection), or germ sporangia (zoosporic infection). Evidence suggests that oospore germination resulting in the formation of sporangia (zoospore-type) is favoured by anaerobic conditions (i.e. saturated soil conditions) (Scharen, 1960).

Primary zoospores, formed within the sporangium, exit and immediately encyst in a cluster at the sporangium tip. The number of zoospores discharging from the sporangium may vary from a few to 100 (Drechsler, 1929). Secondary, kidney-shaped zoospores with tinsel and whiplash flagella, emerge and are attracted towards plant roots in a chemotaxic response. The roots of a variety of plant species, even those non-susceptible to *A. euteiches*, have been shown to attract the motile zoospores indicating that the response is not host specific (Cunningham and Hagedorn, 1962a). The zoospores are attracted towards the region immediately behind the root tip (Cunningham and Hagedorn, 1962b; Yokosawa *et al.*, 1986), where leakage of metabolites is strongest. This results in localised massing of zoospores in selected regions which may increase the inoculum potential[1] (*sensu* Garrett, 1970) of the pathogen.

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[1] Inoculum potential is defined as the energy available for infection of a host at the surface of the infection court

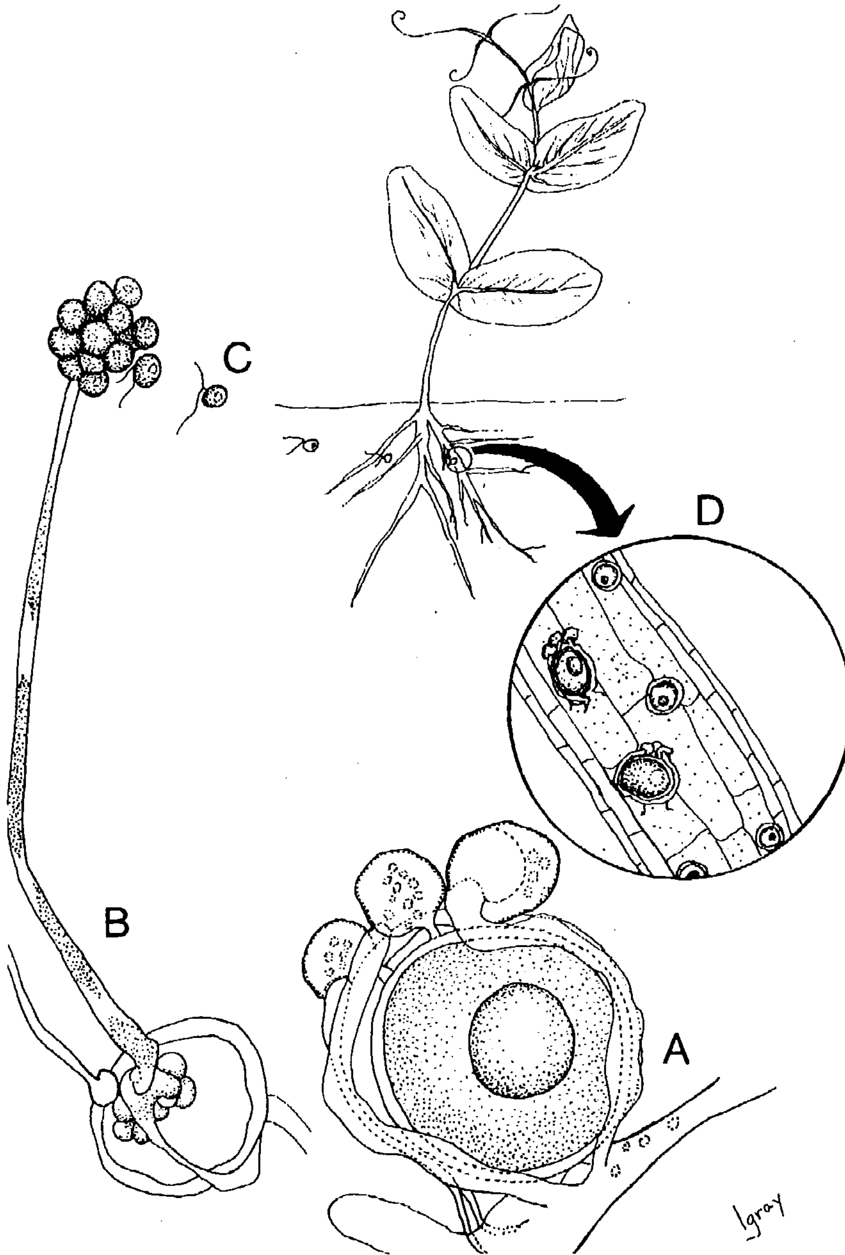

Figure 1.5: Life-cycle of *A. euteiches* on the pea host. A- dormant oospore, B – germination of the oospore to form a sporangium with encysted primary zoospores at the tip, C – secondary zoospores swim towards pea root, D – infection of the root and myceliogenic spread through the root tissue with subsequent formation of further oospores. From Jacobsen and Hopen, 1981.

Upon reaching the root, the secondary zoospores shed their flagella and encyst. The cysts re-germinate via simple germ tubes which directly penetrate the host. Germ tubes usually, but not always, penetrate between the host cells (Cunningham and Hagedorn, 1962b). Fungal mycelium rapidly spreads through the cortex of the root tissues. The vascular cylinder is less vulnerable to attack and may remain functional for water transportation for some time, except in cases of severe infection or adverse environmental conditions (Cunningham and Hagedorn, 1962b). The pathogen has been shown to produce the enzymes cellulase and polygalacturonase which assist in the spread of the fungus through host tissue (Papavizas and Ayres, 1974). Oogonia and antheridia are produced and mature oospores are formed within one to two weeks after infection (Pfender, 1984). As the roots decay, the oospores are released back into the soil.

1.5.3 Factors affecting disease development

The two primary factors affecting disease development are (1) the level of inoculum in the soil and (2) soil moisture. Temperature affects the initiation and development of disease to a lesser extent, but does have a bearing on symptom expression. Other soil factors play only a minor role in Aphanomyces root-rot (Papavizas and Ayers, 1974).

1.5.3.1 Pathogen inoculum

Not surprisingly, the level of pathogenic inoculum, or inoculum density, of *A. euteiches* in field soil can be directly related to disease levels (Boosalis and Scharen, 1959; Chan and Close, 1987a; Oyarzun *et al.*, 1997). The number of oospores per 100 g of field soil and the disease severity were shown to exist in a highly significant, curvilinear relationship (Chan and Close, 1987a) (Figure 1.6).

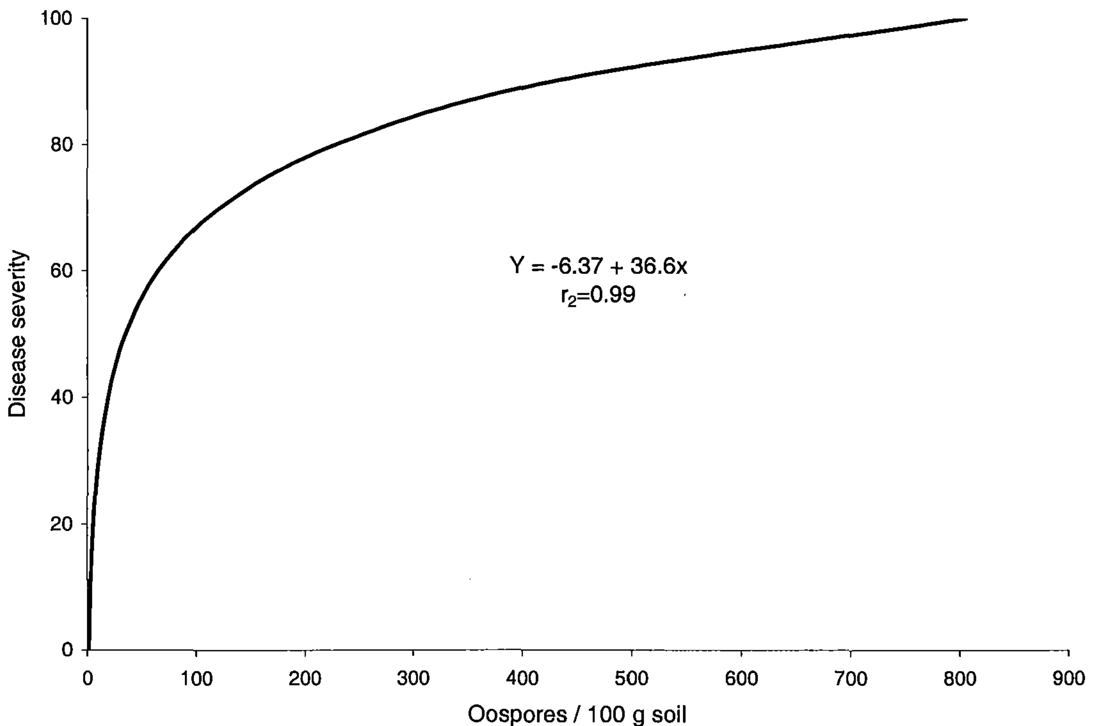

Figure 1.6: Relationship between oospore numbers (*A. euteiches* inoculum) and disease severity determined. Based on the relationship determined by Chan and Close, 1987a.

1.5.3.2 Soil moisture

The presence of soil water is crucial for *A. euteiches* infection, disease development and rapid spread. Soil water stimulates oospore germination, possibly by creating anaerobic conditions (Scharen, 1960), or by increasing levels of leakage of metabolites from pea roots (Kerr, 1964). In addition, free water must be available in the soil to stimulate zoospore release (Hoch and Mitchell, 1973) and to allow the flagellated zoospores to travel in moisture films on soil particles.

Although serious outbreaks of Aphanomyces root-rot are exclusively associated with very wet seasons (Papavizas and Ayers, 1974), only brief periods of soil saturation (24 h) are required for the pathogen to infect the crop (Pfender, 1984). A minimum level for initiation of root rot disease is considered to be about 30% soil water holding capacity (Haenseler, 1926; Smith and Walker, 1941).

1.5.3.3 Temperature

Aphanomyces root-rot can occur at all temperatures conducive to pea growth (Pfender, 1984). However, the optimum temperature for infection is approximately 16°C and for disease development between 20°C and 28°C (Burke and Mitchell, 1968; Burke *et al.*, 1969). Increasing temperature does, however, accentuate symptom expression on diseased plants. As the vascular root tissue is progressively destroyed, the water-transportation capability of the root is lost. Subsequently, during periods of high temperature and plant transpiration, infected plants wilt rapidly due to lack of water turgor.

1.5.3.4 Soil chemical and physical properties

Both chemical and physical soil properties have been shown to have a remarkable lack of association with the incidence of Aphanomyces root-rot on peas (Oyarzun *et al.*, 1998). Soil compaction, resulting in impaired water drainage and thereby favoring infection by *A. euteiches*, is the only factor which has been conclusively correlated with increased disease levels (Fritz *et al.*, 1995; Grath and Håkansson, 1994; Tu and Findlay, 1986).

1.6 Disease control strategies for *Aphanomyces euteiches*

1.6.1 Fungicides

A. euteiches, like other Oomycetes, is physiologically distinct from the true fungi (Erwin and Ribeiro, 1996). The differences, both in biochemical pathways and in cell wall composition, make them resistant to most of the fungicides active against the true fungi. In addition, *A. euteiches* is resistant even to the few agents which are registered for use against the Oomycetous pathogens. For example, neither the systemic acylalinine-type of Oomycete fungicides, such as metalaxyl, nor the ethyl phosphonates, such as fosetyl-Al, or cymoxanil are able to control *A. euteiches* (Bruin and Edginton, 1983). The chemical metalaxyl, active against most Oomycete fungi, is recommended for use in *A. euteiches*-selective media (Pfender *et al.*, 1984), as it suppresses the morphologically similar groups of fungi such as *Pythium* spp.

Kotova and Tsvetkova (1980) found that the fungicide Tachigaren (hydroxyisoxazole or hymexazol) reduced Aphanomyces disease and increased yield of treated peas.

Subsequently, there have been several reports describing the use of this chemical with different levels of success in disease control (Gritton *et al.*, 1995; Jermyn *et al.*, 1982). In Japan, Tachigaren is marketed for control of *Pythium* and *Aphanomyces*-diseases on sugar beet, but it is not registered for use in New Zealand. Currently, there are no available fungicides which are able to consistently control Aphanomyces root-rot in New Zealand.

1.6.2 Herbicides

Herbicides for weed control in peas have been shown to suppress *A. euteiches*. The increase in apparent incidence and severity of the disease worldwide has been associated with the de-registration of certain herbicides for use on peas (John Kraft, pers. comm.). Growers, therefore, have unwittingly been suppressing the disease for many years when using herbicides to which the fungus is susceptible.

Dinitramine herbicides, such as trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*ρ*-toluidine; e.g. Treflan®), dinitramine (N^4,N^4-diethyl-α,α,α-trifluoro-3,5,-dinitrotoluene-2,4-diamine; e.g. Cobex®) and dinoseb (2,4-dinitro-6-*sec*-butylphenyl; e.g. Premerge®) were used commonly as a pre-planting weed control on processing peas. In 1972, about 60% of the pea acreage in Wisconsin, a major pea growing area in the United States, was treated with these herbicides (Bruehl, 1987). This class of herbicides has subsequently been shown, in many reports, to suppress pea root rot disease (Grau and Reiling, 1977; Harvey *et al.*, 1975; Jacobsen and Hopen, 1975, 1981; Sacher *et al.*, 1978; Teasdale *et al.*, 1979b). *A. euteiches* zoospore production was shown to be reduced by dinitroaniline herbicides (Grau, 1977; Teasdale, *et al.*, 1979a).

1.6.3 Host resistance

The breeding of pea cultivars with genetic resistance to *A. euteiches* would probably be the most economic and effective disease control strategy and, therefore, a large amount of time has been spent pursuing this objective. Despite an absence of high-level resistance within the species, a number of partially resistant to tolerant pea breeding lines have been developed (Davis *et al.*, 1976, 1995; Gritton, 1990; King *et al.*, 1981; Kraft, 1981, 1989, 1992; Kraft and Tuck, 1986; Rao *et al.*, 1995; Shehata *et al.*, 1976). However, the transfer of this resistance to agriculturally-acceptable lines was made difficult because some

sources of resistance also had other undesirable traits for node-length, flower colour and hilum colour (Marx *et al.*, 1972). These traits have more recently been separated (i.e. bred apart) allowing the resistance characteristics to be transferred into commercial pea lines (J. Kraft, pers. comm).

Pathogenic variability within *A. euteiches* has also confounded the aforementioned difficulties in breeding resistant pea lines (Beute and Lockwood, 1967; Lockwood, 1960). Currently, therefore, host resistance does not constitute an adequate disease control method. It is likely that commercially-available pea lines with resistance to the pathogen will be released in the near future, but the level of resistance is likely to be low to moderate and may be overcome by strains of *A. euteiches* (J. Kraft, pers comm).

1.6.4 Crop rotation

The effectiveness of crop rotation, one of the oldest methods for control of root-diseases, varies inversely with the length of rotation necessary to obtain control of the disease (Garrett, 1944). Due to the slow decline in the population of *A. euteiches* oospores in field soil, this method of disease control has been of limited value in controlling the disease. Oospores may be viable for up to 10 years after formation (Pfender, 1984) and many alternative host species may be able to sustain the pathogen in the absence of a pea host. Nevertheless, crop rotation can still be used by farmers to control the rate of the build-up of pathogenic inoculum in soils.

In New Zealand, a typical crop rotation in an arable farming system involving peas would have the pea crop follow a cereal such as barley or wheat. On a livestock / mixed livestock and cropping farms, peas would be planted prior to winter feed species such as grasses and brassicas. As well as giving high monetary returns to the farmer, peas are also used to provide a break to assist in control of cereal diseases (e.g. wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Sacc.) v. Arx and Oliver) and for maintaining soil fertility, especially nitrogen (White, 1987).

1.6.5 Green manures and composts

Incorporating the stems, leaves and roots of many plant species into infested soils has been shown to reduce the incidence of Aphanomyces root rot. Two families of plants have been shown to be particularly effective: the Brassicaceae (cruciferous plants) and the Poaceae (oat family). Of the Brassicaceae, cabbage (*Brassica oleracea* var. *capitata* L), kale (*B. oleracea* L. *acephala* DC), mustard (*B. nigra* L.), white mustard (*Sinapis alba* L.), turnip (*B. rapa* subsp. *rapa* L.) and rape (*B. napus* L.) have all exhibited root-rot control efficacy when cultivated into soils (Chan and Close, 1987c; Muehlchen *et al.*, 1990; Papavizas, 1966, 1967; Papavizas and Lewis, 1971). Oats (*Avena sativa* L.), rye (*Secale cereale* L.), corn (*Zea mays* L.) and sudan grass (*Sorghum halepense* L. Pers.), all members of the Poaceae, have also been found to significantly reduce Aphanomyces root rot when used as green manures (Davey and Papavizas, 1961; Fritz *et al.*, 1995; Tu and Findlay, 1986; Tu, 1990, 1992; Wilkins *et al.*, 1998; Williams-Woodward *et al.*, 1987).

Cruciferous plants contain high contents of toxic sulfur-containing compounds such as mercaptans, sulphides and isothiocyanates which are liberated into surrounding soil during their decomposition (Lewis and Papavizas, 1970). These volatile compounds have high toxicity towards *A. euteiches*, inhibiting mycelial growth, zoospore formation, motility and zoospore germination (Lewis and Papavizas, 1971), which may account for the observed disease control.

Like the cruciferous plants, plants in the family Poaceae produce toxic compounds to which *A. euteiches* is sensitive. Principally, saponin compounds, such as avenacin and ß-escin, have been shown to be toxic towards zoospores (Deacon and Mitchell, 1985; Engelkes and Windels, 1994). In addition, these plants may change the biological composition of treated soils. Tu (1990) found higher populations of *Bacillus* and *Pseudomonas* Migula bacteria and lower populations of root-rot causing fungi in green-manure-treated soils.

Certain composts, particularly those containing portions of animal waste (manure), have also been found to be suppressive to Aphanomyces root rot (Walter *et al.*, 1995). The efficacy of these 'active' composts may, again, be attributable to microbiological factors.

1.6.6 Disease avoidance

Disease avoidance is, to date, the only economical, dependable "control" practice for *A. euteiches*. This method involves the "indexing" of field soil to determine the inoculum potential of *A. euteiches* prior to growing a susceptible crop. A practical method was devised by Sherwood and Hagedorn (1958) and by Reiling and King (1960). This assay involves the sampling of soil from across a test field, mixing the samples and growing peas in the soil mix under disease-conducive conditions (i.e. high soil water potential) in a glasshouse. Plants are recovered after several weeks' growth, the roots assessed visually for disease and a disease index score calculated. As Aphanomyces root-rot is mostly determined by the inoculum level of the pathogen in the soil and the presence of free water (which is controlled in the assay), the level of disease may be related to the inoculum levels of *A. euteiches* in the field soil sample. The accuracy of the glasshouse assay was demonstrated by Reiling and King (1960). They repeatedly showed a significant correlation between root-rot levels in the field and the level of root-rot disease in the glasshouse. Hazardous fields may, therefore, be identified and distinguished from non- or slightly-infested fields.

1.7 Biological control

Biological control, in the context of plant pathology, constitutes the "decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plant but excluding man" (Barker, 1987). It is only relatively recently that this strategy has been considered as a potential method for Aphanomyces root-rot control. In part, this is due to the lack of success of traditional control approaches, the worldwide trend towards organic (pesticide free) and sustainable agriculture, and a mounting body of circumstantial evidence from research on soil suppression / receptivity towards *A. euteiches* indicating that the pathogen may be naturally susceptible to soil microorganisms.

1.7.1 *Aphanomyces euteiches*-suppressive soils

Soils suppressive[2] to Aphanomyces root-rot have only recently been described both overseas (Oyarzun *et al.*, 1997, 1998; Persson, 1998; Persson *et al.*, 1999; Worku and Gerhardson, 1996) and in New Zealand (Wakelin *et al.*, 1998). In each case, where the nature of the suppressiveness has been examined, biological factors have been implicated.

Oyarzyn *et al.* (1998) found that reduced soil receptivity[3] towards *A. euteiches* was associated with *Gliocladium* spp. in the rhizoplane and *Acremonium* spp. in the rhizosphere. The effect of green manures on suppression of *A. euteiches* may also be due to microbiological factors. Tu (1990) found increases in the levels of *Bacillus* and *Pseudomonas* bacteria in soils which had received green manure treatments; the same soils showing a significant decrease in Aphanomyces root-rot in the following pea crop. Smolinska (2000) also found an increase in the population of total spore-forming bacteria, fluorescent pseudomonads, actinomycetes and fungi in soils amended with cruciferous plant residues. The increase in the numbers of the spore-forming bacteria persisted for over one year following addition of the residues to soil.

1.7.2 Biological control of *A. euteiches*

Most significant work on biological control of *A. euteiches* has concentrated on the use of soil bacteria as seed treatments. Parke *et al.* (1991) were the first to report attempts to identify bacterial isolates antagonistic to pea root pathogens. They demonstrated biological control activity by *Burkholderia cepacia* (basonym *Pseudomonas cepacia*) Palleroni and Holmes AMMD, *Pseudomonas fluorescens* Migula PRA25 and *Corynebacterium* sp. Lehmann & Newmann 5A against Aphanomyces root-rot and Pythium damping-off. However, when tested under field conditions, disease control by these bacteria was found to be inconsistent (Gritton *et al.*, 1995; King and Parke, 1993).

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[2] Soils in which the disease rating remains low on a host plant grown in that soil in the presence of a pathogen and in favourable environmental conditions (Alabouvette, 1990; Hornby, 1983)

[3] The effect, either suppressive or conducive, of soils on the inoculum potential of pathogen in the soil (Alabouvette *et al.*, 1982).

Dandurand and Knudson (1993) found that a granular formulation of *Trichoderma harzianum* Rifai and a seed-slurry formulation of *P. fluorescens* could control *A. euteiches* on peas grown in sand in growth chambers, but they did not test them under field conditions. Strains of the actinomycete *Streptomyces* sp. Waksmann & Henrici have also exhibited *in vitro* antagonism towards *A. euteiches* (Jones and Samac, 1996; Yuan and Crawford, 1995), but again these have not been tested in the field.

The commercial biological control product Kodiak, a formulation of the bacterium *Bacillus subtilis* (Ehrenberg) Cohn strain GB03 (Gustafson Inc.), has also shown some efficacy against Aphanomyces root-rot in field trials (Kraft and Coffman, 1994). Used principally for control of cotton pathogens such as *Rhizoctonia solani* (Brannen and Kenney, 1997) Kodiak has been accepted as a standard pesticide. Its success is due both to its efficacy and its chemical-like formulation attributes which allow it to be treated by growers as a standard pesticide. This product sets a *de facto* standard for successful commercialisation of biological control agents aimed at control of soil borne plant pathogens and presents an excellent model for the development of specific biological control agents against *A. euteiches*.

1.8 Aim of this project

Due to increasing on-farm losses, a current lack of practical or economic disease-control options, and the increasing focus on sustainable and organic farming of peas in New Zealand, research into control of *A. euteiches* in New Zealand has been given a high-priority by the Foundation for Arable Research (FAR). FAR, therefore, have supported this project with the overall aim to "investigate the potential for biological control of *Aphanomyces euteiches* under local (Canterbury) conditions[4]".

[4] Local pea growing conditions are, of course, likely to be very similar to those overseas. Results, therefore, have a greater significance than the project aim may suggest.

1.8.1 Objectives

A number of objectives were developed which outline the structure of the project and, subsequently, this thesis.

Objective 1: Development of a suitable blueprint for biological control of *A. euteiches* and the selection of a class of organism which would best fit the model.

Objective 2: Isolation of such biological control organisms from local environments and selection for strains with possible biological control activity based on *in vitro* antagonism to *A. euteiches* (both mycelial and zoospore germination / germtube growth inhibition).

Objective 3: Evaluation of biological control strains exhibiting *in vitro* activity in glasshouse trials as a basis for the selection of isolates for field evaluation.

Objective 4: Field evaluation of best bacteria.

Objective 5: Determination of whether biological control agents successful against Aphanomyces root-rot can control other soil borne plant diseases *in vitro*. Isolates exhibiting strong inhibitory actions will be assessed for control of *Pythium ultimum* Trow damping-off of lettuce and *Gaeumannomyces graminis* var. *tritici* (*Ggt* / take-all) on wheat.

Objective 6: Investigation of the potential mode-of-action of effective biological control agents (production of antibiotics and cell-wall-degrading enzymes).

Chapter 2 Isolation, identification, and *in vitro* antagonistic potential of endospore forming bacteria

2.1 Introduction

In many respects, each plant disease may be considered unique from all others, due to the biology of the pathogen (physiology, life-cycle), the host plant, the environment where the disease occurs (including both biotic and abiotic factors), as well as conditions favourable for disease occurrence and development. It follows, therefore, that the development of biological control strategies for each disease, should take into account as many of the pertinent aspects of the overall host / pathogen / environment interactions as possible. This can allow the detection of susceptible phases during the pathogen's life-cycle or during the disease processes which can be targeted using biological control.

When considering biological control of Aphanomyces root-rot disease of peas it is necessary, therefore, to consider the nature of the pathogen, *Aphanomyces euteiches*, the host plant, *Pisum sativum*, and the environment in which the disease occurs, i.e. the soil in the rhizosphere region of the pea plant.

2.1.1 Environment – rhizosphere soil

The life-cycle of *A. euteiches* occurs in the soil directly adjacent to the pea root system. The conditions which are necessary for disease development are the presence of pathogen inoculum, oospores and zoospores, the presence of saturated or near-saturated soil, and a host root, providing chemical triggers for phases of the pathogens life-cycle such as oospore germination and zoospore chemotaxis. The fundamental requirement for an organism capable of biological control of this disease must, therefore, be its ability to successfully establish and survive in soil immediately surrounding the pea root

(rhizosphere soil). As there is good evidence for synergistic co-evolution between plant roots and associated microorganisms (Lebuhn *et al.*, 1997), consideration should be given to the types of microorganisms which are naturally present in, and presumably adapted to, the pea rhizosphere region. Selection of these types of organisms for assessment as biological control agents is likely to increase the probability of obtaining individual isolates adapted to the pea rhizosphere environment.

2.1.2 Pathogen – *Aphanomyces euteiches*

A. euteiches, and other Oomycetous plant pathogens, have a biology quite different from the true fungi (Section 1.4), to the extent that they are no longer classified within the same Kingdom (Barr, 1992). The composition of the cell wall, which in Oomycetes is made of cellulose but in the true fungi is made of chitin, is one of the most significant differences. The selection of biological control agents that possess cellulose-degrading enzymes rather than chitinases would, therefore, be of value.

The complex life cycle of *A. euteiches* may provide opportunities for biological control unique to the Oomycete class of plant pathogens (Martin and Loper, 1999). Disease may be reduced when development of the pathogen or disease are interrupted, for example at oospore germination, zoosporangium formation, zoospore release, zoospore encystment, motile zoospore chemotaxic response, zoospore aggregation on the root surface, and during the penetration of the root tissue (Martin and Loper, 1999). This may occur indirectly as a result of successful colonisation of the rhizosphere soil by other microorganisms, resulting in competition for chemical triggers required for oospore germination, zoospore chemotaxis or by interfering with access to infection sites on roots. Alternatively, biological control can occur directly by parasitism of the pathogen (e.g. of the oospores or hyphae; Sneh *et al.*, 1977) or by chemical antagonism towards the pathogen (e.g. antibiotic production; Carruthers *et al.*, 1994).

2.1.3 The host plant – *Pisum sativum*

In the selection of a biological control agent, it is essential that the researcher take into account the compatibility of that agent with the host plant. In some cases, especially at high inoculation rates, biological control agents have shown phytotoxicity or pathogenicity

towards the host plant (A. Stewart; pers. comm.). Although compatibility of the biological control agent with the host plant can only be assured through testing *in situ*, consideration should be given in the early stages of selection to the nature of the biological control agent, thereby minimising the risk of adverse reactions.

2.1.4 Bacillus: a bacterium for biological control of pea root rot.

From the scientific literature, it appears that endospore-forming bacteria may have potential for biological control of Aphanomyces root-rot disease on pea (Milner *et al.*, 1996; Parke, 1987; Wilkins *et al.*, 1998). The family *Bacillaceae*, which broadly encompasses the spore-forming bacteria, contains the genera *Bacillus* and *Paenibacillus*. These aerobic, rod-shaped bacteria, have many of the attributes considered desirable in a biological control agent. This includes compatibility with pea as a host plant, antagonistic activities against various phytopathogenic fungi, adaptation as a rhizosphere colonist and the capacity for relatively easy commercial formulation.

The rhizosphere soil surrounding pea plants contains numerous microorganisms. In the rhizosphere soil of 6 week old pea plants, Windels and Kommedahl (1982) found bacteria at 2×10^{10} colony forming units (c.f.u.) $gram^{-1}$, fungi at 1.5×10^{6} c.f.u. $gram^{-1}$, and actinomycetes at 1×10^{9} c.f.u. $gram^{-1}$ of soil. Although it is difficult to determine the exact composition of the rhizosphere bacterial community, as it is variable both spatially and temporally on any given plant root, spore-forming bacteria have been found to be a dominant group on pea (Mundt and Hinckle, 1976; Patwari, 1956; Walker *et al.*, 1998). Furthermore, no spore-forming bacteria have been recorded as being pathogenic on pea in New Zealand (Landcare Research, 2001). Paradoxically, species of *Pseudomonas*, a genus which contains organisms with biological control potential, due to their rapid colonisation of root tissue and production of anti-microbial compounds (Weller, 1988), have been shown to be pathogenic to peas (Landcare Research, 2001).

Circumstantial evidence exists which indicates that spore-forming bacteria may be involved in the natural suppression of *A. euteiches*. A decrease in the incidence of Aphanomyces root-rot disease and a concurrent increase in the overall levels of spore-forming bacteria was found to have occurred in soils into which grasses had been incorporated as green manures (Davey and Papavizas, 1961; Williams-Woodward *et al.*,

1997). Although no direct association has been shown between disease reduction and increase in numbers of spore-forming bacteria, it is thought that the microflora do play an integral role in the suppression of disease by decomposing the plant tissues in which the fungus may survive between host plants.

Endospore-forming bacteria, or spore-forming bacteria as they are commonly known, have been found to produce a wide range of metabolites that may be important for biological control purposes. These include peptide and non-peptide antifungal chemicals (Katz and Demain, 1977; Leifert *et al.*, 1995; Milner *et al.*, 1996), biosurfactants (Stanghellini and Miller, 1997) and lytic enzymes, of which glucanases may be of particular importance to oomycetous plant pathogens, (Nielsen and Sörensen, 1997). Production of auxin and indolic compounds, which may act as hormones to stimulate plant growth, have also been shown by strains of *Paenibacillus polymyxa* (Lebuhn *et al.,* 1997; Timmusk *et al.*, 1999).

Endospores are produced when environmental conditions become unfavourable for vegetative growth. Because endospores are able to tolerate large fluctuations in temperature, soil moisture (desiccation), pH, chemical exposure etc they can survive for long periods in inhospitable conditions. Biological control agents able to form endospores, therefore, have attributes that make them suitable for commercial formulation. They allow the formulation of the bacterial agent into a chemical-like powder with storage characteristics equal to or greater than conventional chemical pesticides (Brannen and Kenney, 1997; Brannen, 1998). In addition, a powder formulation of the spores treated onto seeds may provide a convenient delivery system for the bacterium, for use in the spermosphere and rhizosphere environments.

The potential use of *Bacillus* and *Paenibacillus* spp. as biological control agents is evidenced by the successful commercialisation of several strains for use against root-pathogenic fungi. For example, the vast majority of cotton seed planted in the United States, are treated with a strain of *B. subtilis* (Brannen and Kenney, 1997). The product, Kodiak® (Gustafson Inc., Texas, USA), suppresses root-pathogenic fungi, especially *Rhizoctonia* and *Fusarium* spp. on a number of crops. Another strain of *B. subtilis,* marketed as Serenade (AgraQuest Inc., California, USA), is registered for the control of a wide range of Deuteromycetes, Oomycetes, Ascomycetes and bacterial plant pathogens on

both horticultural and vegetable crops (AgraQuest, 2000). It is apparent, therefore, that some isolates of spore-forming bacteria possess the necessary attributes for successful development into commercial reality.

The attributes of endospores that enable them to survive harsh conditions also assist with the development of laboratory procedures for their isolation. Tolerance towards a wide range of vegetatively damaging agents such as heat, ethanol, irradiation, desiccation etc, may, therefore, be used as a tool for the semi-selective isolation of spore-forming bacteria from the large background microflora which exists in soils (Priest, 1989).

2.1.5 Aims

The aims of this work were threefold:

1. to isolate a number of spore-forming bacteria from Canterbury soils,
2. to test the isolates for antagonistic potential against the mycelial and zoospore life-phases of *A. euteiches in vitro*,
3. to determine the identity, to species level, of isolates exhibiting the strongest biological control activity.

2.2 Materials and methods

2.2.1 Isolation of bacteria from agricultural soils

Bacterial isolations were made from five soils, collected from around the Canterbury region, which had previously been characterised by their development of *A. euteiches* pea root rot (Wakelin *et al.*, 1998) as either suppressive (soils 5, 9 and 18; Appendix 1) or non-suppressive (soils 2 and 3; Appendix 1). In addition, isolations were made from a mixed soil sample from the *A. euteiches* disease-indexing unit at the Ministry of Agriculture and Forestry (MAF), Lincoln.

A bulk sample of each soil was taken from numerous positions across the field, thoroughly mixed and a 0.5 g sub-sample taken. Each sub-sample was separately placed into a Universal bottle containing 10 mL of sterile tap water and rapidly agitated on a wrist-action shaker for 10 min to disrupt soil particles and disperse bacterial cells. The samples

were then pasteurised by placing in a 80°C water bath for 20 min to select for endospore-forming bacteria (Priest, 1989). Bacteria were recovered by pipetting 50 μL aliquots of each soil suspension onto five nutrient agar (NA; Merck) plates and incubating overnight at 20°C.

Each isolate was given a two-component reference code; the first number being the soil from which it was isolated and the second the isolate number from that soil. For example, 18·25 was isolate number 25, from soil number 18.

Colonies arising on the Petri plates were purified by transferring to NA plates before storing at –80°C.

2.2.1.1 Storage of cultures in glycerol at –80°C

A single colony of each bacterium was taken from a NA Petri plate and transferred into 10 mL of Nutrient broth (NB; Gibco) in a Universal bottle. The cultures were grown at 25°C whilst being continually agitated at 100 rpm on a reciprocal shaker. After 10 d growth, 1 mL of turbid broth culture was mixed into 1 mL of 50% glycerol in a sterile Eppendorf tube, allowed to rest for 30 min and stored at –80°C.

2.2.2 Isolation of spore-forming bacteria from pea rhizosphere soils

Four pea seeds (cv. Pania), treated with the fungicide Apron C 70 DS (Ciba-Geigy Ltd.; 2 g Kg^{-1} seed), were planted into a 120 mm diameter pot containing soil and grown in a glasshouse with regular watering. After 4 weeks, the plants were removed and the roots washed under running tap water for 1 min to remove soil adhering to the roots. The stems were discarded and the roots were cut in half with a sterile scalpel. The upper and lower halves of the roots were each put into Universal bottles containing 10 mL of sterile distilled water and shaken rapidly for 10 min on a wrist-action shaker. The Universal bottles were then placed into a 80°C water bath for 20 min to destroy vegetative cells. From each bottle, 50 μL aliquots were pipetted onto five NA plates and spread across the surface. The plates were incubated overnight in the dark at 23°C; colonies arising on the Petri plates were sub-cultured onto NA plates. Pure cultures were obtained for each isolate and all isolates were stored on NA slopes at 5°C.

Each isolate was given a two-component reference code; the first part being either PT (top half of the pea root) or PB (bottom half) followed by the isolate number from that segment. For example, PT 10 was isolate number 10 from the top-segment of the pea roots.

2.2.3 Isolation of *Aphanomyces euteiches*

A. euteiches was isolated from infected plant tissue using the baiting method of Parke and Grau (1992). Soil, known to be infested with *A. euteiches*, was collected from the pea disease-indexing unit at MAF, Lincoln. Pea seeds coated with Apron (as before), were planted into 120 mm diameter containers (5 seeds per pot) and filled with the diseased soil. The pots were kept in a greenhouse and the soil kept moist until seedling germination. The soil was then kept at saturation by standing the containers in saucers containing water to a depth of approximately 3 cm. After 4 weeks growth, the seedlings were removed and the roots rinsed under running tap water to remove soil. The roots were surface disinfected in 0.5% NaOCl for 2 min followed by three rinses of sterile water. Sections of disinfected root (approximately 1 cm long) were placed onto cornmeal agar (CMA; Gibco) amended with metalaxyl (3 mg L^{-1}). The plates were incubated at 20°C and sparsely growing hyphae originating from the root segments were transferred to fresh CMA plates. Isolates were identified as *A. euteiches* after microscopic examination of hyphae, oogonia, oospores, zoosporangia, and the pattern of branched hyphae (Scott, 1961). Cultures of *A. euteiches* were stored long-term on CMA at 4°C and at 12°C (Parke and Grau, 1992). Cultures for regular use were maintained on CMA at 20°C and sub-cultured regularly.

2.2.4 Mycelial inhibition assay

All bacteria isolated were assayed for their capacity to suppress mycelial growth of *A. euteiches in vitro*. Bacteria were taken from storage, cultured onto NA until exhibiting strong growth, and streaked at four replicate points around the periphery of a potato-dextrose agar plate (PDA; Gibco) (Figure 2.2). A 5 mm diameter plug of *A. euteiches* (isolate 4·6), taken from the edge of a 3 d culture, was then placed in the centre of the PDA plate. The plates were incubated at 23°C under dark conditions. After 5 d, the culture plates were examined for signs of mycelial inhibition. The distance between the edge of the bacterial colony and the edge of the fungal hyphae was measured at each of the four

inoculation points for each plate, and the average determined. The fungus was considered to be 'inhibited' if it had not grown up to or past the point of bacterial inoculation (i.e. the distance was 0 mm).

2.2.5 Zoospore inhibition assay

Bacterial isolates exhibiting inhibition towards mycelial growth of *A. euteiches* were further assayed for inhibition of zoospore germination.

A. euteiches zoospores were produced using the method of Kraft *et al.* (1994). Six conical flasks, each containing 10 g of pea seed in 200 mL of distilled water, were autoclave sterilised. A 10 mm^2 mycelial plug of *A. euteiches* (isolate 4·6) was taken from the front of a 5 d old culture, transferred into each flask and allowed to grow at room temperature. After 7 d, the mycelium was recovered, washed with sterile water and transferred to a single conical flask containing 100 mL of a minimal salts solution ($CaCl_2 \cdot 2H_2O$ 0.26 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.49 g L^{-1} and KCl 0.074 g L^{-1}). The flask was aerated gently overnight with sterile air from a bench-top pump. The following day, zoospores were recovered in the minimal salts solution and counted with a haemocytometer.

In order to get consistency with results from previous experiments, the assay followed the same methodology developed at The Horticulture and Food Research Institute of New Zealand Ltd. (HortResearch), Lincoln (Wakelin *et al.* 1998). Antagonistic bacteria were grown overnight at 30°C in 10 mL of NB whilst being gently shaken at approximately 100 rpm. The liquid cultures obtained were designated as the 'stock dilutions'. For each isolate, a 100-fold (10^{-2}) dilution was made by transferring 0.1 mL of the stock dilution into a Universal bottle containing 9.9 mL of NB and inverting several times. For all isolates, 0.1 mL drops of both dilutions were each spread across the surface of two PDA plates. After 4 h pre-colonisation time, 0.75 mL of *A. euteiches* zoospores (at 1×10^4 zoospores mL^{-1}) were pipetted onto each plate and spread over the agar surface. Control plates consisted of zoospores challenged against 0.1 mL of NB, zoospores challenged against 0.1 mL sterile water and 0.1 mL of NB with no zoospores added. Petri dishes were incubated in the dark at 23°C. After 3 d, each Petri plate was observed under a compound microscope for germination of zoospores. Four fields of view for each plate were scored for fungal growth and a plate average calculated: 0= no growth, 1= sparse, 2= light, 3=

moderate and 4= heavy (refer to Figure 2.1). A total score, out of a maximum of 32 (2 plates × 4 observations × maximum score of 4 for each observation), was calculated for each dilution.

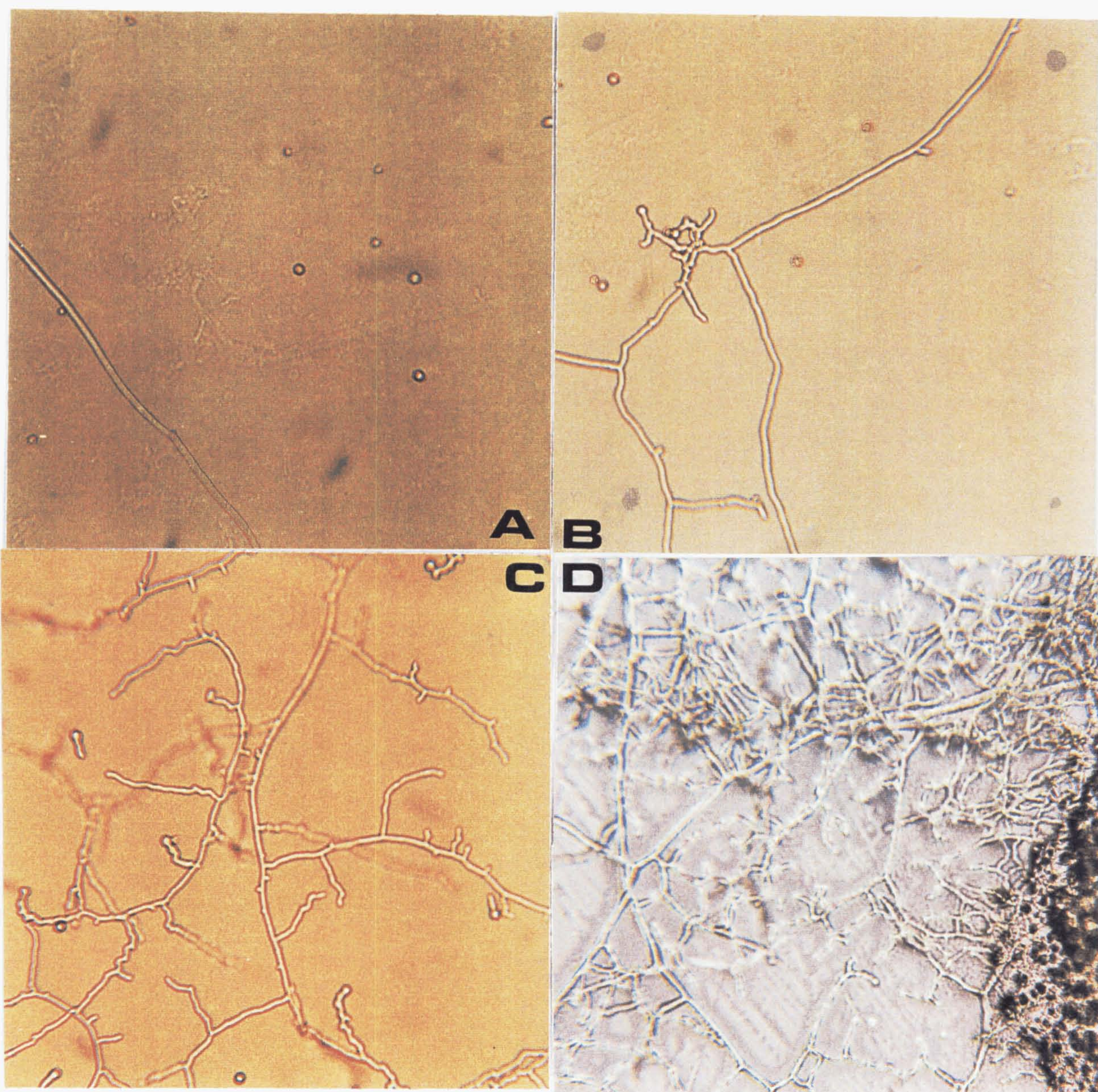

Figure 2.1: Scoring protocols for the zoospore inhibition assay. A= sparse (score =1), B = light (2), C= medium (3), and D= heavy (4). No zoospore growth was scored as 0.

2.2.6 Identification of bacterial isolates

2.2.6.1 Primary characterisation of bacterial isolates exhibiting *in vitro* antagonism

The 12 bacterial isolates which were selected for evaluation in glasshouse bioassays (isolates 15·80, 18·25, 18·94, PT 1, PT 10, PT 69, PB 45 from this assay and isolates MW 9B, MW 10, MW 18 and MW 27 from previous work (Wakelin *et al.*, 1998)) were characterised. Each isolate was Gram typed using the 3% KOH method of Gregersen (1978) and examined microscopically for vegetative cell shape after staining with Safrinin red. In addition, the bacteria were tested for endospore production. After growth on NA amended with 50 mg L^{-1} $MnSO_4$ (Priest, 1989) at 30°C for 10 d, cells of each culture were stained with Malachite green over steam for 6 min and examined microscopically.

2.2.6.2 Full identification of the ten most promising bacterial strains

Bacterial isolates MW 10, MW 12, MW 18, MW 27, 15·80, 18·25, 18·94, PT 1 and PT 10 were fully identified using an API rapid 50 CHB microtubule system (bioMérieux) according to the manufacturers instructions. Each isolate was taken from storage at -80°C and cultured overnight at 30°C on NA. Single colonies of each isolate were sub-cultured onto two NA plates and again cultured overnight at 30°C to ensure vigorous growth. The resulting bacterial growth was scraped from each plate into 1 mL of sterile saline solution (0.85% NaCl in water); only one plate was required for isolates exhibiting heavy overnight growth. The turbidity of each bacterial suspension was adjusted with sterile saline to reach that of the McFarlane standard 2 (bioMérieux) and standardised suspensions were used to inoculate ampoules of API 50 CHB medium. The inoculated medium was then pipetted into each of the 50 carbohydrate-utilisation test strips and the assembled trays incubated at 30°C. Results were taken at 24 and 48 h after incubation. Positive results, indicating successful utilisation of an individual carbohydrate, were identified by a change in colour from red to yellow of the test medium (except for esculin where a positive change was indicated by a colour change to black). The resulting biochemical profile for each isolate was compared with standard profiles and the closest match in each case was taken as the identity of that isolate.

2.2.6.3 Molecular identification of isolates MW 27 and 18·25

The API 50 CHB-based identification of isolates MW 27 and 18·25, which were later extensively tested in glasshouse and field trials, was verified using a molecular approach based on the sequence of the highly specific 16S rRNA gene (Ash *et al.*, 1993).

Extraction and preparation of DNA from bacterial cells

DNA was extracted from bacterial cells using the method of Lawson *et al.* (1989) according to Ash *et al.* (1993). Each isolate, 18·25 and MW 27, was taken from storage at –80°C and cultured onto two NA Petri plates. After 3 d growth at 30°C, the cells were scraped into 5 mL of TES buffer (0.05 M Tris-HCl, 0.005 M ethylene-diamine-tetra-acetic acid EDTA , 0.05 M NaCl, pH 8.0), washed twice by centrifugation at 3220 × g for 10 min in 5 mL TES buffer, and then re-suspended in 2 mL of TES buffer. Lysozyme enzyme (100 μL; Sigma Chemical Co., St. Louis, USA) (10 mg mL^{-1}) was added to each tube, and the cells held on ice for 15 min. Proteinase K (Sigma) and RNase (Sigma) were then added to a final concentration of 50 μg mL^{-1} each and the cells incubated at 65°C for 1 h. Sodium dodecyl sulphate (SDS, 20% w/v) was added to a final concentration of 2% (w/v) and the cells incubated for a further 10 min at room temperature.

DNA extraction was based on a phenol / chloroform method (Lawson *et al.*, 1989). Into each tube, 1.5 mL of phenol and 1.5 mL of chloroform / isoamyl alcohol (24:1, v/v) were added and the solutions gently mixed by inverting. The aqueous phase, containing the DNA fraction, was collected and the procedure repeated three times. Finally, the aqueous phase was extracted with chloroform only and removed to a clean tube. The DNA was precipitated from the solution by adding twice the volume of ice-cold 100% ethanol and 10µl of 7.5 M NaOAc, and collected by centrifugation at 3220 × g for 10 min. The DNA pellet was allowed to dry and then dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.41). The DNA was quantified and purity checked by agarose gel (1%) electrophoresis with a High DNA Mass™ ladder (Life Technologies New Zealand, Ltd.). Bands were visualised under 254 nm UV light after staining in ethidium bromide (0.5 µg mL^{-1}) for approximately 30 min.

PCR amplification of the bacterial 16S rRNA gene

Two universal primers derived from the highly conserved areas of the 16S rRNA molecule U1 (5′ CGT GCC AGC AGC CGC GGT AAT 3′) and U2 (5′ AAG GAG GTG ATC CAG CCG CA 3′) (Ash *et al.*, 1993), generated by Gibco BRL custom primers (Life Technologies), were used to amplify a region of the 16S rRNA molecule from each of the DNA samples prepared previously.

Amplification was performed by a Perkin-Elmer Gene Amp 2400 PCR system (Perkin Elmer Cetus Corp., Norwalk, USA). Each PCR reaction mixture contained 2 μL of sample DNA (10 ng μL^{-1}), 4 μL of each primer (10 μM), 200 μM of each deoxynucleotide triphosphate (dTTP, dATP, dGTP and dCTP), 2.5 U *Taq* polymerase enzyme (Roche Molecular Biochemicals), 5 μL 10 × Buffer (supplied with the *Taq* polymerase enzyme and containing 1.5 mM Mg^{2+}), and 30.5 μL of water. A negative control, containing sterile water in place of the DNA component of the reaction mixture, was included to assess whether any contamination was present. The PCR temperature profile consisted of an initial 3 min denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C and 2 min of primer extension at 72°C. At the conclusion of the PCR cycle, the mixtures were held firstly at 72°C for 7 min then constantly at 4°C until use. PCR products were separated and visualised on a 1% agarose gel (as before).

Cloning of PCR gene products into a plasmid vector

PCR products, approximately 1 Kb in size, were selected from each DNA sample for cloning. Following the manufacturers instructions, 1 μL of PCR product was mixed with 1 μL of plasmid vector (pGEM®-T vector systems, Promega Corporation, Madison, USA), 5 μL of 2 × ligation buffer, 1 μL T4 DNA ligase enzyme and 2 μL of water and incubated overnight at 4°C. Competent cells of *Escherichia coli* INVαF' (Invitrogen Corporation, San Diego, USA) were thawed on ice and 2 μL of ligation mix was added. The cells were returned to ice for a further 20 min then heat-shocked at 42°C for 50 s to initiate plasmid uptake. The cells were cooled on ice for 2 min, transferred into 950 μL of LB broth (Appendix 3), and incubated at 37°C. After 1 h, the cells were collected by centrifugation (400 × g for 5 min), re-suspended in a small volume of remaining broth and spread across the surface of a Petri plate containing LB agar amended with ampicillin (1 $\mu L\ mL^{-1}$ agar; 100 mg mL^{-1}) and 40 μL of 20 mg mL^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-

galactopyranasidase). Non-transformed *E. coli* cells were included as a negative control. LB agar Petri plates were incubated overnight at 37°C to allow bacterial growth to occur and then placed at 4°C for 30 min to stimulate expression of the X-gal phenotype.

Plasmid preparation, DNA sequencing and sequence analysis

Three discrete, white, bacterial colonies were selected from each LB plate, picked-off into 5 mL of LB broth amended with ampicillin (as before), and grown overnight at 37°C. Plasmids preparations were made from each of the overnight cultures using the Wizard® Plus SV Miniprep DNA purification system (Promega) according to the manufacturers instructions. To verify the presence of the insert in each plasmid, the purified plasmid was diluted and subjected to PCR as described before using primers U1 and U2. Having established the presence of the correct sized insert in each plasmid preparation, plasmid samples derived from each of the two bacterial isolates, 18·25 and MW 27, were sent to the University of Waikato DNA sequencing facility (ABI Prism™, automated DNA sequencing machine) for sequence determination from both the SP6 and T7 primer regions present on the p-GEM-T® vector either side of the cloning site. The resultant sequence information was compared against those on the GenBank database (www.ncbi.nlm.nih.gov/blast/blast) using the BlastN option and significant alignments were determined.

2.3 Results

2.3.1 Isolation of bacteria from agricultural soils

A total of 558 bacterial isolates were recovered directly from the pasteurised soil samples. The Aphanomyces-suppressive soils 15, 18 and 9 yielded 107, 134 and 131 isolates, respectively. The non-suppressive soils 2 and 3, and the Aphanomyces disease-indexing soil, yielded 49, 49 and 88 isolates, respectively (Table 2.1).

2.3.2 Isolation of bacteria from pea rhizosphere soils

In total, 146 bacterial isolates were recovered from the pea rhizosphere region. The upper half of the pea rhizosphere yielded 71 different bacterial isolates and the lower half 75 (Table 2.1).

Table 2.1: Sources of bacterial isolates

Soil sample [1]	**Number of individual bacteria isolated**
15 (*A. euteiches* suppressive)	107
18 (*A. euteiches* suppressive)	134
9 (*A. euteiches* suppressive)	131
2 (*A. euteiches* conducive)	49
3 (*A. euteiches* conducive)	49
A. euteiches indexing soil	88
PT (pea rhizosphere, top)	71
PB (pea rhizosphere, bottom)	75
Total	704

[1] Origins of soils are given in Appendix 1

2.3.3 Mycelial inhibition assay

Of the 558 bacterial isolates recovered directly from the soil samples, only three isolates (15·80, 18·25 and 18·94) produced zones of inhibition against *A. euteiches* mycelium in dual-culture bioassays (Table 2.2; Figure 2.2).

Of the 146 bacterial isolates recovered from the rhizosphere-regions of pea plants, 28 were found to inhibit the *in vitro* growth of *A. euteiches* mycelium to different degrees (Table 2.2). These were PB isolates 1, 16, 20, 26, 27, 30, 31, 34, 43, 45, 47, 53, 65, 67, 68 and 71, and PT isolates 1, 2, 4, 5, 9, 10, 17, 26, 27, 45, 58 and 69. The bacterial isolates 15·80 and PB 43 were the most inhibitory.

2.3.4 Zoospore inhibition assay

Each of the 31 effective bacterial isolates (Table 2.2) were able to reduce zoospore germination and subsequent growth, to some extent, when tested *in vitro*. Only seven isolates (15·80, 18·25, 18·94, PB 45, PT 1, PT 10 and PT 69) were inhibitory by 50% or more relative to the control treatments (Table 2.3; Figures 2.3, 2.4). Bacterial isolates 18·25 and 15·80 and PT 69 were the most inhibitory. Nutrient broth had no measurable effect on zoospore germination.

Table 2.2: Bacterial isolates exhibiting inhibition to mycelial growth of *A. euteiches* isolate 4.6 *in vitro*.

Bacterial isolate	Inhibitory distance[1]	Bacterial isolate	Inhibitory distance[1]
18·25	2.50	PB 1	2.75
18·94	1.50	PB 16	1.25
15·80	4.75	PB 20	2.50
PT 1	3.25	PB 26	1.50
PT 2	2.25	PB 27	2.75
PT 4	3.50	PB 30	1.50
PT 5	2.00	PB 31	1.00
PT 9	1.00	PB 34	1.25
PT 10	2.25	PB 43	4.75
PT 17	1.50	PB 45	2.00
PT 26	2.00	PB 47	3.50
PT 27	1.25	PB 53	3.75
PT 45	0.75	PB 65	1.00
PT 58	2.00	PB 67	2.00
PT 69	3.25	PB 68	2.00
		PB 71	1.50

[1] – Distance, in mm, between the edges of the bacterial and *A. euteiches* colonies. Each number is the average of four interaction zones.

Table 2.3: Effect of bacterial isolates on zoospore germination.

Bacterial isolate	**Zoospore germination score[1]**			**Bacterial isolate**	**Zoospore germination score**		
	Stock[2]	**10^{-2} dilution[3]**	**Total score**		**Stock**	**10^{-2} dilution**	**Total score**
18·25	1	5	6	PB 1	18	25	43
18·94	12	19	31	PB 16	27	27	54
15·80	19	0	19	PB 20	28	26	54
PT 1	8	18	26	PB 26	25	25	50
PT 2	26	26	52	PB 27	25	32	57
PT 4	13	22	35	PB 30	29	28	57
PT 5	17	30	47	PB 31	18	19	37
PT 9	22	28	50	PB 34	15	29	44
PT 10	6	15	21	PB 43	24	12	36
PT 17	26	24	50	PB 45	3	25	28
PT 26	13	20	33	PB 47	29	31	60
PT 27	22	24	46	PB 53	30	28	58
PT 45	19	23	42	PB 65	22	26	48
PT 58	17	22	39	PB 67	25	30	55
PT 69	4	15	19	PB 68	26	26	52
NB only	0	0	0	PB 71	16	21	37
NB + Z[4]	32	32	64	Water + Z[5]	32	32	64

[1] – Assessment of zoospore germination on a 0-4 scale. Scores are a total of four plates for each dilution, four observations per plate. Scores range from 0 = total inhibition to 32 = no inhibition of zoospore germination.

[2] – Overnight culture of bacterial isolates

[3] – Overnight bacterial culture diluted 100-fold

[4] – NB + zoospores

[5] – Water + zoospores

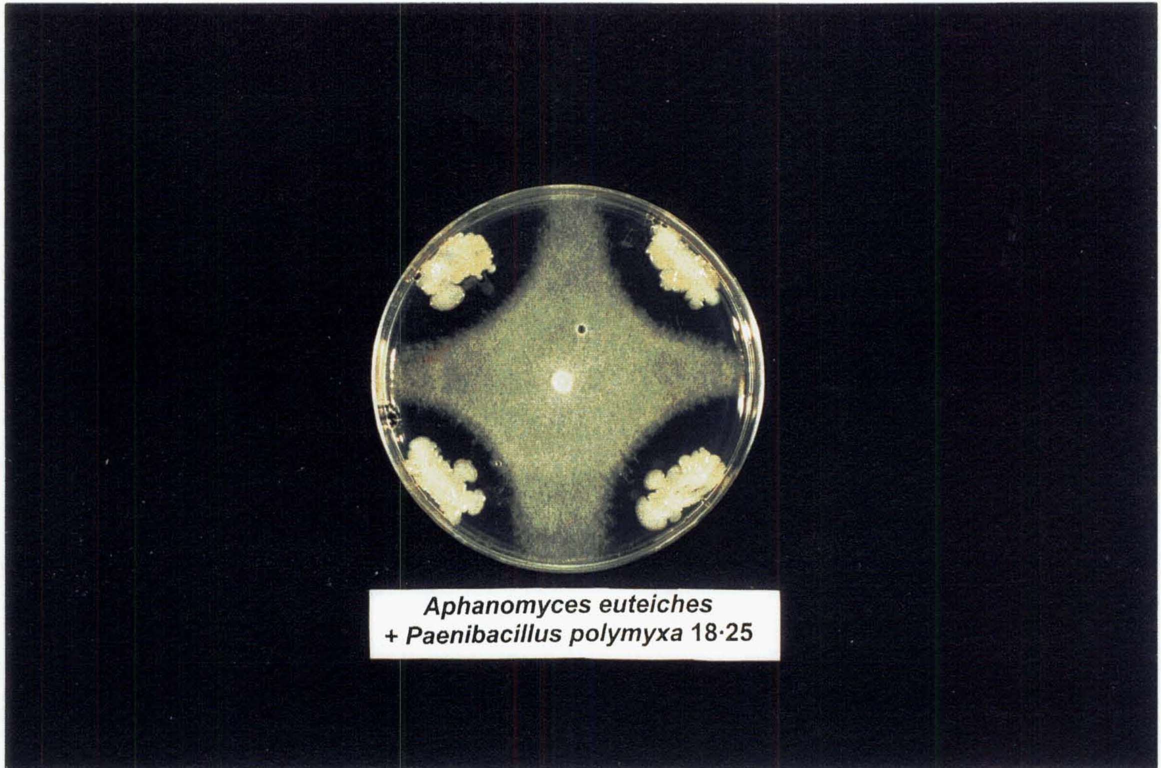

Figure 2.2: P. *polymyxa* 18·25 (corners) inhibiting the mycelial expansion of *A. euteiches.* (Culture conditions: PDA, 23°C, 5 days).

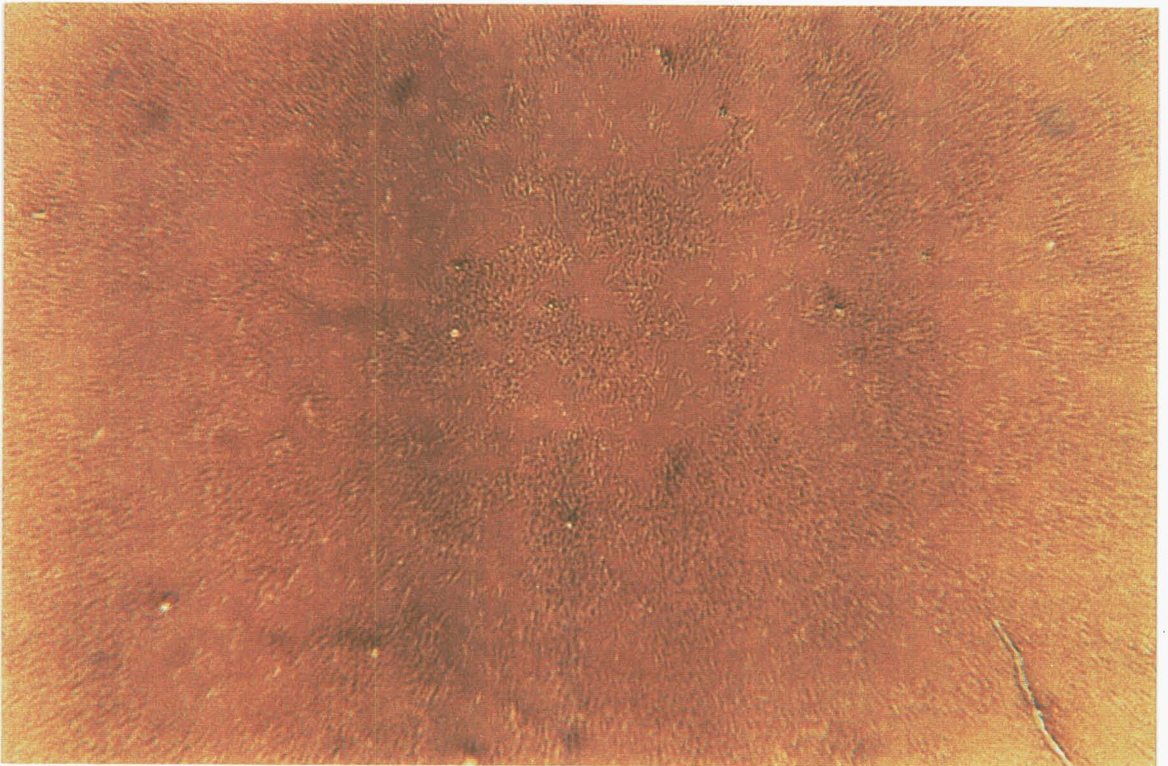

Figure 2.3: Suppression of zoospore germination by bacterial isolate 18·25. Circular bodies are encysted *A. euteiches* zoospores. (Culture conditions: PDA, 23°C, 3 days).

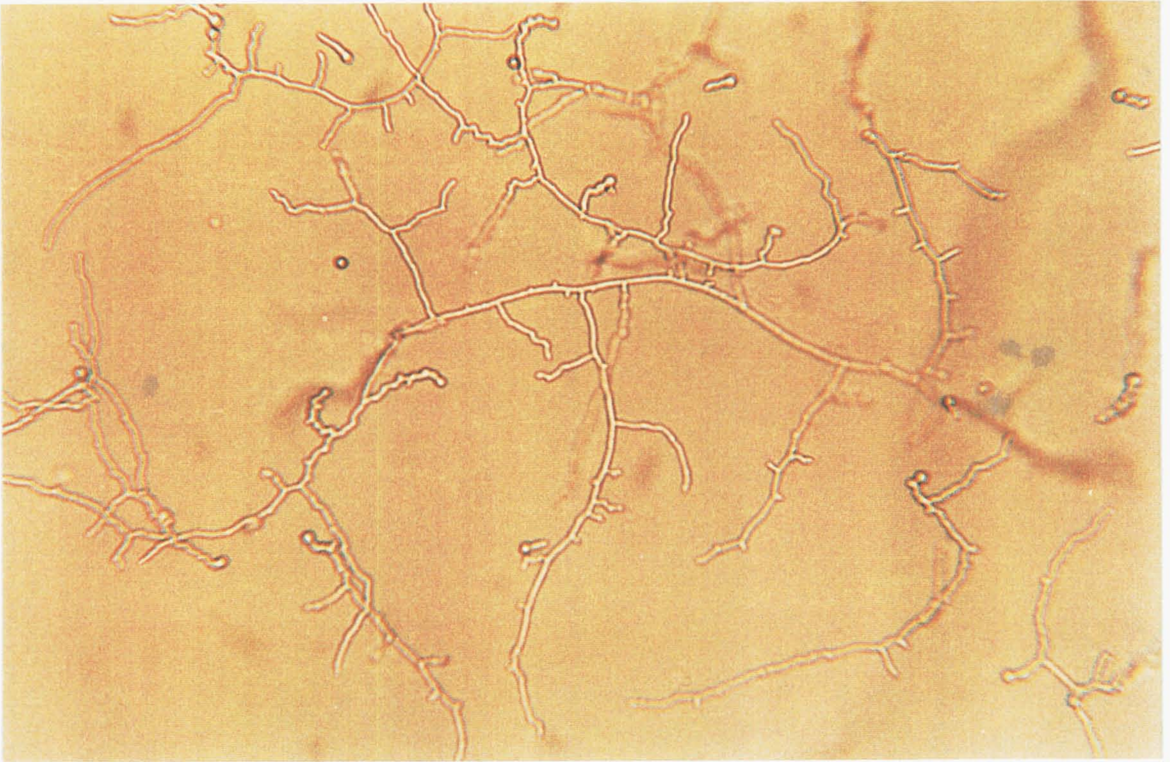

Figure 2.4: Non-suppression of zoospore germination and germ-tube growth by a unidentified bacterial isolate, PB 53. (Culture conditions: PDA, 23°C, 3 days).

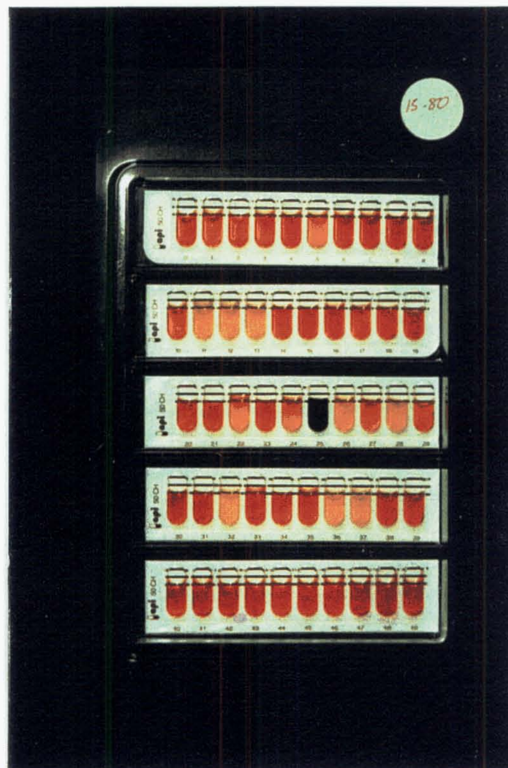

Figure 2.5: Carbohydrate-fermentation profile for bacterial isolate *Bacillus cereus* 15·80.

2.3.5 Identification of bacterial isolates

Each of the 31 bacterial isolates which were inhibitory to the mycelial growth of *A. euteiches* were found to be Gram positive, rod-shaped, endospore-producing bacteria capable of growth under aerobic conditions. Therefore, based on the scheme of Claus and Berkeley (1984), they were assigned to the bacterial genera *Bacillus* and *Paenibacillus.*

Isolates 15·80, 18·25, 18·94, PT 1, PT 10 and PT 69, and isolates MW 10, MW 12, MW 18, MW 27 (*A. euteiches*-antagonistic, spore-forming bacteria supplied by M. Walter, HortResearch Ltd., Lincoln), were fully identified using an API rapid 50 CHB microtubule system (bioMérieux). The identities are given in Table 2.4.

Table 2.4: Bacterial identifications based on multiple carbohydrate fermentation profiles (API 50 CHB).

Isolate number	Identification
15·80	*Bacillus cereus* type 1
18·25	*Paenibacillus polymyxa*
18·94	*Paenibacillus polymyxa*
PT 1	*Paenibacillus macerans*
PT 10	*Bacillus pumilus*
PT 69	*Bacillus subtilis*
MW 10	*Bacillus cereus* type 1
MW 12	*Bacillus pumilus*
MW 18	*Bacillus pumilus*
MW 27	*Bacillus mycoides*

The 16sRNA nucleotide sequence of bacterial isolate 18·25 (Appendix 2), was homologous with that of the *Paenibacillus polymyxa* 16SrRNA gene (GenBank accession number emb|AJ223989.1|PS16SCF43). The 16sRNA nucleotide sequence of bacterial isolate MW 27 (Appendix 2), was homologous with that of the *Bacillus mycoides* 16SrRNA gene (GenBank accession number emb|AJ223989.1|PS16SCF43). In both cases, greater than 99% of nucleotides had a perfect alignment with those contained on the database, thereby confirming their identities.

2.4 Discussion

Spore-forming bacteria were isolated from a broad range of soil samples, both directly from samples of field soil and from soil in the rhizosphere region of pea plants. Abundant spore-forming bacteria were found to be present in all soil samples, indicating that they are a well represented class of organism in agricultural soils. This corroborates other findings, which show that spore-forming bacteria are extremely common and well distributed in soil environments (Priest, 1989).

Isolates of spore-forming bacteria taken from the rhizosphere region of pea plants had a higher frequency of antagonism towards *A. euteiches* than those taken directly from soil: in the rhizosphere soil, 19% of the bacterial isolates were *A. euteiches*-suppressive, and in the non-rhizosphere soil, the proportion was only 0.5%. Similar frequencies of antagonism amongst rhizosphere microorganisms have been observed in earlier work. For example, Pandey *et al.* (1997) found that approximately 18% of the microorganisms isolated from tea-plant rhizospheres were antagonistic to pathogenic fungi and Berg (1996) found that 16 % of the 2,045 bacterial isolates recovered from the rhizospheres of oil-seed rape were antagonistic to *Verticillium dahliae* var. *longisporum* Stark.

The high frequency of antagonistic microorganisms found in the rhizosphere is probably a reflection of the highly competitive nature of this environment (Lynch, 1990). The rhizosphere zone is, by definition, an area of soil enriched in nutrients produced by the plant root, resulting in an abundance of microbial activity. Competitive mechanisms, such as the production of antibiotics, may be an effective mechanism by which a microorganism can capture or retain possession of substrates in such situations (Lynch, 1990). The high proportion of antibiotic producing isolates in the rhizosphere may also result from the selection of specific species of microorganisms, àdapted to grow on the host root exudates etc, which happen to have high rates of antibiotic production. In general, rhizospheres are considered to be useful sources of microorganisms which have biological control activity against root pathogens (Lynch, 1990). This is probably because they can compete effectively for the available resources and are adapted to living in close association with the host plant roots (Lebuhn *et al.*, 1997).

The dual culture method was selected as the initial assay of biological control activity because of its simplicity and the need to screen a large number of isolates. The secondary screen, based on zoospore germination and germ-tube growth suppression, was, in comparison, far more laborious to prepare (especially the zoospore inoculum), prone to contamination, and its analysis was based on an arbitrary scale. However, even given its limitations, the results of this assay were considered to be more reliable for predicting actual field potential of biological control candidates. This is because the production of zoospores, their movement through soil, aggregation at the root surface and germination, represents the most susceptible phases of the disease cycle (Papavizas and Ayers, 1974). The main phase of mycelial growth occurs post-infection, during the colonisation and destruction of the root tissue, but the pathogen does have a short mycelial phase immediately prior to infection: germ tube growth which occurs following zoospore aggregation on the root surface. Suppression of the mycelium, therefore, may lead to reduced ability of the pathogen to infect the plant root.

The suppression of the zoospore phase, however, is likely to have more relevance to potential for disease control than suppression of mycelial growth. Indeed, Teasdale *et al.* (1979a) showed that suppression of Aphanomyces root-rot on peas by dinitroaniline herbicides was related to the greater effect of the chemicals on zoospore activity than on the mycelial growth, although both phases were susceptible. Isolates that are strongly suppressive to both phases, therefore, should have an added advantage with respect to potential for control of the pathogen.

Isolates of spore-forming bacteria inhibitory to both the zoospore and mycelial phases of *A. euteiches* were found in *in vitro* assays. However, the relevance of *in vitro* assays to *in vivo* disease suppression has been questioned (Broadbent *et al.*, 1971; Knudsen *et al.*, 1997). Parke (1987) found a poor correlation between bacterial inhibition of *A. euteiches in vitro* and control of root-rot disease on peas. However, other evidence has supported the use of *in vitro* assays. For example, the artificial removal of the antibiotic producing genes in bacteria, which can be easily selected for in *in vitro*-type tests, resulted in the loss of biological control activity against *A. euteiches* (Carruthers *et al.*, 1994). Regardless of types and levels of anti-pathogen interactions observed *in vitro*, however, control of root diseases *in vivo* will only occur following successful establishment and survival in the root zone of the host plant (Paulitz, 1990). Therefore, there is a definite requirement to

undertake further testing *in vivo* of the biological control agents that perform best under the *in vitro* assays.

In this project, the bacterial isolates *P. macerans* PT 1, *B. pumilus* PT 10, unidentified bacterium PB 45, *B. subtilis* PT 69, *B. cereus* 15·80, *P. polymyxa* 18·25 and *P. polymyxa* 18·94, having exhibited dual modes of pathogen suppression *in vitro,* were selected for evaluation in the glasshouse for suppression of root-rot disease. These two genera (*Bacillus* and *Paenibacillus*) have been widely investigated as potential biological control agents of numerous soil borne plant pathogens. For example, various *Bacillus* spp. have been used for the control of *Aspergillus niger* Tiegh, *Rhizoctonia solani*, *Fusarium* sp., *Pythium* sp., *Botrytis cinerea* Pers., *A. euteiches, Gaeumannomyces graminis* var. *tritici*, and *Paenibacillus* sp. have been used for the control of pathogens such as *Pythium* spp., *G. graminis* var. *tritici,* and *Fusarium oxysporum* Schlecht (Asaka and Shoda, 1996; Brannen and Kenney, 1997; Capper and Campbell, 1986; Handelsman *et al.*, 1990; Hwang *et al.*, 1996; Parke, 1987; Podile and Prakash, 1996; Walker *et al.*, 1998). In many instances, the mode of action of these bacteria, is through the production of anti-fungal compounds such as antibiotics and/or cell wall degrading enzymes (Asaka and Shoda, 1996; Brannen and Kenney, 1997; Campbell, 1983; Leifert *et al.*, 1995; Nielsen and Sörensen, 1997; Podile and Prakash, 1996; Milner *et al.*, 1996). At least one isolate, a strain of *B. subtilis*, has been successfully commercialised, and is now widely available under the label Kodiak® (Gustafson Inc.) (Brannen, 1998; Brannen and Kenney, 1997).

Chapter 3 Glasshouse assessment of selected antagonistic bacteria for control of Aphanomyces-disease on pea

3.1 Introduction

Although selection of isolates based on *in vitro* testing is a useful way to identify pathogen-suppressive strains from a large background of general soil bacteria, it does not address any of the other factors previously identified as being important in the biological control of *A. euteiches* (i.e. compatibility with the pea root and potential to colonise and survive in the rhizosphere soil). However, it is also impractical to use field based trials as a screening approach for biological control activity. To bridge *in vitro* selection based on pathogen antagonism and potential field-based biological control activity, plant-based glasshouse trials are commonly employed (Knudsen *et al.*, 1997).

As well as selecting for isolates with potential to reduce disease, glasshouse assays have the added advantage of being able to identify isolates which have no potential for biological control development. This may result, for example, through incompatibility with the plant-root or soil environments. *In vitro* selection for organisms capable of pathogen suppression may result in selection of strains which cause plant disease; either a primary pathogen or one which acts with another disease causing agent.

Compared with *in vitro* assays, glasshouse trials offer identification of biological control activity based on disease suppression *in planta*, with the soil environment as a component of the assay. The main advantage over field-based evaluation is the potential to develop a system / assay to give a standardised level of disease pressure. This is particularly important for pathogens such as *A. euteiches* where its distribution in field soils is notoriously heterogeneous (J. Kraft; pers. comm.). This allows for the screening of multiple biological control candidates under standardised conditions.

The type of pathogen inoculum used in glasshouse trials may have a considerable bearing on the development of disease and subsequent results of the trial (Simon *et al.*, 1987) and should, therefore, be considered carefully. For glasshouse trials involving *A. euteiches*, artificially-produced inoculum in the form of zoospores (Parke, 1987; Parke *et al.*, 1991) and oospores (Persson *et al.*, 1999) are most commonly used. These forms of inocula, especially zoospores, have the advantage of being easy to produce and provide propagules of even size and infectivity which give reproducible levels of disease. However, as the main reason for conducting glasshouse trials is to accurately represent field conditions, naturally produced inoculum, i.e. infested field soil, may be a better choice. In this form, pathogen inoculum would have the advantages of containing a mixture of pathogenic strains of *A. euteiches* and introducing a range of soil microflora which the biological control agent must cohabit with. This adds a higher level of stringency in the glasshouse trial, the results of which may more accurately predict field performance.

This chapter describes the development of a plant-based glasshouse trial system for assessment of biological control agents against Aphanomyces root-rot of pea, using field soil as a natural source of pathogen inoculum, and its subsequent use to screen spore-forming bacteria for disease control potential.

3.2 Material and methods

3.2.1 Identification of a suitable soil as an inoculum source of *A. euteiches*

3.2.1.1 Soils

Sources of infested soil were: “Chapmans’ block”, Lincoln (formally owned by HortResearch and known to be infested with *A. euteiches*), a farm at Southbridge which had lost peas to a non-identified root disease in the previous season (R. Cawood, HeinzWatties Ltd.; pers. comm.), and as a negative control, a Lincoln farm which had no history of pea cropping. From each field, soil samples were taken from numerous positions, to a depth of 10 cm, and mixed together into a bulk sample. Samples were taken from each of the bulk, mixed samples, and mixed with soil-less potting mixture at ratios of 10:0, 8:2, 6:4, 4:6 and 2:8 (soil : potting mix, volume / volume).

3.2.1.2 Glasshouse assay

For each soil at each dilution rate, four PB¾ bags were filled with the soil / potting mix mixture and planted with six pea seeds (cv. Whero) which had been surface disinfected (0.5% NaOCl for 2 min, followed by 3 rinses with tap water). Each bag was placed into a 2 L plastic container and these were randomly arranged in a glasshouse (Figure 3.2). Each treatment, consisting of a bag containing six pea seeds, was present once in each of four blocks. Initially, the soil / potting mixture media was kept moist to promote seedling germination and growth. However, from 2 weeks after planting until harvest, soil conditions were kept saturated by maintaining a 2-3 cm deep level of water at the bottom of each container, therefore favouring infection and disease by *A. euteiches*.

3.2.1.3 Data collection and analysis

Four weeks after planting, the pea plants were removed from the bags and assessed for disease. The roots were washed free of adhering soil and potting mix and the level of disease scored on a 0-4 scale (Figure 3.1):

0 = No symptoms; roots healthy and white.

1 = Initial symptoms of root rot disease; discolouration, usually a light tan colour, in sections of the root system.

2 = Discolouration of most or all of the root system, usually still of a tan colour. Small watery lesions may be present on the root and around the hypocotyl / epicotyl regions.

3 = Advanced disease symptoms. Dwarfing of the plant and yellowing of the lower leaves. Extensive darkening and discolouration of the root system and extensive lesion formation;

4 = root entirely rotted / plant dead.

As the values for the disease scoring are based on an arbitrary scale (i.e. are not truly quantitative) non-parametric data analysis was used. To this extent, the median disease scores were found for each treatment replicate and the average median disease score determined (i.e. average of the median scores over the replicates). Pair-wise comparisons were made between the nil-control and each other treatment using the Mann-Whitney *U*-test in the SAS computer package.

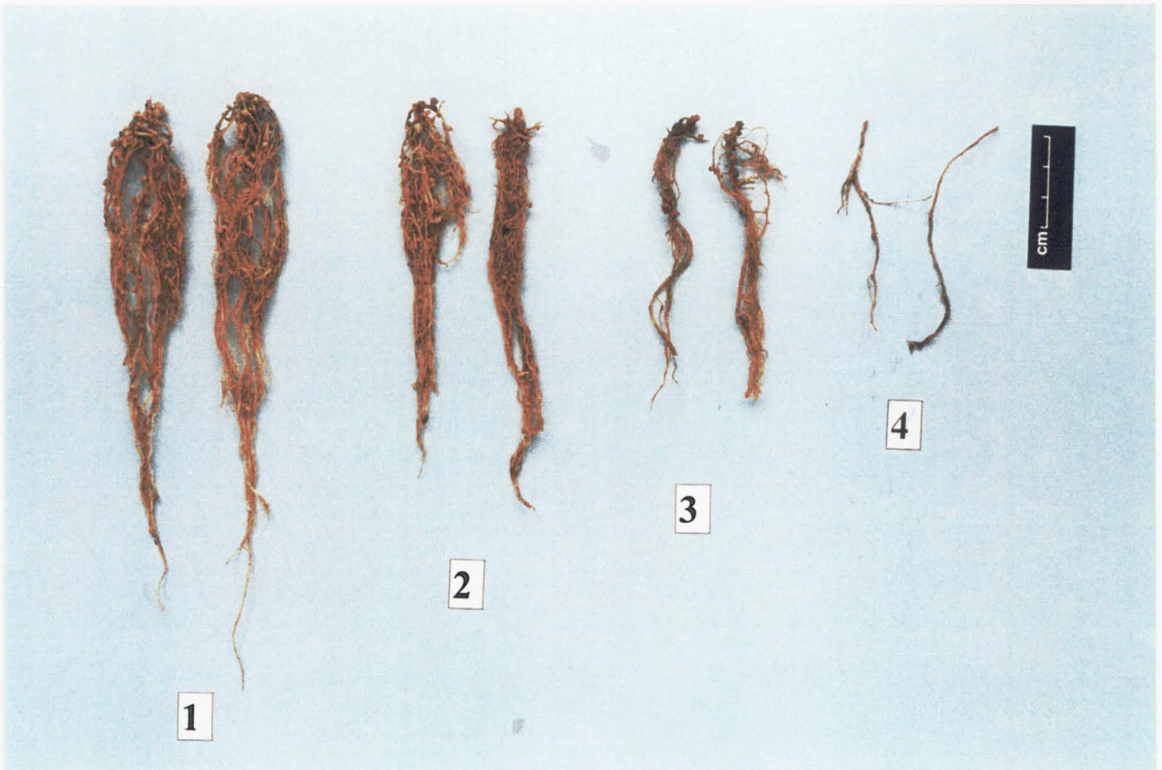

Figure 3.1: Aphanomyces root-rot disease symptoms on pea roots.

Figure 3.2: Typical glasshouse trial setup. Pea seeds are planted into plastic bags containing potting mixture. Each bag is placed into a 2 L 'ice-cream' container. Water is maintained to a 2-3 cm depth in the bottom of the container, saturating the soil and promoting disease.

3.2.2 Effect of soil dilution and watering rates on disease expression in Chapmans' soil

3.2.2.1 Soil

Soil from the Chapmans' block, identified in the previous assay as a good inoculum source of root-rot disease (Section 3.3.1), was collected as described before (Section 3.2.1.1). Samples of the soil were mixed with potting mixture at ratios of 4:6, 6:4 and 8:2 (volume soil : volume potting mix). Each pot then received water at either a high rate (as in the previous experiment) or at a medium rate (watering to keep soil moist but not saturated). Therefore, six treatments were tested.

3.2.2.2 Glasshouse assay

The glasshouse assay was set up in a similar manner to that previously described (Section 3.2.1.2), except that the replication rate was increased to six blocks. Initially, all treatments were watered moderately from above whilst being allowed to drain freely. Two weeks after planting, however, treatments receiving the 'high' watering rate were kept at saturation by standing the planter bags in 2 L containers containing water to a 2-3 cm depth (i.e. as described in Section 3.2.1.1). Treatments receiving 'moderate' watering rate were watered as required to keep the potting mixture moist but not saturated.

3.2.2.3 Data collection and analysis

Four weeks after planting, the pea plants were removed from the bags and assessed for disease as before (Section 3.2.1.3). Sections of root tissue were taken from seedlings exhibiting typical symptoms of root-rot disease, and *A. euteiches* was isolated using the method described in Section 2.2.3 for positive identification. Analysis of variance (ANOVA) was used to determine if the watering regimes affected disease development.

3.2.3 Glasshouse assessment of antagonistic bacteria – trial 1

3.2.3.1 Source of biological control bacteria

The seven bacteria found to be antagonistic to *A. euteiches* in this project (Chapter 2), and five isolates previously identified as *A. euteiches*-suppressive (Wakelin *et al.*, 1998), were assessed under glasshouse conditions prior to field testing. Due to the high number of isolates to be tested, they were divided into two batches and tested for disease suppression over two separate assays. In assay 1, the spore-forming isolates *Paenibacillus polymyxa* 18·25, *P. polymyxa* 18·94, *B. cereus* 15·80, *P. macerans* PT 1, *B. pumilus* PT 10, *B. subtilis* PT 69 and the unidentified isolate PB 45 were evaluated, and in assay 2 the isolates *B. cereus* MW 10, *B. cereus* MW 12, *B. pumilus* MW18, *B. mycoides* MW 27, and the unidentified isolate MW 9B were evaluated.

3.2.3.2 Soil / potting mixture

Soil taken from the Chapmans' Block, HortResearch, Lincoln, was used as a natural inoculum of *A. euteiches*. The soil was sampled as before (Section 3.2.1.1) and diluted 4:6 (v/v) with a potting mixture (SouthHort Ltd., Rolleston) containing neither fertiliser nor fungicides.

3.2.3.3 Seed treatment with bacteria

Bacterial isolates were taken either from storage at -80°C (assay 1) or from storage on NA slopes at 4°C (assay 2) and cultured on five Petri dishes containing NA amended with $MnSO_4$ (30 mg L^{-1}) and glucose (10 g L^{-1}) (Priest, 1989). After 10 days growth at 30°C, bacteria were scraped from the surface of the agar plates into 10 mL of potassium phosphate-buffered saline (PBS; 7 g NaCl, 11.5 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4, H_2O 1 L), were pelleted by centrifugation for 20 min at 16 000 × g, and re-suspended in a further 5 mL of PBS. Each suspension of bacteria was transferred to a sterile Universal bottle and 0.1 g of methyl-cellulose (low substitution; BDH Limited, Poole, England) added as a sticking agent. Thirty-six pea seeds (cv. Whero), surface disinfected as before (Section 3.1.1.2), were placed into each bottle and mixed thoroughly in the suspension for 5 min. The bacterial suspensions and seeds were poured into Petri dishes lined with Whatmans #1 filter paper and the seeds allowed to dry in a laminar-flow cabinet overnight.

3.2.3.4 Other treatments: Biologicals, fungicides and controls

The fungicides Apron and Tachigaren and the biological control agent Kodiak were applied to seeds at recommended rates as experimental standards. Tachigaren (hydroxyisoxazole) was applied at 5 mL Kg^{-1} seed, 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 and Kodiak (Gustafson; *Bacillus subtilis* GB03) was applied at 2 g Kg^{-1} seed. Moisture on the pea seeds following surface disinfestion allowed the dry products to stick to them. Two control treatments were included in each assay; pea seeds surface disinfected only (nil-control) and surface-disinfected pea seeds treated with methyl-cellulose in PBS (carrier control).

3.2.3.5 Determination of bacterial load per seed

The number of bacterial c.f.u. $seed^{-1}$ was determined. Five pea seeds, randomly selected from each treatment, were placed into a Universal bottle containing 10 mL of PBS and shaken vigorously on a wrist-action shaker for 20 min. A series of dilutions were made from each treatment (to 10^{-6}) and 0.1 mL of each dilution was 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.

3.2.3.6 Growth of peas

Plastic planter bags (PB¾) were filled with the soil mix (Section 3.2.3.2), planted with six treated pea seeds, and placed inside 2 L plastic containers in the greenhouse. After germination, each pot was thinned to five seedlings. The pots were kept moist by overhead watering for the first 3 weeks after planting. Thereafter, the pots were kept saturated as before (Section 3.2.1.2), for a further 2 weeks.

3.2.3.7 Experimental design

Treatments were arranged in a randomised-block design in the glasshouse. There were 12 treatments in assay one and 10 in assay two. Each treatment was randomly represented once (a pot containing five treated pea seeds) in each of five blocks.

3.2.3.8 Data collection and analysis

The germination rate of peas in each treatment was determined 14 days after planting. At the conclusion of each assay (5 weeks after planting) the pea plants were removed from the pots and assessed for disease. The roots of each plant were washed under running water to remove all soil and the level of disease scored visually on a 0-4 scale (Section 3.2.1.3.). Data analysis on the disease score data was carried out as described before (Section 3.2.1.3).

3.2.4 Glasshouse assessment of antagonistic bacteria – trial 2

The experiment was repeated using the same protocol as previously described with minor modifications. To increase the load per seed of the slower-growing isolates, *P. polymyxa* 18·94 and *P. polymyxa* 18·25 were cultured on to five Petri plates each and the other isolates on to three. Visual disease assessments were made and the resultant data analysed as before (Section 3.2.1.3). In addition, roots were weighed after blotting dry with paper towels and the number of oospores per gram of root tissue measured. For each plant, a one gram sample of frozen root tissue was comminuted in 10 mL of sterile water with an Ultra-Turrax probe macerator and the number of oospores were counted with a haemocytometer under a compound microscope. Twenty counts were made for each sample and the average number of oospores determined. Dàta were analysed using ANOVA and treatment means separated with LSD. Pearsons correlation coefficients were calculated to determine the presence of any relationships between the variables measured.

3.3 Results

3.3.1 Identification of a suitable soil as an inoculum source of *A. euteiches*

Soil type affected the development of disease on pea roots (P=0.0001): only peas planted in the Chapmans' soil developed appreciable levels of root rot disease under glasshouse conditions (Table 3.1). Peas planted in the soil from Southbridge or from the farm which had no previous pea cropping history (no-pea soil), only exhibited very slight root-rot disease symptoms, such as small areas of light discoloration on the roots. Chapmans' soil was, therefore, selected for further development as an inoculum source of *A. euteiches* for glasshouse assays. Dilution of the soils with different ratios of potting mixture reduced disease expression (P=0.0312).

Table 3.1. : Root-disease scores[1] for peas in three soil types at different ratios with potting mixture.

Soil : potting mixture	Chapmans' soil	Southbridge soil	No-pea soil
10 : 0	2.75	1.00	0.50
8 : 2	3.00	1.00	0.50
6 : 4	2.75	0.75	0.25
4 : 6	3.00	0.25	0.25
2 : 8	2.00	0.25	0.00

[1] Average median disease score. Medians were taken from four replicates of six plants each on a 0-4 disease severity scale (Section 3.2.1.3).

3.3.2 Effect of dilution and watering rates on disease expression (Chapmans soil)

The most significant factor affecting disease expression in the Chapmans soil was the amount of water the plants received (P=0.0001). Peas in the high water treatment developed appreciable levels of disease whilst those in the moderate water treatment had low levels of disease symptoms (Table 3.2; Figure 3.3). In contrast to the previous experiment (Section 3.3.1), dilution of the soil with potting mixture was found to have little effect on the development of root rot disease on peas (P=0.1112). Disease symptoms on pea roots were typical of those expected when *A. euteiches* is the major pathogen, i.e. honey to tan coloured, water-soaked lesions and stunting of the plant.

Figure 3.3: Effect of watering on disease expression in Chapmans' soil. "High" watered plants (top) have darkening and reduction of the root mass, and yellowing of the lower leaves. "Medium" watered plants (below) exhibited early root rot symptoms (honey coloured discoloration).

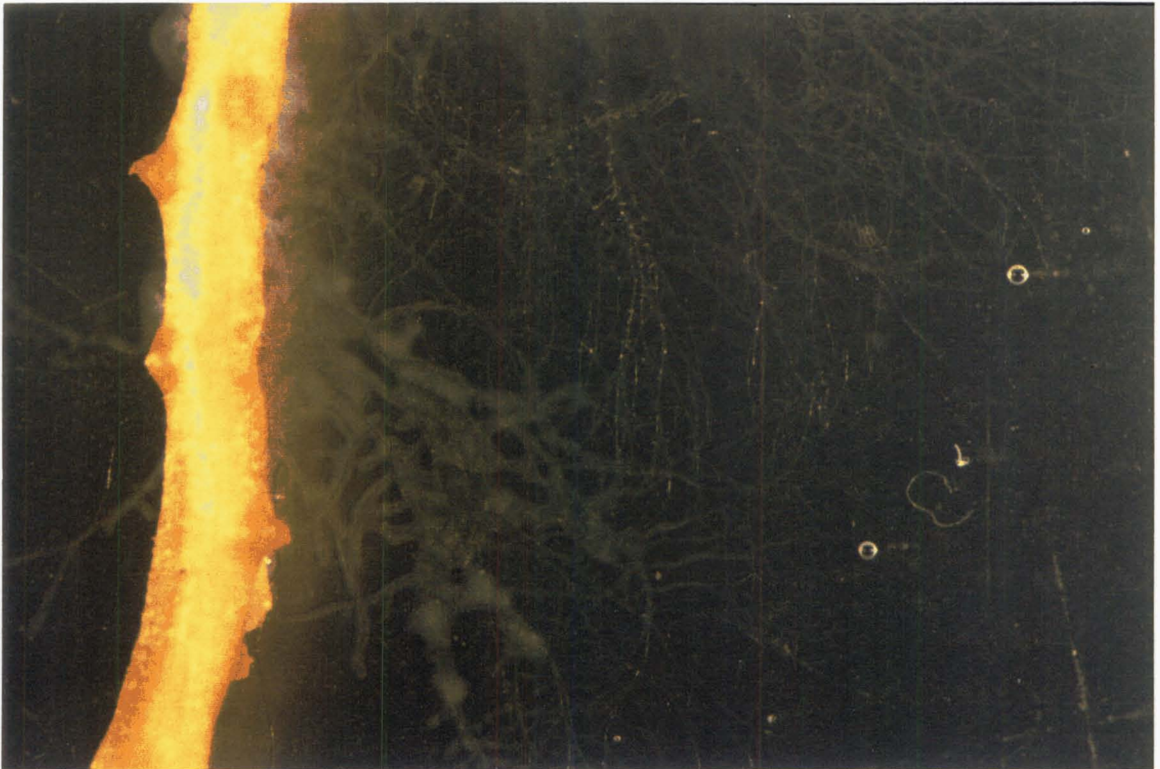

Figure 3.4: Isolation of *A. euteiches* from infected pea root tissue onto CMA. Bacterial contamination can be observed around most of the hyphal growth adjacent to the section of root.

Table 3.2: Effect of watering and soil dilution on disease development on pea roots planted in Chapmans' soil.

Treatment	Soil : potting mixture ratio		
	4:6	**6:4**	**8:2**
High watering[1]	2.5[2]	3.0	3.25
Moderate watering[1]	0.0	0.0	0.75

[1] For definitions, refer to Section 3.2.2.2

[2] Average median disease score. Medians were taken from six replicates of six plants each on a 0-4 disease severity scale (Section 3.2.1.3).

Aphanomyces euteiches was recovered from root tissue exhibiting characteristic symptoms of Aphanomyces root-rot. Identification was based on the ability of the isolates to grow on metalaxyl-amended agar (Figure 3.4), and microscopic examination of hyphae, oogonia and oospores as described by Scott (1961).

3.3.3 Glasshouse assessment of antagonistic bacteria – trial 1

3.3.3.1 Assay one

The results of glasshouse trial 1, assay one, are given in Table 3.3.

The c.f.u. seed^{-1} was different amongst treatments (Table 3.3). Control treatments, including fungicides, had up to 100 c.f.u. per seed. On treatments receiving bacterial formulations (including Kodiak), bacterial counts ranged from 3×10^4 c.f.u. seed^{-1} (*P. polymyxa* 18·25) to 5×10^8 c.f.u. seed^{-1} (unidentified bacterium PB 45).

Apron and *B. cereus* 15·80-treated plants were found to have significantly lower disease scores when compared pair-wise (Mann-Whitney *U*-test) with the nil-control (Table 3.3). The median disease score for the nil-control plants was 2.0, whereas for the Apron and *B. cereus* 15·80 treatments it was 1.4.

Treatment of pea seeds did not affect seed germination ($P<0.05$).

3.3.3.2 Assay two

The results of glasshouse trial 1, assay two, are given in Table 3.3.

As in the previous assay, the bacterial loading per seed was different amongst treatments (Table 3.3). Control treatments, including fungicides, had up to 2×10^3 c.f.u. seed^{-1}. On treatments receiving bacterial formulations (including Kodiak), bacterial counts ranged from 1×10^4 (unidentified bacterium MW 9b) to 5×10^9 (*B. mycoides* MW 27) c.f.u. seed^{-1}.

Although pea seed treatment reduced the median disease scores of pea plants relative to the nil-control, no differences were found to be significant when pair-wise comparisons were made (Mann-Whitney *U*-test; α=0.05). Nevertheless, seed treatment with the bacterial isolate *B. pumilus* MW 18 had an equivalent effect on disease reduction as did the fungicide Apron (scores of 1.6) and treatment with *B. mycoides* MW 27 was more effective than the fungicide Tachigaren and the commercial biological control product Kodiak (Table 3.3).

Treatment of pea seeds did not affect seed germination ($P<0.05$).

Table 3.3 Results from glasshouse trial 1, assays one and two.

Treatment	C.f.u. / seed[1]	Average disease score[2]
Assay one		
Nil-treated	2×10^1	2.0
Buffer and sticker	1×10^2	2.0
B. pumilus PT 10	8×10^6	2.0
P. macerans PT 1	8×10^6	1.9
Unidentified bacterium PB 45	5×10^8	1.8
P. polymyxa 18·94	3×10^5	1.8
P. polymyxa 18·25	3×10^4	1.8
Tachigaren	0	1.8
B. subtilis PT 69	1.5×10^8	1.8
B. subtilis 'Kodiak'	9×10^6	1.6
Apron C70SD	1×10^2	1.4*
B. cereus 15·80	3×10^8	1.4*
Assay two		
Nil-treated	2×10^2	2.0
Buffer and sticker	2×10^2	2.0
Unidentified bacterium MW 9B	1×10^4	2.0
B. cereus MW 10	2.5×10^7	2.0
B. pumilus MW 12	8×10^8	1.8
Tachigaren	2×10^3	1.8
B. subtilis 'Kodiak'	1.5×10^8	1.8
B. mycoides MW 27	5×10^9	1.8
Apron C70SD	1.5×10^3	1.6
B. pumilus MW 18	1.5×10^8	1.6

[1] Bacterial colony forming units per seed.

[2] Average median disease score. Medians were taken from five replicates of five plants each on a 0-4 disease severity scale (Section 3.2.1.3).

* Significantly different ($P<0.05$) from the nil-control (Mann-Whitney *U*-test).

3.3.4 Glasshouse assessment of antagonistic bacteria – trial 2

3.3.4.1 Assay one

The results for glasshouse trial 2, assay one, are given in Table 3.4.

Bacterial loadings per seed did not differ as much as in trial 1, with c.f.u. counts ranging from 1.6×10^6 (*P. polymyxa* 18·94) to 4×10^8 seed^{-1} (*P. macerans* PT 1). The bacterial load for the control treatments ranged from 300 to 400 c.f.u. seed^{-1}.

Overall, there were no significant differences in root rot disease scores for the different treatments ($P<0.05$). However, all treatments except for *B. subtilis* PT 69 and *B. pumilus* PT 10 reduced the levels of disease relative to both the buffer and sticker control and the nil-control (Table 3.4).

Seed treatment significantly affected the mean root weight ($P=0.015$). Peas treated with the fungicide Apron had significantly higher root weights than all other treatments, and those treated with *P. polymyxa* 18·25 were significantly higher than those of *B. subtilis* PT 69, for which disease scores were highest and root weights were lowest (Table 3.4).

Seed treatment also significantly affected the number of oospores per gram of root tissue ($P=0.012$) (Figure 3.5). The number of oospores per gram of root tissue was significantly lower in peas treated with *B. pumilus* PT 10, *P. polymyxa* 18·25, *P. macerans* PT 1 and Tachigaren than the buffer control and *B. subtilis* PT 69 treated peas. Peas treated with the fungicide Apron, *P. polymyxa* 18·25, *P. macerans* PT 1 and Tachigaren consistently ranked better (i.e. less root disease, fewer oospores gram^{-1} of root and greater total root weight) than other treatments, including controls (Table 3.4). However, it was difficult to demonstrate significant levels of differences between the best treatments and others.

A significant positive correlation ($P=0.0081$; $R^2=0.34$) was found between the disease score and oospore number gram^{-1} of root weight. The root weight and disease score were found to be significantly negatively correlated ($P=0.001$; $R^2=-0.56$), as were the root weight and oospore numbers gram^{-1} of root weight ($P=0.04$; $R^2=-0.27$).

3.3.4.2 Assay two

The results for glasshouse trial 2, assay two, are given in Table 3.4.

As in the previous assay, there was less difference in the bacterial loading per seed amongst the treatments receiving bacterial inoculation than in trial one. Amongst the controls, the c.f.u. seed^{-1} ranged from 'not detectable', i.e. no bacterial colonies emerging on the Petri dishes, to 500. Amongst the bacterial treatments, including Kodiak, the c.f.u. counts ranged from 1.6×10^6 (*B. mycoides* MW 27) to 2×10^8 seed^{-1} (*B. pumilus* MW 12).

Seed treatment with Apron, Kodiak and *B. pumilus* MW 18 significantly reduced the median disease score relative to the nil-control plants (Mann-Whitney *U*-test; α=0.05). The median disease score of plants receiving the fungicide (Apron) treatment was 0.4, for plants receiving either of the two bacterial treatments (Kodiak or *B. pumilus* MW 18) 1.0, and for the nil-control plants, 1.8. Although plants treated with *B. mycoides* MW 27 also had a median disease score of 1.0, the large variation between treatment replicates, compared with Kodiak or *B. pumilus* MW 18, meant that this treatment was not significantly different from the nil-control.

The weight of pea roots was affected by seed treatment (P=0.0001). Plants treated with the fungicides Apron and Tachigaren had significantly heavier roots (approximately double) than those of both controls. The root weights of *B. pumilus* MW 12 and *B. mycoides* MW 27 were significantly heavier than the buffer and sticker control, but not the nil-control treatment.

Although there was a large variation in the number of oospores per gram of root tissue between treatments (from 470 spores g^{-1} for Apron, to 70 spores g^{-1} for *B. mycoides* MW 27) (Table 3.4), the difference between treatments was not significant (P=0.22). Overall, the numbers of oospores per gram of root tissue did not correlate with either the disease score or root weights. A significant (P=0.0001) negative correlation (-0.76) was found between the average disease scores and root weights.

Several treatments effective at reducing the disease score and increasing root weights relative to control treatments, such as Apron and Tachigaren, did not reduce the oospore counts per gram of root tissue. *Bacillus mycoides* MW 27 and *B. pumilus* MW 12 were effective across all variables measured; but not necessarily at statistically significant levels.

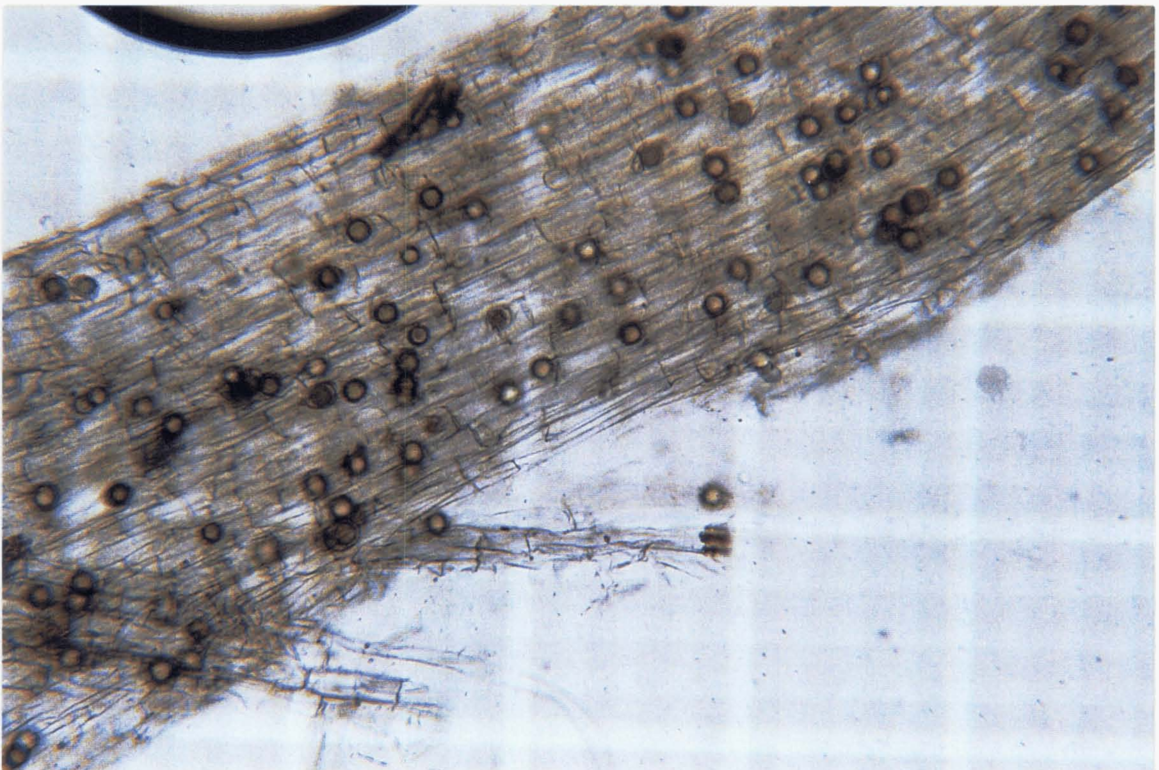

Figure 3.5: Oospores of *A. euteiches* amongst homogenised pea root tissue on a haemocytometer.

Table 3.4: Results from the 1999 glasshouse trial 2, assays one and two.

Treatment	C.f.u. / seed[1]	Disease score[2]	Root weight (g)	Oospores g^{-1} root
Assay 1				
Nil-treatment	3×10^2	1.2	0.93 bc	450 bc
Buffer and sticker	3×10^2	1.6	0.96 bc	540 ab
B. subtilis PT 69	9×10^7	1.8	0.72 c	816 a
B. pumilus PT 10	2×10^8	1.4	0.98 bc	150 c
Bacterium PB 45	5×10^7	1.2	0.80 bc	350 bc
P. polymyxa 18-94	1.6×10^6	1.6	0.78 bc	280 bc
B. cereus 15-80	5.2×10^7	1.0	1.05 bc	370 bc
B. subtilis 'Kodiak'	2.5×10^7	1.0	1.02 bc	260 bc
Tachigaren	2×10^2	1.2	1.08 bc	190 c
P. macerans PT 1	4×10^8	0.8	1.09 bc	180 c
P. polymyxa 18-25	3×10^6	1.0	1.13 b	160 c
Apron C70SD	4×10^2	0.6	1.58 a	230 bc
LSD (*P*<0.05)			0.39	330
Assay 2				
Nil-treatment	not detected	1.8	0.82 cde	410
Buffer and sticker	not detected	1.8	0.60 e	350
Bacterium MW 9B	6.2×10^7	1.4	0.75 de	180
B. cereus MW 10	6×10^6	1.4	0.84 cde	110
B. subtilis 'Kodiak'	6.5×10^7	1.0*	0.82 cde	320
B. pumilus MW 18	7×10^6	1.0*	0.86 bcde	380
Tachigaren	2×10^2	1.2	1.17 b	250
B. pumilus MW 12	2×10^8	0.8	1.08 bc	170
B. mycoides MW 27	1.6×10^6	1.0	0.98 bcd	70
Apron C70SD	5×10^2	0.4*	1.51 a	470
LSD (*P*<0.05)			0. 31	

[1] Bacterial c.f.u. $seed^{-1}$.
[2] Average median disease score (Section 3.2.1.3). Values based on 5 replicates of 5 plants.
* Treatments significantly (P<0.05) different from the nil control using the Mann-Whitney *U*-test.
Treatment means followed by the same letters are not significantly different (LSD; α=0.05).

3.4 Discussion

A biological control assay was developed under glasshouse conditions to select isolates for ongoing evaluation under field conditions.

During the development of the assay, it was necessary to screen several soils for the presence of the root-rot pathogen, *A. euteiches*. From three soils, Chapmans' soil was selected as a source of inoculum for future trials. This soil had previously been found to harbour the disease (Wakelin *et al.*, 1998). Recovery of *A. euteiches* from the roots of peas grown in the soil re-affirmed the presence of the pathogen.

The development of small necrotic lesions on the roots of peas grown in the Southbridge soil may have been attributable to many root-rotting fungi, bacteria, or nematodes (Hagedorn, 1984). As the saturated soil conditions under which the peas were grown are known to favour Aphanomyces root-rot (Parke and Grau, 1992), which was common on the roots of peas grown in the Chapmans' soil under identical conditions, it is likely that *A. euteiches* was absent from this soil.

Although the highest disease levels resulted from the highest ratio of soil to potting mixture, an undesirable caking effect occurred in the planter bags with increasing proportions of soil. This made the separation of weak, diseased roots from the mixture difficult. In addition, higher soil:potting mixture ratios may have provided too much disease pressure, making it difficult to observe biological control activity by the bacterial treatments. The 4:6 ratio of soil to potting mixture was therefore selected as the best for the glasshouse screening of bacteria.

The presence of free soil water is necessary for the pathogen to grow and cause disease (Papavizas and Ayers, 1974; Pfender, 1984). Therefore, it was not surprising that the high watering regime significantly increased disease development.

Taken together, Chapmans' soil at the 4:6 dilution rate, along with the high watering rate, was chosen as the optimum conditions to test the bacterial isolates. When non-treated peas were planted in such conditions, they had discolouration of most or all of the root system and usually exhibited some stunting compared with plants in pathogen-free soil. When examined microscopically, their roots were found to contain approximately 400 oospores $gram^{-1}$ of tissue.

In this study, soil was found to provide a uniform source of pathogen inoculum. Chapmans soil at the 4:6 dilution rate, caused non-treated peas to develop approximately 50% root-rot disease symptoms, which was found to be quite consistent between assays. In other glasshouse trials involving *A. euteiches,* artificially produced propagules of the fungus, i.e. oospores or zoospores, were used as a source of pathogen inoculum (Parke *et al.*, 1991; Parke and Grau, 1992; Persson *et al.*, 1999). The rationale behind using artificially as opposed to naturally produced inoculum, is to provide reproducible levels of disease pressure. However, as shown by this work, this can also be achieved by using infested soil. Moreover, the presence of different strains of *A. euteiches* and other pathogenic fungi in soil, provides a disease pressure more similar to that experienced in the field. Therefore, results from glasshouse trials using soil as a source of pathogenic inoculum should correlate more closely with field results than those achieved from using artificially produced inoculum. The major disadvantage of using soil, however, was the introduction of other pathogens which confounded efforts to determine the level of Aphanomyces root rot disease.

The numbers of cells inoculated onto seeds is likely to affect a bacterium's potential for biological control activity. For each bacterial isolate, this activity is likely to range between a threshold level under which no activity will occur and a maximum, over which there may be no further increase in biological control activity (saturation level) or plants may even be damaged by the bacteria. Parke *et al.* (1991), when attempting biological control of *A. euteiches*, applied a *Pseudomonas* sp. at 10^7 to 10^8 c.f.u. pea $seed^{-1}$. In this study, however, several isolates (especially *P. polymyxa* 18·25 and 18·94) were applied at only 10^4 to 10^6; well below the dose given for the *Pseudomonas* sp., and probably too low to be effective for *Bacillus* or *Paenibacillus* sp. as they are generally considered to have a slower growth rate than *Pseudomonas* sp. (Weller, 1988). The large variation in c.f.u.'s between bacterial treatments in the first glasshouse trial was surprising as approximately

equal overall masses of bacterial product (scrapings from the surface of Petri dishes) were used to inoculate seeds. It was subsequently surmised that the low c.f.u. loadings from the *P. polymyxa* treatments was due to the production of large quantities of exo-polysaccharide which resulted in a dilution effect. Furthermore, the excess production of the exo-polysaccharide was stimulated by repeated re-streaking of the bacterial cells on Petri plates; a process used to fully utilise the surface of the agar by each bacterium. In the second glasshouse trial, this was overcome to a large extent by streaking the polysaccharide-producing bacterial isolates only once on each Petri plate, and by having a larger number of plates per isolate than other bacterial isolates. The higher inoculation rate of these isolates in the second glasshouse trial may have led to their increased disease control efficacy.

In the first glasshouse trial, biological control activity of the bacterial isolates alone against Aphanomyces root-rot was difficult to demonstrate due to the presence of other pathogens. The reduced level of root rot shown in treatments using the fungicide Apron indicates that other root pathogens were present (Jermyn *et al.*, 1982). Their presence was later confirmed when recoveries of pathogenic fungi were made from pea roots grown in this soil (Section 4.3.1). As *A. euteiches* often occurs with other pathogenic fungi in a disease complex (Oyarzun and Van Loon, 1989; Tu, 1987), the presence of other pathogenic fungi was expected, but not to the extent observed in this trial.

The determination of oospore numbers per gram of root tissue in the second glasshouse trial allowed the partitioning of *A. euteiches*-incited disease from that caused by other pathogens. When comparing disease scores with oospore counts, it can be seen that, for most treatments, reduction of disease symptoms is associated with a reduction in oospore counts. Apron, however, reduced disease symptoms only; no reduction in oospore numbers was apparent which confirms its reported lack of activity against *A. euteiches*. Counting oospores per gram of root tissue, therefore, provided a very good way of determining the level of Aphanomyces root-rot disease within a multiple pathogen disease complex. In addition, it provided a quantitative measurement of *A. euteiches* infection. The method has been used previously for determining the level of resistance within pea breeding lines (Kraft and Boge, 1995) and for assessing soil suppressiveness towards the disease (Persson *et al.*, 1999). The method is, however, very time consuming and is possibly not suitable for trials in which the pathogen causes either low or high disease

pressures. At low disease pressure, the number of spores produced within the root may be too low to accurately measure, except perhaps following a separation or enrichment step, or the fungus may be present myceliogenically, resulting in an underestimation of the disease. At high disease pressure, the roots, especially the fine lateral ones, are often degraded entirely, releasing the spores into the soil resulting in an underestimation of the disease level.

Visual disease assessment, however, also has limitations when assessing Aphanomyces root-rot. As discussed before, *A. euteiches* often causes disease in a complex involving other pathogens, the proportion of which may depend on environmental factors, relative levels of inoculum and the levels and types of genetic resistance within the pea. As the symptoms caused by each pathogen are often quite similar, and are further confounded following infection by multiple pathogens, the disease scoring system based on visual symptoms can be very limited. In addition, assessment of disease on a scale is not truly quantitative, as the scores assigned to disease symptoms are an arbitrary estimation of the level of disease.

The specific detection of *A. euteiches*, either as inoculum in soil or in pea roots as a measure of infection, may be facilitated through the use of polyclonal antibodies (Kraft and Boge, 1994; Petersen *et al.*, 1996) or, more recently, specific PCR-technology (Vandermark *et al.*, 2000). Although both methods are specific for the pathogen, they both have similar limits to their use. Most significantly, both methods require standardising against conventional disease assessment parameters (visual disease scores or oospore numbers) before they can be used qualitatively. In addition, they both require specific technical expertise, equipment (PCR machine, *A. euteiches* specific primers or antibodies), and are relatively expensive to use. In their favour, they are both highly specific towards the pathogen and, once an assay standard has been developed, may quickly be able to quantify the disease.

Root weight was highly significantly negatively correlated with the visual disease assessments in both assays of trial 2 and, as such, was useful in describing the level of infection. However, as with the visual disease scoring assessments, the method was only useful in describing the level of overall disease rather than that caused by *A. euteiches*

only. This was reflected in the relatively weak correlation found between oospore numbers per gram of root and root weight in the same assays.

Each of the methods used for measurement of disease, therefore, had limitations. Although oospore counts were the most valuable in accurately describing the level of *A. euteiches* infection, their determination was too time consuming to be recommended for large-scale trials. In conditions where disease caused by *A. euteiches* is strongly favoured, for example where soil is saturated and / or when the inoculum of the pathogen is high, root weight can quickly and adequately describe the disease. In trials described in this work, however, the measurement of several different parameters gave a better overall picture of the root disease than would have been evident by measurement of any one parameter alone.

The only requirements for Aphanomyces root rot disease to be expressed are the presence of the host plant, favourable environmental conditions and the inoculum of the pathogen. Therefore, where peas are to be grown, long-term management of the disease is dependent upon reducing inoculum in the soil. Subsequently, a reduction in the number of oospores per gram of root tissue, which would ultimately result in a reduction in the build-up of the pathogen in the soil, is of great significance. In addition, this could have benefits not only for the currently affected plants, but for subsequent crops. The best seed treatments for reducing the oospore numbers in the roots were the bacterial isolates *B. pumilus* PT 10 and MW 12, *B. mycoides* MW 27, *B. cereus* MW 10, *P. macerans* PT 1 and *P. polymyxa* 18·25. However, only *B. pumilus* MW 12, *B. mycoides* MW 27, *P. macerans* PT 1 and *P. polymyxa* 18·25 were also effective at reducing visual root rot disease symptoms.

Although the commercial biological control agent Kodiak has shown great promise for control of *A. euteiches* in trials overseas (J. Kraft; Unpublished. Pers. comm.; Gritton *et al.*, 1995), it was relatively ineffective in these trials giving less disease control than some of the other bacterial isolates tested. This may have been due to unsuitable environmental conditions in the glasshouse trials as experienced with the product by Wilkins *et al.* (1998).

The fungicide Tachigaren has also exhibited variable degrees of efficacy against *A. euteiches* on pea (Gritton *et al.*, 1995; Jermyn *et al.*, 1982; King and Parke, 1993; Kotova and Tsvetkova, 1980). In these trials, the fungicide performed in a similar manner

to Kodiak: always reducing the level of disease (all parameters) relative to the nil-control treatment, although not always at a significant level, and not as effectively as the best bacterial treatments.

Parke *et al.* (1991) applied rhizosphere bacteria to pea seeds and evaluated them in a growth chamber assay for control of *Pythium* damping-off and *A. euteiches* root-rot. They found several bacterial isolates, identified as species of *Pseudomonas*, *Bacillus*, *Corynebacterium*, and *Flavobacterium*, significantly and consistently improved pea emergence and / or yield. However, direct comparisons cannot be made between the relative efficacies of their isolates and the bacteria evaluated in the current study, because their initial trials were conducted in sterile media into which the pathogen was artificially inoculated (zoospores), and they presented no actual data on plant emergence or disease severity.

Kommendahl and Windels (1978) evaluated 22 bacterial isolates, originally isolated from pea rhizospheres, as seed treatments for control of *A. euteiches, F. oxysporum, F. solani* and *R. solani* root diseases on peas in trials which used soil as a natural source of inoculum. They found that seven isolates improved plant stand compared with the nil-control (40% stand), with one isolate being equally effective as the Captan treatment (80% stand). However, about half of the isolates tested actually reduced the plant stand relative to the nil control. None of their bacterial treatments were able to reduce levels of root rot symptoms below that of the non-treated control.

The bacterial isolates tested in this study were able to reduce the level of Aphanomyces root rot disease when planted in a naturally infested soil and, therefore, have definite potential as biological control agents. Although plant stand (emergence) was not determined *per se*, no deleterious effects were observed after treatment of the pea seed with the bacteria described.

The bacterial isolates which demonstrated clear potential for biological control across both glasshouse trials were *B. mycoides* MW 27, *B. cereus* 15·80 and *P. polymyxa* 18·25 (taking into account its low rate of application in the first trial), each of which was subsequently selected for field evaluation. However, the disease control abilities of several others, *B. subtilis* PT 69, *B. pumilus* MW 12, *P. macerans* PT 1 and *B. pumilus* MW 18, and *P. polymyxa* 18·94, was variable. As only a limited number of isolates could be tested in the field, further selection within the 'variable' group of isolates was required. On purely arbitrary grounds, *B. pumilus* MW 18 was selected on ease of culturability, *B. subtilis* PT 69 was included as a species representative, and *P. polymyxa* 18·94 as further representative of the *Paenibacillus* genus.

In summary, the following isolates were selected for field testing:

1. *Bacillus cereus* 15·80
2. *Bacillus mycoides* MW 27
3. *Bacillus subtilis* PT 69
4. *Bacillus pumilus* MW 18
5. *Paenibacillus polymyxa* 18·25
6. *Paenibacillus polymyxa* 18·94

Failing efficacy of the above isolates in the field, the bacterial isolates *B. pumilus* MW 12 and *P. macerans* PT 1 and *B. pumilus* PT 10, which also exhibited some disease suppressive characteristics in the glasshouse, should be evaluated.

Chapter 4 Field trial assessment of spore-forming bacteria for control of Aphanomyces root rot of peas

4.1 Introduction

A general prerequisite for the biological control of soil borne plant diseases, is the establishment and survival of the biological control agent in the rhizosphere region of a crop plant by the biological control agent (Brown, 1974; Weller, 1988). In the glasshouse, where conditions are generally both relatively uniform and conducive to microbial growth, biological control agents can be selected for disease control efficacy. However, field soils are variable in physical and biological composition and are exposed to variable environmental conditions such as temperature and soil type. Thus, compared with the glasshouse, the field is a far more challenging environment in which the potential biological control agent must grow. This often results in poor performance of ill-adapted strains (Lewis and Papavizas, 1991). Evaluation of biological control agents under field conditions is, therefore, the only method by which reliable assessments can be made about their potential for disease control (Knudsen *et al.*, 1997). Unfortunately, such trials are often difficult and expensive to set up, maintain and assess compared to glasshouse or laboratory assays. In addition, the variability inherent in the field environment compared with that encountered in the glasshouse, means that a higher degree of replication is often required in order to determine significant effects.

Biological control of *A. euteiches* was investigated by Parke *et al.* (1991) who identified the Gram-negative bacteria *Burkholderia cepacia* AMMD and *P. fluorescens* Migula PRA25 as potential biological control agents of *A. euteiches* under growth chamber conditions. A preliminary field trial evaluation showed that the bacteria increased seedling stand and yield under moderate to high disease pressure conditions. However, when these bacteria were subsequently tested under a range of field conditions (Gritton *et al.*, 1995;

King and Parke, 1993), their efficacy was found to be variable. Although a patent was obtained for *Burkholderia cepacia* against *A. euteiches* (Parke, 1993), it has not yet been developed commercially as a successful biological control agent. Reasons for this delay may include its restricted agricultural usage due its potential to act as a human pathogen, especially in patients with cystic-fibrosis (Govan and Deretic, 1996). Furthermore, the short shelf-life (survival) of many *Pseudomonas*-like biological control agents may be a restriction to their commercialisation (Brannen and Kenney, 1997; Nemec 1997). Conversely, spore-forming bacteria generally have a long shelf-life in formulation, and can maintain their presence in the soil under adverse conditions (Weller, 1988).

When considering biological control of root-rot of peas, it must be recognised that the disease usually occurs in association with other root pathogens (Oyarzyn and Van Loon, 1989; Tu, 1987). The pathogen *A. euteiches*, rarely, if ever, acts alone when initiating root rot on peas, but rather with other fungi such as *Fusarium oxysporum* and *Pythium* spp. In any given pea trial, the amount and type of disease which develops on roots will be affected by numerous factors such as the presence of different pathogens, environmental conditions which may favour some pathogens over others, and the presence of genetic resistance in the host plant to one or more of the pathogens. The complexity of pea root rot disease expression and development and the complexity of the soil environment can also affect the activity of the biological control agents in unforeseen ways. It is necessary, therefore, to screen as many biological control candidates from glasshouse trials as practical, in field conditions. This will increase the chances of identifying one or more field-active isolates.

This chapter describes a series of field trials, the chronology of which is outlined in Figure 4.1. The initial trial was carried out to assess the potential of six spore-forming bacteria (*B. subtilis* PT 69, *B. pumilus* MW 18, *B. mycoides* MW 27, *B. cereus* 15·80, *P. polymyxa* 18·25, and *P. polymyxa* 18·94) for potential biological control activity. Subsequent trials focused on the potential for enhancement of activity by integrating the two best bacteria, *B. mycoides* MW 27 and *P. polymyxa* 18·25, with fungicides or combining them together. In the final trial, several commercially-developed formulations of the bacterium *B. mycoides* MW 27 were investigated for disease control efficacy.

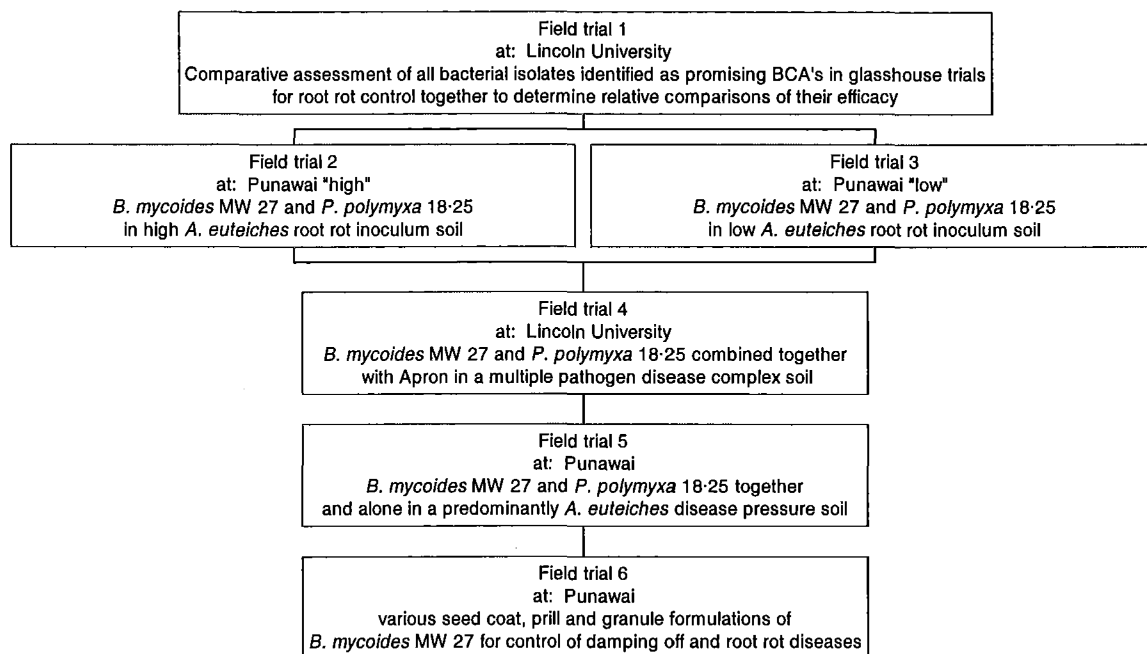

Figure 4.1: Flow diagram outlining a series of field experiments evaluating spore-forming bacteria for pea root rot control.

4.2 Materials and methods

Summary of the location, experimental design, dates of planting and harvest and the types of data collected for field trials 1-6 is given in Table 4.1. Table 4.2. outlines the treatments which were included in each trial.

4.2.1 Location of the field trial sites

Field trials 1 and 4 were carried out at the Field Services Centre, Lincoln University (S 43°38'60", E 172°28'05"). To create an *A. euteiches* infested site, an area of soil, 4.9 m long × 4.2 m wide × 15 cm deep was excavated and replaced with infested soil from the Chapmans' farm (Section 3.2.1; Templeton fine sandy loam soil).

Field trials 2, 5 and 6 were carried out in high *A. euteiches* indexed fields on a farm at Punawai, mid-Canterbury (S 43°46'40", E 171°35'06"; Eyre stony silt loam soil). Field

trial 3 was carried out in a low *A. euteiches* index field directly adjacent to trial 2 (approximately 15 m apart).

4.2.2 Rolled towel bioassay

Rolled towel bioassays were carried out on each of the three soils (Lincoln, Punawai high *A. euteiches* and Punawai low *A. euteiches*) to determine the level of *A. euteiches* inoculum (Williams-Woodward *et al.*, 1998). From random positions across each site, six to ten, 500 ml samples of soil were taken to a depth of 10 cm with a garden-trowel and mixed together thoroughly. Approximately 1 Kg of the mixed sample was dried in the glasshouse for 10 d and sieved through a 2 mm screen. Surface disinfected pea seeds (0.5% NaOCl for 5 min followed by three rinses in sterile distilled water) were germinated on autoclaved-sterilised vermiculite for 5 days. Four seedlings were placed onto a moistened laboratory paper towel (Hygenix Royale 25.4 cm × 26.7 cm) with 1 cm^3 of the tested soil (~ 0.89 g) placed onto the section of root immediately below the seed (Figure 4.2). A second paper towel was placed over the seedlings and wetted thoroughly. Plastic wrap was then placed over the towels and the entire 'sandwich' rolled into tubes. Ten assays, totaling 40 pea seedlings, were prepared and placed together in a plastic bag. The entire bag was stood upright in a beaker and filled to just below the level of the seed with an aqueous solution of PCNB (pentachloronitrobenzene; Terrachlor PCNB, 75% WP at 1 g L^{-1} water). After 21 days, the seedlings were examined for typical symptoms of Aphanomyces root-rot (honey / yellow discolouration, softening / collapse of the root tissue). Isolations from pea root tissue was made onto agar (Section 2.2.3) to verify the presence of the fungus.

4.2.3 Pea seed and treatment

4.2.3.1 Cultivars

Two pea cultivars were used over the course of the trials: Whero and Dwarf Massey. Whero, a purple flowered field or maple pea, was used in field trial one following its previous use in glasshouse trials. In trials 2 and 3, however, the cultivar Dwarf Massey, a white flowered processing pea, was used to avoid contamination via cross pollination with an adjacent field of peas being grown for certified seed. This cultivar was subsequently used in future trials.

4.2.3.2 Treatment of pea seeds with biological control bacteria

Prior to treatment, all pea seeds were surface-disinfected as described in Section 4.2.2.

Bacterial isolates (Table 4.2) were taken from storage at –80°C, cultured onto NA Petri dishes and incubated at 25°C. After resuming strong growth, isolates were sub-cultured onto multiple NA +Mn plates or, for bacterial isolates *P. polymyxa* 18·25 and 18·94, PDA.

For field trials 1, 2 and 3 the procedure for inoculating pea seeds remained similar. In trial 1, six plates of each bacterial isolate were grown for 10 d at 25°C. In trials 2 and 3, 15 plates of *B. mycoides* MW 27 and 30 plates of the slower-growing *P. polymyxa* 18·25 were grown at 25°C. After 10 - 14 d, the bacterial growth on each plate was scraped into PPBS and collected by centrifugation at 16 000 × g for 20 min. The bacterial pellets were re-suspended into 20 mL of PPBS, and 0.5 g of methyl-cellulose was added to thicken the suspension. Pea seeds were thoroughly coated with the bacterial suspensions in Schott bottles, tipped onto a deep Petri dish lined with filter paper (Whatmans #1) and allowed to dry overnight in a laminar flow cabinet.

The growth of bacterial isolates for field trials 4, 5 and 6 followed the same procedure as above. However, following collection of the bacterial cells via centrifugation, the samples were freeze dried and ground into a powder using a mortar and pestle. In field trial 4, methyl-cellulose and glucose were added to the bacterial powders at 2% w/w each. Moist pea seeds (following surface disinfestion) were thoroughly coated with the bacterial powder by shaking in a plastic bag. Seeds receiving combined bacterium-Apron treatments were similarly coated after mixing the samples together.

In field trial 5, powder formulations of the bacterium were prepared and the c.f.u. gram^{-1} determined using the standard dilution series procedure. A portion of the *B. mycoides* MW 27 bacterial powder, which was found to have a much higher c.f.u. count than that of *P. polymyxa* 18·25, was diluted with chalk talc ($CaCO_3$) to give approximately equal rates. The formulations containing the two bacterial isolates were mixed together and inoculated onto pea seeds as for trial 4. *Paenibacillus polymyxa* 18·25 alone, *B. mycoides* MW 27

undiluted (high rate), and *B. mycoides* MW 27 diluted (low rate) were also applied as seed treatments.

Field trial 6 assessed the relative performance of different formulations of the bacterium *B. mycoides* MW 27. A seed coat formulation (Lincoln seed coat formulation) was made using the procedure described before. This isolate was cultured onto 30 NA +Mn Petri dishes for 14 d at 27°C and the growth recovered into PPBS 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. The $CaCO_3$ (chalk talc) was included because it appeared to have a stimulatory effect in the previous trial (perhaps by regulating local pH reaction). Methyl-cellulose was used as a sticking agent and glucose as an initial carbon source for the bacterium.

In addition to the Lincoln seed coat formulation, formulations of *B. mycoides* MW 27 were produced by AgResearch New Zealand Ltd (AgResearch), Lincoln. The isolate 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 NB. 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 suspension, containing 2×10^8 c.f.u. mL^{-1} was then supplied to Dr Von Johnson, AgResearch, for formulation into prills (Figure 4.3), 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.

4.2.3.3 Control treatments

In each trial, a nil-control consisting of non-treated, surface-disinfected pea seed, was included. In addition, each field trial included the fungicide Apron C 70 SD (Ciba-Geigy; 350 g Kg^{-1} metalaxyl and 350 g Kg^{-1} captan) applied at 2 g Kg^{-1} seed.

The commercial biological control product Kodiak, a formulation of *B. subtilis* GB03 (Gustafson Inc.) applied at 2 g Kg^{-1} seed, was included in trials 1-4.

In trial 6, a control comprising only the carrier used in the Lincoln seed coat formulation ($CaCO_3$, methyl-cellulose and glucose) was also included.

4.2.3.4 Determination of bacterial colony forming units

The numbers of c.f.u. per seed or gram of prill or granule formulations were determined using a serial dilution technique similar to that described before in Section 3.1.3.5. As a modification, the surfactant Tween 80 (polyoxyethylene (20) sorbitan mono-oleate; BDH Limited) was added at 2 drops L^{-1}. In addition, vortexing was used to mix samples, compared with a wrist-action shaker previously, as it was found to result in more discrete colony formation following plating.

The c.f.u. of bacteria in the prill and granule formulations of *B. mycoides* MW 27, used in field trial 6 were frequently determined over a 70 day period to establish bacterial shelf life.

4.2.4 Experimental design

Two types of experimental designs were used throughout the trials: complete randomised block design and a Latin-square design. As the distribution of *A. euteiches* in soil, and the subsequent disease pressure, is often very variable, the Latin-square type of experimental design was considered preferential. However, limitations in space and / or time made it difficult to use this design in each trial. Subsequently, trials 1, 4 and 6 were set up using the randomised block design.

Aside from the overall design (randomised block or Latin square), each trial followed a similar layout. Each treatment was replicated once in each block. Each replicate consisted of 36 pea seeds planted in a 6 × 6 grid at a depth of 5 cm. A 10 cm spacing was left between seeds. The distance between seedlings in adjoining treatments was at least 20 cm. A typical field trial layout is shown in Figure 4.4.

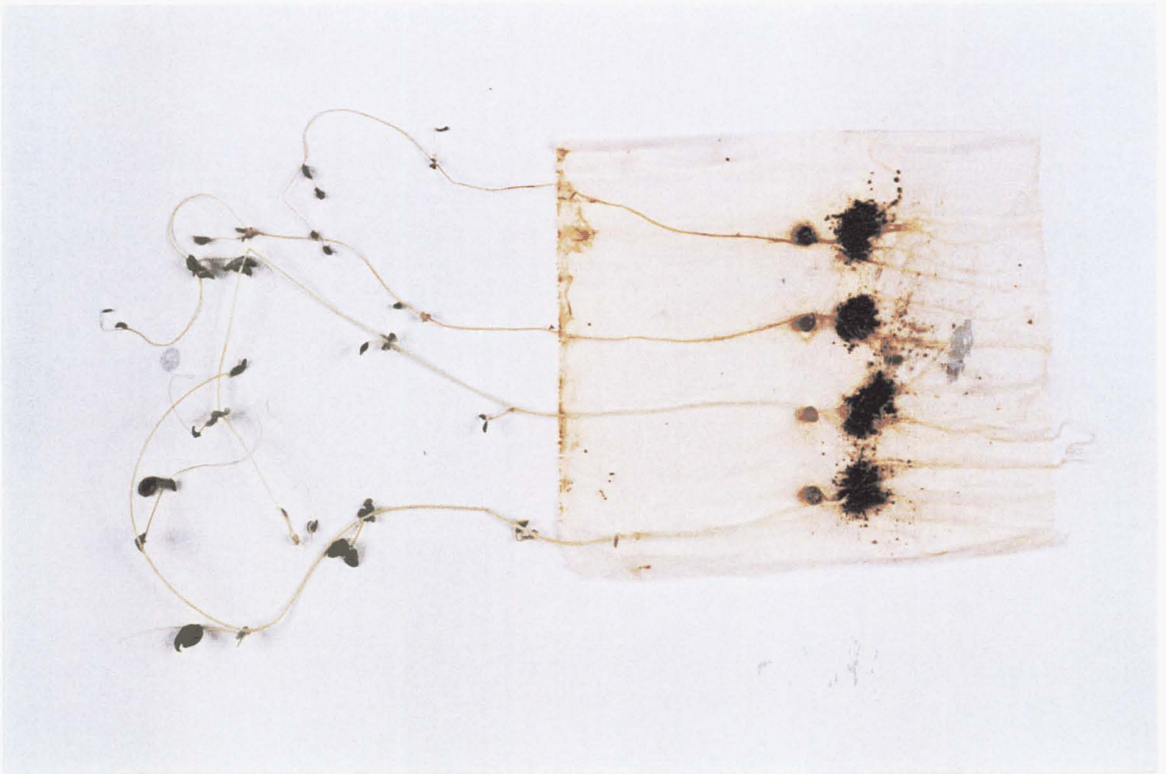

Figure 4.2: Rolled towel bioassay.

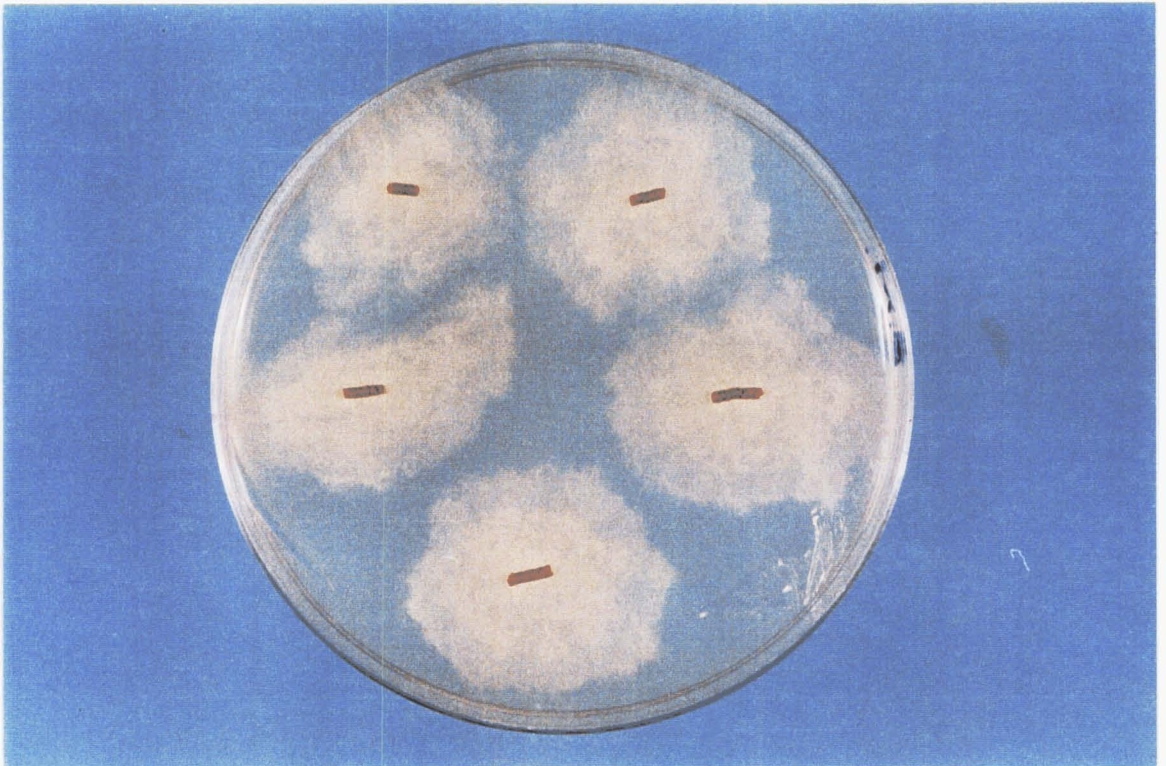

Figure 4.3: Colonies of *Bacillus mycoides* MW 27 growing out from the prills generated by Dr Von Johnson, AgResearch, Lincoln, for use in field trial 6.

Table 4.1: Summary information for field trials 1-6.

Field trial	Location[1]	Planting date	Harvest date	Design[2]	Trt[3]	Rep[4]	Assessments[5]
Field trial 1	Lincoln	4/02/1999	8/04/1999	RBD	9	4	S D
Field trial 2	Punawai 'high'	28/10/1999	28/12/1999	L-Sq	5	5	S D RW L PN APW PY
Field trial 3	Punawai 'low'	28/10/1999	9/01/2000	L-Sq	5	5	S D RW L PN APW PY
Field trial 4	Lincoln	15/02/2000	26/04/2000	RBD	6	4	S D RW L
Field trial 5	Punawai 'high'	15/03/2000	23/05/2000	L-Sq	6	6	S D RW L
Field trial 6	Punawai 'high'	04/10/2000	27/12/2000	RBD	6	5	S L PN PY TPY

[1] Lincoln = Lincoln University field trial site; Punawai 'high' or 'low' = high or low *A. euteiches*-indexed fields at Punawai

[2] RBD = complete randomised block design; L-Sq = Latin-square design

[3] Number of treatments

[4] Number of replicates

[5] S = plot stand; D = visual score of the level of root rot disease; RW = root weight (g) after blotting dry; L = plant length (cm); PN = number of pods per plant; APW = average pods weight (g); PY = weight (yield) of pods per plant (g); TPY = total yield per plot (yield of pods per plant × number of plants per plot) (g).

Table 4.2: Summary of treatments used in field trials 1-6.

Field trial 1	Field trial 2	Field trial 3	Field trial 4	Field trial 5	Field trial 6
Nil-treatment[1]	Nil-treatment	Nil-treatment	Nil-treatment	Nil-treatment	Nil-control
Apron C70SD	Apron C70SD	Apron C70SD	Apron C70SD	Apron C70SD	Apron C70SD
Kodiak	Kodiak	Kodiak	*B. mycoides* MW 27	*P. polymyxa* 18·25	Carrier-control
P. polymyxa 18·25	*P. polymyxa* 18·25	*P. polymyxa* 18·25	*P. polymyxa* 18·25	*B. mycoides* MW 27 (low)[2]	*B. mycoides* MW 27 SC Lincoln
B. mycoides MW 27	*B. mycoides* MW 27	*B. mycoides* MW 27	*P. polymyxa* 18·25 + Apron C70SD	*B. mycoides* MW 27 (high)[3]	*B. mycoides* MW 27 SC AgResearch
B. pumilus MW 18			*B. mycoides* MW 27 + Apron C70SD	*B. mycoides* MW 27 + *P. polymyxa* 18·25	*B. mycoides* MW 27 Prill
B. cereus 15·80					*B. mycoides* MW 27 Granule
B. subtilis PT 69					
P. polymyxa 18·94					

[1] Surface disinfected pea seed only

[2] Diluted with $CaCO_3$ to the same c.f.u. per seed of the *P. polymyxa* 18·25 treatment

[3] Applied at the maximum rate

[4] Seed coat treatment made at Lincoln University, or AgResearch, Lincoln.

4.2.5 Preparation and cultivation of the trial sites

Preparation of the soil prior to planting for field trials 1, 4 and 5 was carried out by hand. A shovel was used to cultivate the soil to a depth of approximately 25 cm, approximately 3 weeks prior to planting. This was repeated one to two days prior to the planting of the trials. At the time of planting, a garden fork was used to break up any remaining clods in each micro-plot area.

Field trial sites 2 and 3 were cultivated using standard farming techniques (ploughing, rolling and disking) by the farm manager, John Snowdon.

Field trial 6 was cultivated to a depth of approximately 20 cm using a rotary-hoe (Figure 4.5). 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 to remove remaining clods immediately prior to planting.

4.2.6 Data collection and analysis

For each trial, the soil temperature at 10 cm depth was measured using a Tiny-tag data-logger (Orion Components Ltd., Chichester, England).

The different parameters which were measured or calculated for each field trial are given in Table 4.1. Plants awaiting assessment following recovery from the field sites were stored in large plastic bags at 10°C to minimise water loss and progressive disease development.

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 for each treatment replicate between 19 to 21 days after planting.

The plant length, in cm, was measured from the attachment point of the cotyledons to the tip of the plant (i.e. primarily the above-ground portion of the plant).

The fresh root weight was measured after washing the roots under running water and blotting dry between a double layer of paper towels.

The number of pods per plant were counted and total weight of pods (plant yield) were determined in some trials. The average pod weight per plant was calculated by dividing the total pod weight by the number of pods on a plant by plant basis.

The total plot yield (TPY) was calculated for field trial 6. To determine this factor, the 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 whether pea seed treatments significantly affected the yield parameters. In field trials 1-4, for which methods and formulations were still being refined, the Fishers LSD was used as the *post hoc* test to separate treatment means. In trials 5 and 6, however, the more conservative Duncans' test was used.

Visual assessments of the level of root rot disease symptoms were made in field trials 1-5. The procedure for scoring roots and the subsequent data analysis is described in detail in section 3.1.1.3. The roots were given a score on a 0-4 scale of increasing disease severity but because the scale was arbitrarily generated, i.e. was not truly quantitative, non-parametric data analysis was applied. For each treatment replicate, the median disease score was determined and pair-wise comparisons were made between controls and each of the other treatments using the Mann-Whitney *U*-test.

In field trials 5 and 6, where multiple factors were measured and significant differences between treatments were found, Pearsons correlation co-efficients were determined.

Figure 4.4: Typical field trial layout (trial 4; Lincoln University) showing replicate plots layout. Netting has been erected over the site to protect from bird and rabbit damage.

Figure 4.5: Cultivation of soil for field trial 6 (Punawai) using a rotary hoe.

4.2.7 Control of other pests and diseases

In field trial 1, rabbits and especially birds were a problem. Therefore, in subsequent trials, netting was erected on scaffolding over the trial sites (Figure 4.4). Field trials 2 and 3, however, were planted at the same time adjacent to a commercial pea field and, therefore, the abundant food source resulted in less damage to the trial area.

In field trial 1, powdery mildew disease, caused by *Erysiphe pisi*, was controlled with a single application of the fungicide Alto 100SL (cyproconazole 100 g L^{-1}; Sandoz) approximately 5 weeks after planting.

Slugs and snails were controlled using Mesurol (20 g Kg^{-1} methiocarb; Bayer NZ Ltd.).

Roundup (glyphosate, 480 g l^{-1} a.i.; Monsanto U.S.A.) was sprayed over all field trial sites approximately 10 weeks prior to cultivation to destroy weeds. Except for trial 6, weed control post planting was carried out by hand. In trial 6, Gallant (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.

4.2.8 Recovery of fungi from pea roots grown at the Lincoln field trial site

To identify the nature of the root-rot disease complex present in the Lincoln field trial soil, a qualitative survey of the species of plant pathogenic fungi present in the pea roots was undertaken. Sections of diseased roots, originating from field trial 1, were surface sterilised in 0.5% NaOCl for 2 min, rinsed three times with sterile distilled water, and plated on to PDA and CMA. Hyphae emerging from the sections of pea roots were separately sub-cultured onto PDA or CMA and grown at 20°C under dark conditions until sporulation occurred. *Fusarium* spp. were identified according to Booth (1977); other Deuteromycetous fungi were identified according to Barnett *et al.* (1997). Oomycetous-type fungi were tested for growth on CMA with and without metalaxyl (3 mg L^{-1}). Those which were able to grow were identified as before (Section 2.2.3). Two isolates of Oomycetous fungi which were found to be inhibited by metalaxyl were identified to generic level by Dr Geoff White (CMI; England).

4.3 Results

4.3.1 Field trial 1 – Comparative assessment of all six bacterial isolates

Non-treated control plants exhibited low to medium levels of root rot disease: the average median level of root infection was only 1.5 (Table 4.3). The roots of the control plants exhibited the typical early root rot disease symptoms: multiple small lesions on the roots with discolouration varying from light tan to brown.

Relative to the nil-control, all pea seed treatments reduced the average median disease score (Table 4.3). However, pair-wise comparisons between treatments and the control, using the Mann-Whitney *U*-test found no significant differences at α=0.05.

Table 4.3: Initial field-screen of several bacterial isolates for biological control of pea root rot disease under field conditions, Lincoln University.

Treatment	C.f.u. / seed	% Stand[1]	Mean disease score[2]
Nil-control	0	97.9	1.50
B. pumilus MW 18	9.5×10^6	95.1	1.25
Apron C70SD	0	94.4	1.25
B. subtilis PT 69	7.1×10^7	95.1	1.00
B. cereus 15·80	1.3×10^7	93.7	1.00
Kodiak	2.5×10^7	96.5	1.00
P. polymyxa 18·94	4.8×10^5	98.6	1.00
P. polymyxa 18·25	5.0×10^6	93.1	1.00
B. mycoides MW 27	5.0×10^7	95.9	1.00

[1] Percentage plants present per treatment replicate (average of 4 replicate plots planted with 36 seeds each). Determined 19 days after planting.

[2] Average of median disease scores for each treatment replicate. Median disease scores based on visual assessment of the level of root rot disease on a 0-4 disease severity scale.

The plant stand was found to be very high for all treatments, indicating both that there was little or no seed or seedling disease pressure and that seed treatment had no negative effect

on germination or seedling growth (Table 4.3). No significant differences were found between treatments using ANOVA.

The c.f.u. counts differed between bacterial treatments, ranging from 4.8×10^5 to 7.1×10^7 c.f.u. $seed^{-1}$. However, no overall trend was obvious between the rate of inoculation and plant stand or disease level scores.

Results from the rolled-towel bioassay showed that a very high level of *A. euteiches* inoculum was present in the field soil. However, isolations of fungi from infected pea roots following the destructive sampling of the field trial resulted in a poor isolation frequency of *A. euteiches*. Rather, the disease-causing fungi *Fusarium oxysporum* and *F. solani* (Mart.) Sacc. were recovered at a high rate. Other pathogenic fungi isolated included *Thielaviopsis basicola* (Berk. & Br.) Ferr. and *Pythium* spp. Therefore, although inoculum of *A. euteiches* was present at the trial site, conditions were not favourable for its expression compared with other root rot causing fungi.

Selection of bacteria for ongoing field evaluation based solely on this trial was not possible because the median disease score system was not sensitive enough, resulting in the six most effective treatments having the same score. Therefore, the results of the glasshouse trials, in particular the concentration of oospores per gram of root, were taken into account. Upon consideration of all the data, the bacterial isolates *B. mycoides* MW 27 and *P. polymyxa* 18·25 were selected for ongoing testing.

4.3.2 Field trials 2 and 3: Punawai high and low disease index sites

The c.f.u. counts for seeds inoculated with the bacterium *P. polymyxa* 18·25 was approximately 10^5 c.f.u. per seed, which was considerably lower than the two other bacterial treatments, *B. mycoides* and Kodiak, which had counts of approximately 10^7 c.f.u. $seed^{-1}$. No bacteria were recovered from seeds which were surface-disinfected only (nil-control) or treated with Apron.

In the rolled towel bioassay, soil from the high disease site resulted in 66.7% incidence of root rot whereas soil from the low disease site resulted in 10% root rot incidence.

In the field trials, the average median disease score for the non-treated plants in the high disease soil was 1.6 and in the low disease soil was 1.4 (Table 4.4). However, although the difference in disease level was small, there were large differences in the yield per plant (10.2 g $plant^{-1}$ compared with 33.04 g $plant^{-1}$ for high and low index soil respectively). The increase in yield resulted from both increases in mean pod weight and number of pods per plant.

Treatment of pea seed with the fungicide Apron or any of the biological control agents did not affect ($P>0.05$) the percentage plant stand, root disease score, root weight, plant length or the number of pods per plant in either of the two field trials.

In field trial 2 (high disease site), pea seed treatment was found to affect the average pod weight ($P=0.01$). The *B. mycoides* MW 27 treatment produced significantly heavier pods than all other treatments except for Kodiak. However, this did not translate into a significant increase in plant yield.

In field trial 3 (low disease site), seed treatment did not affect the average pod weight per plant. However, total yield of pods per plant were significantly reduced ($P=0.04$) following treatment with the bacterial isolates Kodiak and *P. polymyxa* 18·25.

The soil temperature over the course of the field trial fluctuated around 15°C; never exceeding 20°C or falling below 10°C (Figure 4.8).

Table 4.4: Results from field trials 2 and 3: *B. mycoides* MW 27 and *P. polymyxa* 18·25 under high and low disease pressures.

Treatment	C.f.u. / seed[1]	% Plant stand	Disease score[2]	Root weight (g)	Plant length[3] (cm)	Pods / plant	Mean pod weight (g)	Yield of pods (g) per plant
High *Aphanomyces* disease-pressure site (field trial 2)								
B. mycoides MW 27	5.3×10^7	77.8	1.8	0.90	60.6	4.2	2.71 a	12.2
P. polymyxa 18·25	4.4×10^5	75.6	1.4	1.00	61.5	4.0	2.10 c	10.1
Kodiak	2.9×10^7	83.3	1.6	0.96	58.4	4.1	2.44 a b	11.6
Apron C70SD	0	76.1	2.0	0.82	56.5	3.7	2.26 b c	9.9
Control	0	81.1	1.6	0.76	59.4	3.9	2.39 b c	10.9
							LSD = 0.31	
Low *Aphanomyces* disease-pressure site (field trial 3)								
B. mycoides MW 27	5.3×10^7	79.4	1.0	1.34	60.1	5.7	5.19	31.0 a b
P. polymyxa 18·25	4.4×10^5	85.6	1.2	1.36	62.0	5.7	4.94	29.1 b
Kodiak	2.9×10^7	85.6	1.0	1.30	61.8	5.6	4.96	28.7 b
Apron C70SD	0	85.0	1.0	1.56	60.5	5.6	5.34	30.9 a b
Control	0	81.7	1.4	1.49	62.3	6.1	5.35	33.0 a
								LSD = 2.79

[1] Bacterial colony forming units [2] Average of the median, visually assessed disease score (0-4 range of increasing disease).
[3] Cotyledon to top.
Values based on the average of 25 plants per plot, with 5 replicate plots per treatment.
Treatment means followed by the same letter are not significantly different at α=0.05 using Fishers Least Significant Difference Test.

4.3.3 Field trial 4 – Bacteria in combination with Apron

The level of root rot disease occurring in field trial 4, at Lincoln, was higher than that of the previous trials. In the non-treated control plants, the average median disease was 2.79 (Table 4.5). Plants affected to this degree had extensive darkening of most or all of the root system. However, pair-wise comparisons using the Mann-Whitney *U*-test found that none of the seed treatments significantly reduced the level of disease relative to the nil-control (α=0.05). Nor were there any significant differences between the Apron-only and Apron combined with bacteria treatments (α=0.05).

In addition to the overall lack of disease control effects by seed coating, no differences were found on the percentage plant stand (P=0.7008), plant length (P=0.9649) or root weight (P=0.5554) (Table 4.5).

Table 4.5: Results from field trial 4 assessing the efficacy of combining *P. polymyxa* 18·25 and *B. mycoides* MW 27 with the fungicide Apron at reducing root-rot disease, at the Lincoln field trial site.

Treatment	**C.f.u.[1]/ seed**	**% Plant stand**	**Plant length[2] (cm)**	**Root weight (g)**	**Disease score[5]**
Control	30	96.8	85.0	0.26	2.79
MW 27[3]	2.2×10^7	95.2	88.1	0.25	2.64
18·25[4]	4.8×10^6	96.0	87.8	0.27	2.43
Apron C70SD	0	94.8	85.8	0.41	2.57
MW 27 + Apron C70SD	4.9×10^7	94.4	87.0	0.27	2.50
18·25 + Apron C70SD	3×10^6	95.6	88.2	0.24	2.71

[1] Colony forming units of bacteria [2] Cotyledon to top

[3] *Bacillus mycoides* MW 27 [4] *Paenibacillus polymyxa* 18·25

[5] Average of the median, visually assessed disease score (0-4 range of increasing disease)

Values based on the average of 25 plants per replicate, 4 replicates per treatment.

Due to a late-season planting of the trial, the soil temperature was initially quite high but decreased steadily over the course of the trial to a low of approximately 7°C. As growth and activity of soil bacteria generally decreases with reducing temperature, the potential biological activity of the bacterial treatments was probably diminished by the end of the trial period.

4.3.4 Field trial 5 – *B. mycoides* MW 27 and *P. polymyxa* 18·25 individually and combined

For *B. mycoides* MW 27, the low and high rates of pea seed treatment were found to contain 1×10^8 c.f.u. per seed and 6.5×10^5 c.f.u. per seed, respectively. The low rate of *B. mycoides* MW 27 inoculation was similar to that of *P. polymyxa* 18·25 treated seed (4.8 $\times 10^5$ c.f.u. per seed). When combined together, the two bacteria were each coated onto pea seed at 2.7×10^5 c.f.u. per seed, giving a similar combined rate as with *B. mycoides* MW 27 at the low rate. Apron and nil-control pea seeds had from 200 – 700 c.f.u. per seed.

The disease pressure exerted on pea plants in field trial 5 was similar to that in field trial 3, which was set up in a similar inoculum-pressure soil. Non-treated control plants had an average median disease score of 2 (Table 4.6) and exhibited root disease symptoms accordingly (Section 3.1.1.3; Figure 3.1).

Analysis of the results found that seed treatments had no effect on the level of root disease relative to the nil-control. However, root weight was affected by seed treatments (P=0.012). Apron and *B. mycoides* MW 27 (low rate) treatments gave significantly higher root weights than all other treatments. The root weights of peas treated with *B. mycoides* MW 27 at the low rate was significantly higher than those treated with the same bacterium at the higher rate.

Table 4.6: Results from field trial 5 which assessed the effect of combining *P. polymyxa* 18·25 and *B. mycoides* MW 27 on *Aphanomyces* root-rot disease of pea under field conditions at Punawai.

Treatment	C.f.u. / seed[1]	% Plant stand	Plant length[2] (cm)	Root weight (g)	Disease score[7]
Apron C70SD	700	87.0 a	40.4 a	0.94 a	1.67
MW 27 low[3]	6.5×10^5	73.1 c	38.4 b	0.95 a	1.75
MW 27 high[4]	1×10^8	81.0 abc	38.0 bc	0.82 b	1.83
Control	200	72.7 c	37.3 bc	0.81 b	2.00
18·25 + MW 27[5]	2.7×10^5	76.4 bc	36.1 c	0.80 b	2.00
18·25[6]	4.8×10^5	83.3 ab	36.2 c	0.77 b	1.92
Duncan's value		8.998	1.920	0.1298	

[1] Colony forming units of bacteria [2] Cotyledon to tip

[3] *B. mycoides* MW 27 applied at approximately the same rate as *P. polymyxa* 18·25

[4] *B. mycoides* MW 27 applied at the maximum rate

[5] *B. mycoides* MW 27 and *P. polymyxa* 18·25 combined

[6] *P. polymyxa* 18·25

[7] Average of the median, visually assessed disease score (0-4 range of increasing disease).

Values based on the average of 20 plants per replicate, 6 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

The percentage plant stand varied between treatments (P=0.006). Apron and *P. polymyxa* 18·25 treatments gave significantly higher plant stands than the nil-control and the *B. mycoides* MW 27 (low rate) treatments.

Plant length, which is reduced with increasing severity of root rot diseases (Hagedorn, 1984), also varied between treatments (P=0.0005). Peas treated with the fungicide Apron were significantly longer than all other treatments and those treated with *B. mycoides* MW 27 (low rate) were significantly longer than the combined bacterial treatment and the *P. polymyxa* 18·25-alone treatment, but not the nil-control.

However, no association was found between the level of root rot disease and the plant stand (Table 4.7) indicating that the two disease pressures were distinct. This ties in well with the observation that *P. polymyxa* 18·25 significantly affected plant stand but not root disease or yield. Both the plant stand and disease scores were strongly correlated with the root weight and plant length (Table 4.7).

Table 4.7: Correlation coefficients and probability levels for variables assessed in field trial 5.

	Plant length	**Root weight**	**Disease score**
Plant stand	r^2=0.387	r^2=0.331	r^2=-0.161
	P=0.02	P=0.005	P=0.35
Plant length		r^2=0.691	r^2=-0.549
		P=0.0001	P=0.0005
Root weight			r^2=-0.823
			P=0.00001

Pearson correlation coefficients / Prob > |R| under Ho: Rho=0.

Given the strong, highly significant associations between the factors measured, the measurement of plant length or root weight could provide a better, easier method for predicting the level of root rot disease than the disease scores which are determined by visual root rot symptoms. These plant growth parameters were considered to be more sensitive indicators of the effects of root disease than a visual disease score, for which small variations were found to translate into large yield differences in trials 2 and 3 (Table 4.4). As a result, root disease assessments were not done for trial 6, only plant growth and yield parameters were measured. In addition, assessment of visual disease symptoms is time-consuming and would be relatively variable between researchers compared with measurement of the other factors.

The temperature of the soil at the trial site was initially approximately 15°C (Figure 4.8). However, during the course of the field trial the temperature continually dropped, reaching only 5°C at harvest. The low, declining soil temperature resulted from a very-late season (March) planting of the trial.

4.3.5 Field trial 6: 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 up to 70 d after the initial manufacture (Figure 4.7). 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 earlier trials did not result in any bacterial contamination, therefore, the contaminating bacteria probably originated from the glucose or $CaCO_3$ carrier components.

Overall, different formulations of the bacterial treatments significantly affected plant stand ($P<0.01$), number of pods per plant ($P<0.01$), yield of pods per plant ($P<0.01$), total plot yield, ($P<0.01$), and nearly significant differences in plant length ($P=0.054$) (Table 4.8).

The *B. mycoides* MW 27 prill formulation was the most effective treatment for increasing the number of pods and yield of pods per plant, both of which were found to be significantly higher ($P\leq0.05$) than the nil-control treatment (Figure 4.6). This treatment, however, did not have a significant effect on plant stand ($\alpha=0.05$) compared with the nil-control. Conversely, peas treated with the fungicide 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 increases per plant. 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 may be reversed.

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, which was significantly better than the Lincoln seed coat formulation (α=0.05).

Figure 4.6: Treatments were found to have a significant effect on both the number of pods per plant and the yield per plant in field trial 6. Each group of pea pods originated from 25 randomly selected pea plants harvested from the different plots. Treatments, from left to right, were *B. mycoides* MW 27 prill, Apron, *B. mycoides* MW 27 seed coat (AgResearch), nil-control, and *B. mycoides* MW 27 seed coat (Lincoln).

Table 4.8: Results of field trial 6 – testing formulations of *B. mycoides* MW 27

Treatment	C.f.u. / seed[1]	Percentage plot stand[2]	Length[3] (cm)	Pods per plant	Pod weight (yield) per plant (g)	Yield of pods per plot (g)[4]
Apron C70SD	870	92.8 a	39.21	2.78 b	15.07 ab	503.2 a
B. mycoides prill	1.5×10^6	79.4 bc	37.49	3.26 a	16.86 a	482.2 ab
B. mycoides SC A[5]	4.0×10^4	86.1 ab	37.28	2.77 b	14.13 bc	438.0 abc
B. mycoides granule	4.3×10^5	81.7 bc	39.08	2.76 b	14.30 b	420.3 bcd
Control	4.0×10^1	77.2 c	38.03	2.59 b	14.37 b	399.6 cd
Carrier[6]	4.9×10^4	83.3 bc	37.53	2.65 b	13.41 bc	426.3 cd
B. mycoides SC L[5]	6.7×10^7	83.3 bc	33.62	2.41 b	11.83 c	355.0 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] – Cotyledon to tip

[4]- Yield of pods per plot: Average yield per plant multiplied by the percentage plot stand

[5]- SC L – Lincoln seed coat formulation; SC A – AgResearch seed coat formulation

[6]- 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 4.9: Pearson correlation coefficients for plant length, number of pods per plant and the yield per plant measured in field trial 6.

	Length	Pods / plant	Yield / plant	Yield / plot[1]
Stand[1]	r^2=0.184	r^2=-0.028	r^2=-0.028	r^2=0.488
	P=0.29	P=0.43	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 4.9) showed that there was no significant association between the plot stand and any of the other parameters measured except for the yield per plot, which was calculated using the relevant plot stand figure. All other factors, however, were strongly correlated. Increase in plant length was associated with an increase in the number of pods per plant and, as expected, increases in either of these factors were associated with increasing yield values.

The initial soil temperature at the trial site was approximately 10°C (Figure 4.8). During the course of the trial the temperature gradually increased, reaching 20-25°C at harvest. This soil temperature profile is consistent with that expected for normal pea growing conditions (trial ran 4th October – 27th December, 2000).

As with field trials 1-5, soil moisture level were not able to be determined. However, heavy rainfall occurred immediately after plating, resulting in saturated soil conditions for at least 2 days, and wet conditions for approximately one week thereafter (J. Snowden, pers. comm.). Further heavy rainfall occurred at least twice during the early to mid growth phase (J. Snowden, pers. comm.).

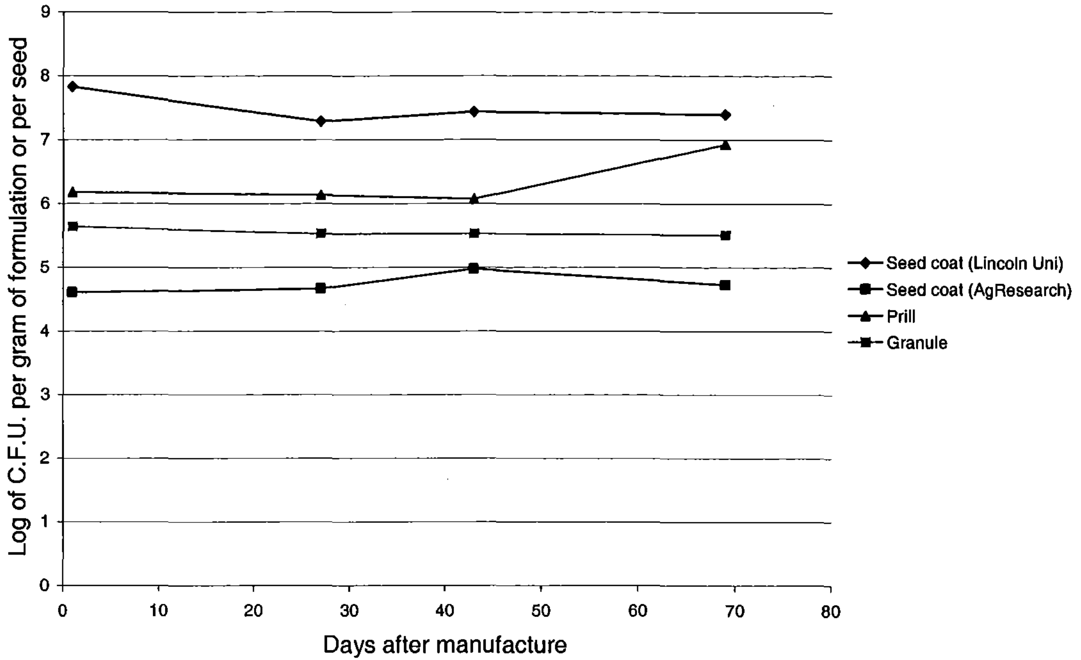

Figure 4.7: Longevity of the bacterium *B. mycoides* MW 27 in the different formulations used in field trial 6.

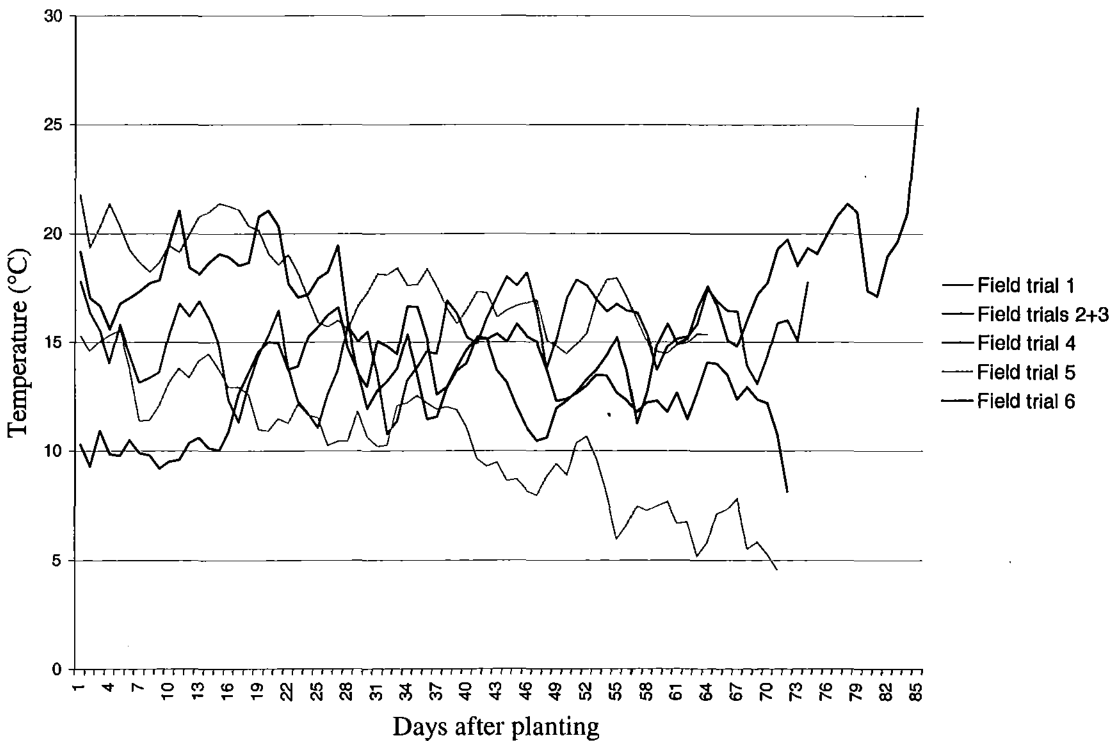

Figure 4.8: Daily average soil temperatures, recorded at 10 cm depth, for the six field trials.

4.4 Discussion

A series of field trials were used to evaluate the potential for biological control of Aphanomyces root-rot of pea using spore-forming bacteria. The six bacterial isolates initially tested were selected because of their activity against the pathogen *in vitro* and in glasshouse trials (Chapters 2 and 3). These bacteria were *B. subtilis* PT 69, *B. pumilus* MW 18, *B. mycoides* MW 27, *B. cereus* 15·80, *P. polymyxa* 18·25 and *P. polymyxa* 18·94.

In the first field trial, all bacteria were found to reduce the disease score relative to the nil-control, but not at significant levels. In part, this was due to the low number of treatment replication (4 replicates) used in the trial and the use of a disease scoring system based on the median disease score values. However, despite the equal ranking of the five best isolates, it was clear that plants treated with *B. mycoides* MW 27 performed best in the field trial. This isolate also effectively reduced disease symptoms in the glasshouse and was subsequently selected for ongoing evaluation. Selecting a second isolate for ongoing testing was more difficult. Ultimately, *P. polymyxa* 18·25 was selected, based on its control of oospore build up in pea roots in the glasshouse and strong *in vitro* anti-fungal activity.

In the first field trial, the presence of other root rotting pathogens, which caused similar symptoms to *A. euteiches*, proved to be a confounding factor. Despite a moderate level of *A. euteiches* inoculum in the soil, as determined by the rolled towel bioassay, the disease pressure was found to be mostly non-*A. euteiches*. Therefore, as in the glasshouse trials, it was impossible to determine if the disease reduction occurred through control of *A. euteiches* or other root rot pathogens without measuring oospore numbers in the root tissue.

Other researchers have suggested that environmental differences in the seasons over which biological control trials were conducted may favour different root pathogens, leading to variability in the results (Kommendahl and Windels; 1978). This variability would be inherent in such trials unless important factors, in particular watering, can be controlled through the use of irrigation. While investigating biological control of pea root rot using the bacteria *Burkholderia cepacia, Pseudomonas fluorescens* and *Corynebacterium* spp, Parke *et al.* (1991) found that poor biological control efficacy in non-irrigated sites was due to low disease expression. However, at irrigated sites, the disease pressure was

moderate to high, and significant levels of disease control and yield increases were observed.

Due to the lack of significant *A. euteiches* disease expression at the Lincoln field trial site, a farm at Punawai, Mid Canterbury, was selected for future trials. Rolled towel bioassays from soil tests indicated that one site on the farm contained a moderate level of *A. euteiches*-inoculum and another site, directly adjacent, a low level of inoculum. This allowed the running of two adjacent, concurrent trials, which were identical in all respects except for the *A. euteiches*-inoculum pressure. The results of trials at these sites yielded only a small difference in the average median disease score levels, however, the overall differences in yield based factors between the two trials was approximately threefold. This showed that the median disease scoring system was not sufficiently sensitive relative to the effect of root disease on plant growth and productivity. A similar observation was made by Parke *et al.* (1991) who in addition to a visual root disease score, also measured other factors such as plant stand, dry mass and yield. This resulted in an indication of both the disease level *per se*, and yield parameters. Other assessment parameters, such as determining the cumulative build up of the disease throughout a trial (Bowers and Parke, 1990 and 1993), provide better resolution of when, and at what level, the disease occurs. However, this necessitates regular, repetitive sampling of the trial and is more suited towards epidemiological studies.

Only two significant treatment effects were observed in field trials 2 and 3 at Punawai (high and low inoculum soils). In the high inoculum pressure trial, *B. mycoides* MW 27 was found to significantly ($P \leq 0.05$) increase the mean pod weight relative to the nil-control. In the low disease pressure soil, *B. mycoides* MW 27 had no significant ($P > 0.05$) effect relative to the nil-control, while treatment of pea seeds with *P. polymyxa* 18·25 significantly reduced the overall plant yield. A similar effect was observed by Kommedahl and Windels (1978) who reported that some of their unidentified bacterial isolates, which were antagonistic to fungal pathogens *in vitro*, were pathogenic and caused stand reductions *in situ*. Although reduction in plant stand by *P. polymyxa* 18·25 was not observed in any of the trials, it is possible that under non or low disease pressure conditions, application of this bacterium to pea seeds may result in yield-based losses, perhaps through physiological changes induced upon the pea plant. In contrast, significant

deleterious effects resulting from application of *B. mycoides* MW 27 to pea seed were never observed.

Combining bacterial biocontrol agents with the fungicides captan and metalaxyl has been investigated previously in an attempt to broaden their spectrum of activity and to enhance their biological control efficacy (Hwang *et al.*, 1996; Parke *et al.*, 1991), with mixed results. Parke *et al.* (1991) found no synergistic effect with isolates of *Pseudomonas* and *Burkholderia* with captan, whilst Hwang *et al.* (1996) found that *B. subtilis* and *P. polymyxa* combined with a half-recommended application rate of metalaxyl gave increased protection against damping-off of pea compared to individual treatments. In field trial 4, which was run at the Lincoln University field site area, the fungicide Apron, which includes both metalaxyl and Captan, was tested alone and in combination with *B. mycoides* MW 27 and *P. polymyxa* 18·25 for disease control. However, none of the treatments affected the level of pea root rot disease. Given that the overall disease expression in the trial was quite high, and that Apron had no effect on disease despite being active against most root rot pathogens, it was concluded that the predominant disease pressure was from *A. euteiches*. However, due to lack of significant rainfall this was not expressed until late in the trial period by which time low soil temperatures were probably limiting bacterial activity.

In field trial 5, the possibility of synergistic action between the bacterial isolates *B. mycoides* MW 27 and *P. polymyxa* 18·25 was investigated. The isolates were combined in equal proportions after first diluting *B. mycoides* MW 27 with a carrier to approximate the lower c.f.u. count of *P. polymyxa* 18·25. The combined bacterial treatment was not found to be significantly better than the bacterial treatments alone. Singly, the treatments of *P. polymyxa* 18·25 and *B. mycoides* MW 27 (undiluted rate) were also ineffective at reducing root rot disease. However, pea seeds treated with *B. mycoides* MW 27 at the low rate did show a significant increase in the fresh root-weight compared with nil-control plants. This treatment was also the most effective at reducing the root-rot disease score and also increased the plant length. A similar effect was found by Paul *et al.* (1995) who applied both *B. mycoides* and *Streptomyces* spp. to cucumber seeds for control of Pythium damping-off. They found that the biological control agents were less effective together than when applied to the seeds alone. This lack of synergy may result from inhibition of one bacterium by the metabolites, such as antibiotics, of the other. Strains of *P. polymyxa*

are known to produce a number of antibiotics active against other bacteria (Zuber *et al.*, 1993).

The increase in the efficacy of the bacterium when applied at the low rate may reside in either the differences in the dose (c.f.u. seed^{-1}) or in the carrier (the low rate contained $CaCO_3$). Ordinarily, unless the dose (inoculation rate) of a biological control agent reaches phytotoxic levels, increasing the dose should be associated with an increase in the level of biological control activity, not a decrease as was found. This was not the case in field trial 5. Therefore, it is possible that the greater efficacy of *B. mycoides* MW 27 at the low inoculation rate was due to the chemicals in which it was formulated. As $CaCO_3$ has been found to have little effect on the disease itself (Groth *et al.*, 1979; Lewis, 1977), especially at the low rate at which it was used, the effect of the compound was probably related to increasing the bacterial efficacy rather than reducing the activity of the pathogen. This view is supported by the work of Tu (1992), where $CaCO_3$ applied to soil stimulated the development of saprophytic bacteria, leading to a reduced level of pea root rot disease. Vidhyasekaran and Muthamilan (1999) also found that the biological control efficacy of *Pseudomonas fluorescens* was increased following incorporation of $CaCO_3$ into the carrier to regulate acidity.

In the final field trial, different formulations of the bacterium *B. mycoides* MW 27 were tested for disease control efficacy. The environmental conditions during the trial were more typical of average Canterbury conditions than those of previous trials. 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 differences were observed between the various formulations, with the prill formulation of *B. mycoides* MW 27 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 g for the prill treatment. Only the fungicide treatment, Apron, had a higher yield of pods per plot than the prill treatment but this resulted from a greater pea survival rate (plot stand), due to damping-off disease control, not from 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).

A number of other microorganisms have been shown to increase pea emergence. These include the fungi *Penicillium oxalicum* Currie and Them, *Aspergillus* spp. and *Trichoderma* spp. (Kommedahl and Windels, 1978), the spore-forming bacteria *B. subtilis* and *P. polymyxa*, *Bacillus* spp. (Kommedahl and Windels, 1978; Parke, 1987) and the Gram-negative bacteria *Burkholderia cepacia*, *P. fluorescens, Corynebacterium spp.* (Parke, 1987; Parke *et al.*, 1991). The most dramatic increases were shown by Parke *et al.* (1991), who described increases up to 40% in pea emergence following seed treatment by *Pseudomonas fluorescens* and *Burkholderia cepacia.* However, the damping-off disease pressure under which these bacterial isolates were tested was much greater than those generated in this study, allowing for greater capacity for control efficacy.

In this study, the bacterium *B. mycoides* MW 27 exhibited significant levels of disease control in some trials but not in others. Compared with the relatively consistent biological control results achieved by Parke *et al.* (1991), the biological control efficacy of this isolate appears low. However, trials 1-5 were beset by unanticipated problems which confounded the effects of the treatment bacteria, for example low rain fall and so low disease pressure. At the Punawai field site, irrigation was not available and at the Lincoln site, results were confounded by high numbers of other root rotting pathogens. However, in trial 6 the higher levels of soil moisture resulted in the level of disease pressure more typical of pea production in Canterbury in spring. These were the types of conditions Parke *et al.* (1991) were able to reproducibly produce through irrigation. When tested under non-irrigated conditions, the bacterial isolates of Parke *et al.* (1991) were also found to have inconsistent biological control efficacy. Similar effects were reported by Kommedahl and Windels (1978).

Further development of the prill and seed coat formulation of *B. mycoides* MW 27 may lead to increased activity (Dr Von Johnson; pers. comm.). In particular, there is scope for increasing the dosage level of the bacterium in the formulations, and manipulation of the various carrier components to provide better conditions for growth. The c.f.u. data showed that all types of formulations had a shelf-life of up to 70 d, despite being stored under ambient conditions. Conversely, the living biomass in formulations of non-spore forming bacteria, such as *Pseudomonas* and *Serratia* spp. usually decline rapidly (Nemec, 1997).

In conclusion, there is potential for the development of the bacterium *B. mycoides* MW 27 as a biological control agent for control of soil-borne pea diseases. Future experiments should focus on further development of the prill formulation of *B. mycoides* MW 27, particularly towards increasing the c.f.u. per gram of prill, and integration of the prill and seed coating treatments. If future field trials are to be conducted, a strong emphasis should be placed on providing irrigation so that disease can be both initiated and maintained. In addition, the potential integration of the fungicide with the bacterium should be revisited. This should initially be carried out in glasshouse trials in which the growth medium is inoculated with a number of root-rot pathogens alone, and in combination, to differentiate the effects between Aphanomyces and non-Aphanomyces disease control by the treatments.

Bacillus mycoides has also been reported to have a wide range of biological control activity in the scientific literature. For example, strains of the bacterium have been used to control needle rust (*Melampsora medusae* Thüm) of Douglas fir (McBride, 1969), *Alternaria helianthi* (Hansf.) Tub. *et* Nish. on sunflower (Kong *et al.*, 1997), take-all of wheat (Capper and Campbell, 1986), *Pythium mamillatum* damping-off (Paul *et al.*, 1995) and *Fusarium oxysporum* wilt of cucumber (Hammad and El-Mohandes, 1999).

Chapter 5 Biological control of wheat take-all disease and lettuce damping-off in the glasshouse with spore-forming bacteria.

5.1 Introduction

The Oomycete fungus *Pythium ultimum* occurs in agricultural soils worldwide but is more common in countries such as New Zealand that have a temperate climate (Martin, 1992) where it causes disease on a wide range of host plant species (Domsch *et al.*, 1980; Martin, 1992). One of the most severe diseases caused by the fungus is damping-off of vegetable seedlings. Losses are typically incurred through poor seed germination (pre-emergent damping-off), lack of seedling establishment (post-emergent damping-off) and poor seedling vigour (root-rot). In cool, moist heavy soils with high levels of organic material, the pathogen can cause large economic losses in susceptible varieties. Control of the disease is commonly achieved with fungicide-treated seeds. However, the pathogen is only susceptible to a narrow range of fungicides (Bruin and Edgington, 1983), such as the acylalinine-type of chemicals. Because they are expensive, their use is often uneconomic for low-value crops. In addition, the use of soil fungicides is currently causing concern because of toxicity to non-target organisms and the resulting disruption to soil ecosystems.

The trials described earlier in this study (Chapter 4) demonstrated that some of the biological control isolates reduced incidence of damping off of pea plants in the field. These bacteria, therefore, may have activity against damping-off diseases on other vegetable crop species and root diseases such as take-all of wheat.

Take-all disease of wheat which is caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), results in serious economic loss in cereals worldwide (Mathre, 1992). No commercial varieties of wheat show resistance to the disease and there are currently no effective fungicides registered for it. However, research into soils showing suppressiveness to take-all, has identified a number of potential biological control organisms (Mathre, 1992). Most research has been conducted into the use of *Trichoderma koningii* Oudem (Simon, 1989), a sterile red fungus (Sivasithamparam, 1998), *Pseudomonas* spp. (Ryder and Rovira, 1993; Weller *et al.*, 1988), *B. mycoides* (Capper and Campbell, 1986) and *B. pumilus* (Capper and Campbell, 1986; Weller, 1988) as biological control agents of the disease, using a variety of different mechanisms. *Trichoderma koningii* was shown to both inhibit *Ggt* through the production of antibiotic compounds and through competitive saprophytic colonisation of the soil harboring the pathogen (Simon *et al.*, 1989). The sterile red fungus also produced antibiotic compounds, but unlike *T. koningii* it was found to colonise the cortex region within wheat roots, thus restricting ectotrophic growth of the pathogen (Sivasithamparam, 1998). *Pseudomonas* spp. produced a range of antibiotic compounds and can compete for iron through siderophore production (Ryder and Rovira, 1993; Weller *et al.*, 1988). Likewise, *Bacillus pumilus* and *B. mycoides* inhibited growth of *Ggt in vitro* through the production of antifungal compounds such as toxins and cell wall degrading enzymes (Capper and Campbell, 1986).

In this chapter, isolates of spore-forming bacteria, selected for their *in vitro* anti-fungal activity (Chapter 2) and the disease suppressive isolates *B. mycoides* MW 27 and *P. polymyxa* 18·25, were tested for control of *P. ultimum* damping-off of lettuce and *Ggt* ‘take-all’ disease on wheat in glasshouse trials. Based on literature reports of strong antagonism by *B. pumilus,* a strain of this bacterium (PT 10) was included in the take-all assay.

5.2 Materials and methods

5.2.1 Biological control of *Gaeumannomyces graminis* var. *tritici* in the glasshouse

A preliminary glasshouse trial was carried out to determine if some spore-forming bacteria which had previously exhibited control of soil-borne plant pathogens (Chapters Two, Three and Four) had potential for biological control of wheat take-all disease.

5.2.1.1 Preparation of pathogen inoculum

Preparation of pathogen inoculum followed the method of Hollins *et al.* (1986). Seven bags, each containing 500 g of sand / maizemeal substrate (500 g dry river sand, 15 g maizemeal, 65 mL water) were autoclaved twice for 1 h periods over 2 d. Each bag of cooled substrate was seeded with ten, 1 cm diameter plugs of actively growing *Ggt* (strain Williams #2; Dr M. Cromey, Crop and Food Research) and incubated for 4 weeks at 22°C.

5.2.1.2 Treatments

Seven treatments were tested for take-all disease control:

1. Pathogen control – naked wheat seed in pathogen-amended potting mixture
2. No-pathogen control (as above but with no pathogen in the potting mixture)
3. Fluquinconazole (fungicide) –treated wheat seed
4. *B. mycoides* MW 27 –treated wheat seed
5. *P. polymyxa* 18·25 –treated wheat seed
6. *B. pumilus* PT 10 –treated wheat seed
7. *B. mycoides* MW 27 prill – 0.15 g prill placed below each naked wheat seed

Wheat seed, cv. Otane, was used throughout the experiment.

Treatment of seed with the fungicide, fluquinconazole (0.15 g a.i. Kg^{-1}), was carried out by Dr. M. Cromey, Crop and Food Research, Lincoln.

Bacterial seed treatment followed the earlier protocol for treatment of pea seeds (Section 4.2.3.2); protocols for *B. mycoides* MW 27 were used for *B. pumilus* PT 10. The freeze-dried bacteria were mixed with 2% (w/w) methyl-cellulose, 2% (w/w) glucose and 1% (w/w) $CaCO_3$, ground into a fine powder and treated onto moistened wheat seeds at maximum carrying capacity. The prill formulation of *B. mycoides* MW 27 was identical to that used before (Section 4.2.3.2).

5.2.1.3 Experiment design

The experiment was carried out in the glasshouse in plastic planter bags. Each PB 8 bag was half-filled with '3 month' potting mixture (80% composted bark, 20% WAP 5 crusher dust, containing Osmocote Plus 15-4.8-10.2 and Dolomite lime at 2 Kg m^{-3} each). A 1 cm deep layer of pathogen infested potting mixture (inoculum : potting mixture, 1: 6 v/v) was laid over the top, followed by a further 5 cm layer of pathogen-free potting mixture. Six wheat seeds were planted per bag, to a depth of approximately 3 cm. For the no-pathogen control, non-infested sand / maizemeal was used instead of the *Ggt* –inoculated sand / maizemeal inoculum.

Each of the seven treatments was randomly represented twice in each of six blocks in the glasshouse (randomised block design). One replicate was used for the determination of the level of root disease and the other for yield determinations.

5.2.1.4 Data collection and analysis

After 5 weeks, one replicate per block of each of the treatments was taken back to the laboratory for evaluation of take-all disease (presence of black vascular lesions on the roots and blackening of the stem base). However, the use of potting mixture as a growth substrate instead of sand (Hollins *et al.*, 1986) made the recovery of the fibrous roots nearly impossible. Therefore, this assessment could not be completed. The remaining plants (replicate two) were grown to maturity and assessment made of the plant length, numbers of seed heads per plant, and the dry weight of seed heads.

ANOVA was used to determine whether significant treatment effects occurred, and Duncan's multiple-range test was used to show significant differences between treatments.

5.2.2 Use of spore-forming bacteria for control of *Pythium ultimum* damping-off of lettuce

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

5.2.2.1 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 on corn-meal agar (CMA; Gibco) 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 amended with $CaCO_3$ at 3 g L^{-1} and cholesterol at 30 mg L^{-1} (Appendix 3). 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 resulting 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 found to be 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.

5.2.2.2 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 (cv. 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.

5.2.2.3 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).

5.2.2.4 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 which 0.1 ml aliquots were taken and spread across the surface of NA plates. The Petri dishes were incubated overnight at 25°C, and the number of bacterial colonies counted.

5.2.2.5 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 (Figure 5.4). 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.

Each germination tray was placed in a 2 L plastic 'ice-cream' container (Figure 5.3). 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.

5.2.2.6 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.

5.3 Results

5.3.1 Biological control of *Gaeumannomyces graminis* var. *tritici* in the glasshouse

At maturity, wheat grown in take-all amended potting mixture exhibited typical signs of infection, specifically 'premature senescence of the ear' (Figure 5.2). This resulted in lowering of the yield per plant ($P \leq 0.05$). Decrease in yield was a result of both a reduction in the number of heads per plant (Table 5.1) and the weight per head (take-all infected heads did not 'fill' and were, therefore ,very light).

Treatment of wheat seed affected the length ($P= 0.009$), numbers of heads ($P=0.01$) and total yield ($P=0.006$) of the wheat plants.

Treatment of wheat seeds with *B. pumilus* PT 10 and *B. mycoides* MW 27 (prill), and those grown in potting mixture without *Ggt* inoculum, resulted in significantly more heads per plant ($\alpha=0.05$) compared to the pathogen control.

Bacillus pumilus-treated plants, and those grown in the pathogen-free potting mixture, had significantly higher ($\alpha=0.05$) yields per plant than the nil-control (Figure 5.1). The yields from wheat treated with *Bacillus pumilus* PT 10 and grown under take-all disease pressure was not significantly different ($\alpha=0.05$) to those grown in the absence of the pathogen (Table 5.1; Figure 5.2)

Table 5.1: Effect of different seed treatments on the length, head number and head yield of wheat plants grown in potting mixture amended with the take-all fungus, *Gaeumannomyces graminis* var. *tritici*.

	C.F.U. / seed[1]	Plant length (cm)[2]	Heads / plant	Total yield / plant (g)[3]
No-pathogen	6	64.32 a	2.85 a	8.89 a
B. pumilus PT 10	2.6×10^8	58.00 abc	2.88 a	8.23 ab
B. mycoides MW 27 (prill)	2.4×10^5	58.57 ab	2.24 a	5.99 bc
B. mycoides MW 27	1.5×10^8	55.31 bc	2.04 b	4.91 c
P. polymyxa 18·25	8.8×10^7	51.67 c	2.05 b	4.15 c
fluquinconazole	1.3×10^4	56.84 bc	2.45 a b	6.11 bc
Pathogen only	6	52.86 bc	1.96 b	4.58 c
LSD (α=0.05)		6.389	0.607	2.708

[1] Colony forming units of bacteria per seed or prill applied to each seed

[2] From 1 cm above soil level

[3] Oven-dry weight

Treatment means followed the same letter are not significantly different (LSD; α=0.05)

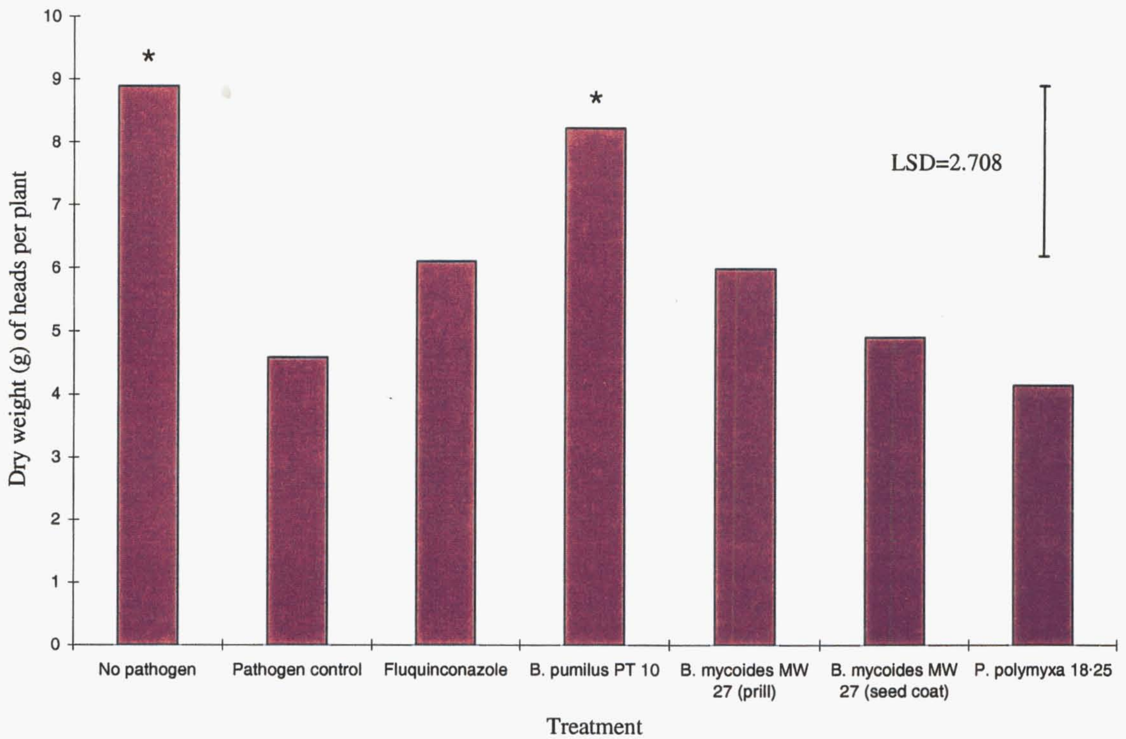

Figure 5.1: Effect of seed treatment on yield per plant (total dry head weight) of wheat grown in potting mixture containing the take-all pathogen, *Gaeumannomyces graminis* var. *tritici*. * Denotes significant difference compared with the pathogen control at α=0.05.

Figure 5.2: Wheat plants near maturity exhibiting symptoms of take-all disease. White-heads (pale coloured amongst the healthy green ones) have no or few viable grains.

5.3.2 *Pythium ultimum* trial 1: glasshouse

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 5.2). 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 rate 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 5.2).

Table 5.2: 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 ($\alpha=0.05$)	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 (LSD; $\alpha=0.05$)

Figure 5.3: Set-up of the lettuce damping-off trial. Each container holds a seedling-germination tray in which 25 treated lettuce seeds were planted.

Figure 5.4: Effect of treatment of lettuce seeds on germination in *Pythium ultimum*-amended potting mixture in a growth cabinet trial. Pathogen-free control left, *B. mycoides*-treated seed centre, and pathogen-only control right.

5.3.3 *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 *Pythium ultimum* had significantly higher levels of germination than all other treatments (Table 5.3). 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 5.3). Lettuce seeds treated with *B. mycoides* MW 27 had a significantly lower level of germination than those treated with *P. polymyxa* 18·25.

Table 5.3: 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. / seed**[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 (LSD; α=0.05)

5.4 Discussion

In this study, three spore-forming bacteria were evaluated in a glasshouse trial for control of take-all disease. Selection of isolates was based on either their efficacy for Aphanomyces disease control in the field (*B. mycoides* MW 27), their suppressiveness towards *Ggt* and other fungi *in vitro* (*P. polymyxa* 18·25 and *B. pumilus* PT 10) or evidence in the scientific literature of their successful use against take-all disease (*B. mycoides* and *B. pumilus*).

In the glasshouse trial, treatment of wheat seeds with the bacterium *B. pumilus* PT 10 was found to result in significant increases in plant productivity compared with the non-treated control, when they were grown in take-all amended potting mixture. The yield per plant was increased 80% from 4.58 g $plant^{-1}$ to 8.23 g $plant^{-1}$. Non-treated plants were found to have significantly ($P \leq 0.05$) fewer heads per plant and more white (senescent) heads that

are typical take-all symptoms. Treatment of wheat seeds with the prill formulation of *B. mycoides* MW 27 also resulted in a significant increase in the number of heads per plant. Although this resulted in a 9% increase in yield, greater experimental replication (only six replicates per treatment were used) would be needed to demonstrate significant differences over the nil-control at $\alpha=0.05$. These results complement examples of biological control of take-all in the scientific literature. Under ideal conditions, application of *B. mycoides* and *B. pumilus* as a soil drench by Capper and Campbell (1986) resulted in the doubling of spring wheat yield and halving of disease incidence in the field. In other trials, however, where disease incidence was low or soil conditions dry, no benefits were observed.

Both *B. mycoides* and *B. pumilus* have been shown to cause lysis of the hyphae of *Ggt* growing on wheat roots (Faull and Capper, 1979), which may account for some of their biological control activity. In addition, the bacterium *Bacillus pumilus* was found to produce cell-wall degrading enzymes and anti-fungal compounds (Leifert *et al.*, 1995; Nielsen and Sörensen, 1997), and has been shown to stimulate defense responses on treated plants (Benhamou *et al.*, 1996).

The prill formulation of *B. mycoides* MW 27 was found to be more effective than the seed-coat formulation at increasing wheat yield; similar findings to those for control of Aphanomyces root-rot disease (Chapter 4). The increased efficacy of the prill compared with the seed coat formulation may be due to a number of factors. Firstly, the prill formulation contains a number of different components, the identity of which are proprietary (AgResearch Ltd., Lincoln), which control the release (temporal) of the biological control agent, buffer its local environment (pH) and provide a nutrient source to promote its growth and allow greater potential for colonisation of the rhizosphere and suppression of disease. In addition, the prill treatment is well placed spatially, i.e. directly below the seeds, for root colonisation compared with the seed coat treatment.

Despite exhibiting strong *in vitro* activity against *Ggt* (Chapter 6), *P. polymyxa* 18·25 was found to be ineffective against the disease. The lack of field activity by this bacterium may be related to its slow growth rate. This was probably of no consequence in the *in vitro* assays, but could have been critical *in situ* where colonisation of root is thought to be a prerequisite for control of soil-borne diseases such as take-all (Weller, 1988).

Treatment of wheat seed with the fungicide fluquinconazole resulted in a yield increase of 40% over the nil-control treatment, however the difference was not significant. Overseas, the fungicide has recently been found to be effective for control of cereal root diseases (Löchel *et al.*, 1998, Stock *et al.*, 1998; Dawson and Bateman, 2000). In over 50 field trials, the fungicide treatments have resulted in average yield increases of 10% for wheat grown under different take-all disease pressures (Löchel *et al.*, 1998). In this glasshouse trial, yield increases for the fluquinconazole treatment were significantly lower than those for the *B. pumilus* treatment. However, experience in this and other studies has shown that glasshouse trials can only indicate potential, and that numerous field trials are required to confirm efficacy in disease control.

The glasshouse trial, therefore, should be repeated to determine whether the yield increases from the bacterial treatment are reproducible. In addition to the yield information, data on the number of senescent heads per plant and level of root disease should be taken to demonstrate control of the disease on the root and subsequent effect on yield. In this case, sand should be used in addition to a potting mixture treatment, as it can be easily washed from the roots, allowing visual disease assessments (Ryder and Rovira, 1993). If disease reduction by the bacterial isolates is confirmed, field trials should be conducted.

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 actually increased damping-off significantly. Given the large amount of variation both between and within the treatments, the trial was repeated using a slightly modified protocol. In the second 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}, 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 pathogen inoculum had been added was approximately 55%. A large proportion of the variation and disease expression in the trial was clearly generated independently of the *P. ultimum* inoculum and may well have created greater effects than did the trial treatments. The abilities of the spore-forming bacterial isolates to control damping-off disease were, therefore, difficult to determine. 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, be a basis from which to 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 previous trials (Chapter 4).

There are many examples in the literature describing the use of spore-forming bacteria for the control of *Pythium* damping-off of vegetable diseases. For example, Hwang *et al.* (1996) demonstrated the control of *P. ultimum* and *P. irregulare* Buisman damping-off of pea in field trials using *B. subtilis* and *P. 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 this type of disease, and given the difficulties described in the previous 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. 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), therefore 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.

In conclusion, these experiments have demonstrated the potential of spore-forming bacteria, particularly *B. mycoides* MW 27 and *B. pumilus* PT 10, for the control of wheat take-all disease. In addition, prospects for the control of *Pythium*-type damping-off diseases were investigated and, although not successful, rationale and protocols have been developed for future investigation.

Chapter 6 *In vitro* investigation into the anti-fungal activity of *B. mycoides* MW 27 and *P. polymyxa* 18·25.

6.1 Introduction

Bacterial biological control agents may limit the severity of soil-borne plant diseases by either pathogen suppression, or by modifying the host plant or its relationship with the pathogen (Chet *et al.*, 1990; Weller, 1988). Pathogen suppression usually occurs through the production of antibiotics, lytic enzymes or siderophores by the bacteria, or by competition for substrates or niche exclusion. The bacteria may also affect the interactions between the host plant and pathogen through production of plant growth promoting metabolites (e.g. phytohormones), release of nutrients or minerals which increase plant growth, or induction or priming of plant resistance responses.

In the scientific literature, there are many examples of the above mechanisms of disease suppression pertaining to isolates of *Bacillus* and *Paenibacillus*. These bacteria have been shown to produce a wide number of antibiotic compounds implicated in biological control (Dijksterhuis *et al.*, 1999; Leifert *et al.*, 1995; Pandey *et al.*, 1997; Walker *et al.*, 1998), as well as hydrolytic enzymes or lytic compounds capable of degrading fungal cell walls (Nielsen and Sörensen, 1997; Campbell, 1983). In addition, they have demonstrated mechanisms of plant growth promotion via hormone production (Lebuhn *et al.*, 1997) or release of nutrients and minerals to plant roots (Brown, 1974).

The initial selection of *B. mycoides* MW 27 and *P. polymyxa* 18·25, which were used extensively in field trials against Aphanomyces root-rot disease on peas (Chapter 4), was based on their observed suppression of the pathogen, *A. euteiches,* in *in vitro* assays (Chapter 2) and glasshouse trials (Chapter 3). This *in vitro* suppression, observed as zones of inhibition, is likely to have resulted from production of cell-wall degrading enzymes (hydrolytic enzymes), anti-fungal agents (antibiotics) or a combination of both.

The cell wall of the 'true-fungi' is predominantly composed of chitin, a polymer of *N*-acetyl-β-D-glucosamine. In contrast, the cell wall of the Oomycete fungi is principally cellulose, a β-1,4-glucose polymer (Ruiz-Herrera, 1992). The investigation of cell wall degrading enzymes, therefore, should focus on chitinase and cellulase and, to a lesser extent, β-1,3-glucanase which is a major storage carbohydrate for fungi.

The anti-fungal compounds produced by *Bacillus* and *Paenibacillus* are predominantly peptidal (Hiraoka *et al.*, 1992; Katz and Demain, 1977; Lebbadi *et al.*, 1994; Pichard *et al.*, 1995). In addition, their production often occurs in conjunction with the sporulation process, the genes for each being closely linked (Sandoff, 1972). To successfully isolate such compounds, the bacterial cultures must be encouraged to sporulate profusely, and then appropriate techniques may be used for the recovery of peptides or small proteins.

6.2 Material and methods

6.2.1 Production of cell wall-degrading enzymes by *P. polymyxa* 18·25 and *B. mycoides* MW 27

The bacteria *P. polymyxa* 18·25 and *B. mycoides* MW 27 were tested for production of cell wall-degrading enzymes using chromagenic substrates (i.e. substrates linked to a dye). Upon degradation of the substrate, the dye is released and can be observed diffusing through the agar (Thorn, 1993). *β*-1,3-glucanase activity was assayed using the substrate laminarin-azure, β-1,4-glucanase activity was assayed with cellulose-azure and chitinase activity was tested using remazol brilliant violet-linked chitin.

In an expansion of the method used by Thorn (1993), Universal bottles were filled with 15 mL of basal medium (agar 15 g L^{-1}, yeast extract 1.0 g L^{-1}, KH_2PO_4 1.25 g L^{-1}, $MgSO_4.7H_2O$ 0.625 g L^{-1} and peptone 0.625 g L^{-1}), autoclave-sterilised and allowed to cool. Under aseptic conditions, 1.5 mL of overlay medium (agar 15 g L^{-1}, malt extract 5 g L^{-1}, yeast extract 1 g L^{-1} and either cellulose, laminarin or chitin substrates at 20 g L^{-1}) was pipetted over the basal medium. After being allowed to set overnight, the surface of the overlay medium was streaked with *P. polymyxa* 18·25, *B. mycoides* MW 27, or left as a nil-control. The bottles were incubated at 30°C and checked daily for signs of enzymatic activity; three replicate bottles were set up for each treatment.

6.2.2 Determination of the anti-fungal spectrum of activity of *P. polymyxa* 18·25 and *B. mycoides* MW 27

The spectrum of inhibition exhibited by the bacterial isolates *P. polymyxa* 18·25 and *B. mycoides* MW 27 against a wide range of plant pathogenic fungi was determined using a dual-culture bioassay technique (Section 2.2.4).

Isolates of *Botrytis cinerea* Pers., *Fusarium culmorum* (W.G.Sm.) Sacc., *F. graminearum* Schwabe, *F. oxysporum* Schlecht, *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C.Snyder & H.N.Hansen, *F. oxysporum* f. sp. *pisi* (C.J.J.Hall) W.C.Snyder & H.N.Hansen, *F. nivale* (Fr.) Ces., *Phytophthora cinnamomi* Rands, *Pythium ultimum, Sclerotinia minor* Jagger, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Sclerotium cepivorum* Berk., *Sclerotium rolfsii*

Sacc., and *Rhizoctonia solani* were taken from the Lincoln University culture collection. An isolate of *Ggt* (Williams # 2) was obtained from Dr M. Cromey (New Zealand Institute for Crop and Food Research Ltd.) and *Pythium irregulare* Buisman was obtained from Dr N. Waipara (AgResearch Ltd.). Isolates of *F. oxysporum* f. sp. *pisi*, *Thielaviopsis basicola, A. euteiches* and *Pythium* spp. were isolated from diseased pea roots (Section 4.2.1.10). Dual culture assays were carried out on PDA or, for the Oomycetes, CMA, using a similar method as described previously (Section 2.2.4). Observations were made after 10 d incubation at 20°C under a 12 h photo-period. Each Petri dish had four replicate interaction zones.

6.2.3 Assay of antibiotic activity by *P. polymyxa* 18·25

6.2.3.1 Bioassay technique

For each bioassay, a 5 mm diameter plug of test fungus, either *Ggt* or *A. euteiches,* was taken from the edge of an actively growing culture and placed in the centre of a PDA or CMA (respectively) Petri dish. Two, 5 mm diameter wells were cut from the agar approximately 30 mm either side of the fungal inoculum and the solution to be bioassayed was pipetted into the wells. The plates were incubated at 25°C until the fungus had reached or grown over the test wells. For each fungus / test solution combination, duplicate plates were set up. The ability of the fungus to grow up to or over the area containing the test solution was assessed.

6.2.3.2 Isolation of anti-fungal compounds from broth culture

The production of antibiotics by *P. polymyxa* 18·25 was tested in six different liquid culture media: nutrient broth (NB), potato dextrose broth (PDB), *Bacillus* fermentation broth (BFB; Dr Von Johnson, AgResearch Ltd.; Appendix 3), tryptose soy broth (TSB; Kurusu and Ohba, 1987; Appendix 3), tryptone glucose yeast extract broth (TGYE; Appendix 3), and cabbage broth (CB10; Leifert *et al.*, 1995; Appendix 3). For each broth, 100 mL was put into a 2 L flask and inoculated with 10 mL of *P. polymyxa* 18·25 suspension. The *P. polymyxa* 18·25 suspension was generated by culturing the bacterium on PDA plates for 10 d at 30°C, and scraping the cells into 10 mL of sterile water.

Inoculated flasks were incubated at 25°C, in dark conditions whilst being gently shaken at 100 r.p.m.

Samples of culture broth were taken at 0, 3, 5, 7, 10 and 14 d after inoculation. At each sampling, 5 mL of broth was removed and centrifuged at 10 000 × g for 30 min. The pellet and supernatant were then placed into separate Universal bottles, autoclave sterilised, and bio-assayed for activity against *Ggt* and *A. euteiches* (Section 6.2.3.1).

6.2.3.3 Precipitation of anti-fungal compounds from broth culture

Two techniques were used to precipitate anti-fungal compound/s from PDB culture: ammonium sulphate precipitation (Copeland, 1994; Lebbadi *et al.*, 1994), and HCl precipitation (Hiraoka *et al.*, 1992; McKeen *et al.*, 1986). In both cases, 1000 mL 14 d PDB cultures of *P. polymyxa* 18·25 were grown as before (Section 6.2.3.2). The broth was prepared for the precipitation procedures by centrifuging off the solid material at 16 500 × g for 20 min at 4°C.

The ammonium sulphate procedure followed that described by Copeland (1994). The pre-cooled broth supernatant (4°C) was placed into a beaker and continually stirred with a magnetic flea whilst ammonium sulphate was slowly added. Two fractionation steps were used: 30% (16.6 g ammonium sulphate 100 mL^{-1} broth) and 80% saturation (32.6 g 100ml^{-1}). Metabolites precipitated from solution were collected at each step by centrifugation at 10 000 × g for 30 min. The precipitates were re-suspended into 5 mL of distilled water, autoclave-sterilised and bio-assayed as described before (Section 6.2.3.1).

In the HCl method, concentrated HCl was added slowly to the cooled supernatants of broth cultures in a similar manner to that described above. HCl was added until pH 2 was reached and then the precipitate was collected, re-suspended and tested for inhibition against *Ggt* and *A. euteiches* (Section 6.2.3.1).

6.2.3.4 Extraction of anti-fungal compounds from solid culture

The presence of an antibiotic(s) compound in the solid medium on which *P. polymyxa* 18·25 had been cultured was determined. The bacteria were taken from an actively

growing culture and streaked over one-third (against an edge) of a PDA Petri plate, leaving the rest bare. The Petri plate was incubated for 7 d at 30°C, whereupon it was cut with a scalpel into three sections: one containing the bacterial growth, one adjacent to the bacterial growth, and one distant from the bacterial growth. The agar from each section was placed into a Universal bottle, autoclaved sterilised, and tested for inhibition against *Ggt* and *A. euteiches* (Section 6.2.3.1).

An attempt was made to extract the antibiotic(s) from agar cultures using the solvents ethanol, methanol, acetone, and water (Howell and Stipanovic, 1979). For each solvent, 10 PDA Petri plates were inoculated with *P. polymyxa* 18·25 and incubated for 10 d at 30°C. Bacterial growth was scraped from the surface of the agar prior to its use. The agar and 100 mL of solvent were comminuted in a Waring blender for 2 min, transferred into a glass beaker and allowed to stand overnight at room temperature. The resulting slurry was strained through a double layer of muslin cloth and centrifuged at 10 000 × g for 30 min. For the ethanol, acetone, and methanol solvent extractions, the resultant liquid was reduced using a rotary evaporator leaving an aqueous solution. The resultant liquid extracts were tested for inhibition against *Ggt* and *A. euteiches* (Section 6.2.3.1).

Electrophoresis was also used to extract anti-fungal compounds from solid agar. *Paenibacillus polymyxa* 18·25 was sub-cultured onto 10 PDA plates and incubated at 30°C. After 10 d, the bacterial growth was scraped from the surface, the agar collected into a Schott bottle and autoclave-sterilised. The molten agar was poured into a horizontal mini-gel box and covered with TAE buffer. Electrophoresis was carried out for 1 h at 100 volts after which the buffer was recovered from each end of the gel box (i.e. at the positive and negative terminals), and the agar collected. Portions of the buffers and the agar gel were put into Universal bottles, re-autoclaved and tested for inhibition against *Ggt* and *A. euteiches* (Section 6.2.3.1).

6.2.3.5 Extraction of anti-fungal compounds from freeze-dried culture

When grown on PDA, *Paenibacillus polymyxa* 18·25 produced large quantities of a viscous, turbid, exo-polysaccharide material. This material was collected from the surface of 20 PDA cultures, which had grown for 14 d at 30°C under dark conditions, and was freeze-dried. The resulting material, which had a consistency similar to thick fibrous

paper, was autoclave-sterilised and plated in dual-culture against both *A. euteiches* and *Ggt* to determine the presence of anti-fungal activity. Two methods were then used in an attempt to extract the active fraction.

Firstly, 1 g of the freeze-dried substrate was placed into a Universal bottle with 10 mL of distilled water and left overnight. A 1 mL portion of the water was removed from the bottle, autoclave-sterilised and bio-assayed for anti-fungal activity (Section 6.2.3.1). The remaining material was then autoclaved (i.e. water and substrate autoclaved together), and bio-assayed for anti-fungal activity.

In a second experiment, 10 mL of ethanol was added to 1 g of the freeze-dried substrate and allowed to sit overnight. The solid and liquid fractions were separated by centrifugation at 10 000 × g for 20 min and the liquid fraction reduced to dryness *in vacuo*. The resulting precipitate was re-suspended in 5 mL of distilled water, which was autoclave-sterilised and tested for inhibition against *Ggt* and *A. euteiches* (Section 6.2.3.1).

6.3 Results

6.3.1 Production of cell wall-degrading enzymes by *P. polymyxa* 18·25 and *B. mycoides* MW 27

Paenibacillus polymyxa 18·25 was found to degrade cellulose and chitin but not laminarin in the crude, *in vitro* assay system whereas the bacterium *B. mycoides* MW 27 did not degrade any of the substrates tested (Figure 6.1).

6.3.2 Anti-fungal spectrum of activity

In dual-culture assays, *P. polymyxa* 18·25 inhibited the growth of the Ascomycete fungi *Botrytis cinerea, Gaeumannomyces graminis* var. *tritici* (Figure 6.2), *Sclerotinia sclerotiorum*, and *Sclerotium cepivorum*, the Basidiomycete *Rhizoctonia solani*, the Deuteromycetes *Fusarium culmorum, F. graminearum, F. oxysporum* (Figure 6.2), *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *pisi*, *F. nivale*, *Thielaviopsis basicola*, the Oomycetes *Aphanomyces euteiches, Phytophthora cinnamomi, Pythium irregulare* and *Pythium* spp., and the sterile fungus *Sclerotinia minor*. However, the bacterial isolate did

not inhibit the growth of the Basidiomycete *Sclerotium rolfsii* or the Oomycete *Pythium ultimum*.

Bacillus mycoides MW 27 had slight transient inhibition towards *Fusarium culmorum* and *A. euteiches*, but was not inhibitory towards any of the other fungi assayed.

Figure 6.1: Degradation of cellulose by *P. polymyxa* 18·25 (right) but not by *B. mycoides* MW 27 (left).

6.3.3 Production of an antibiotic compound by *P. polymyxa* 18·25

6.3.3.1 Isolation of anti-fungal compounds from broth culture

Anti-fungal activity was not found in the supernatant fractions of any of the six broth cultures of *P. polymyxa* 18·25, but was found in all of the autoclaved pellet fractions which contained the bacterial spores / cells from 5-14 d old cultures.

6.3.3.2 Precipitation of anti-fungal compounds from broth culture

Anti-fungal activity was not found with either the 30% or 80% ammonium sulphate precipitate fractions, or from the HCl treatment precipitate.

6.3.3.3 Isolation of anti-fungal compounds from solid culture

Anti-fungal activity, against both *Ggt* and *A. euteiches*, was found using the autoclaved agar taken from directly underneath the bacterial growth and from the agar directly adjacent to the bacterial growth. The limit for detection of anti-fungal activity, was approximately 3 cm from the edge of the bacterial growth.

No anti-fungal activity was found in the water, ethanol, methanol or acetone extractions made from agar on which *P. polymyxa* 18·25 had been cultured.

No anti-fungal activity was found in the buffer solution following electrophoresis of agar on which the bacterium had been grown. However, the agar recovered after electrophoresis was found to have anti-fungal activity when autoclaved and challenged against *Ggt* and *A. euteiches*.

6.3.3.4 Isolation of anti-fungal compounds from freeze-dried culture

Freeze-dried bacterial growth was found to inhibit the growth of *Ggt* and *A. euteiches* following autoclaving. However, neither water nor ethanol extractions demonstrated anti-fungal activity. Autoclaving the freeze-dried sample in water changed its consistency to a thick slimy paste. Although this was found to have anti-fungal properties, its consistency made it unsuitable for further purification and extraction procedures.

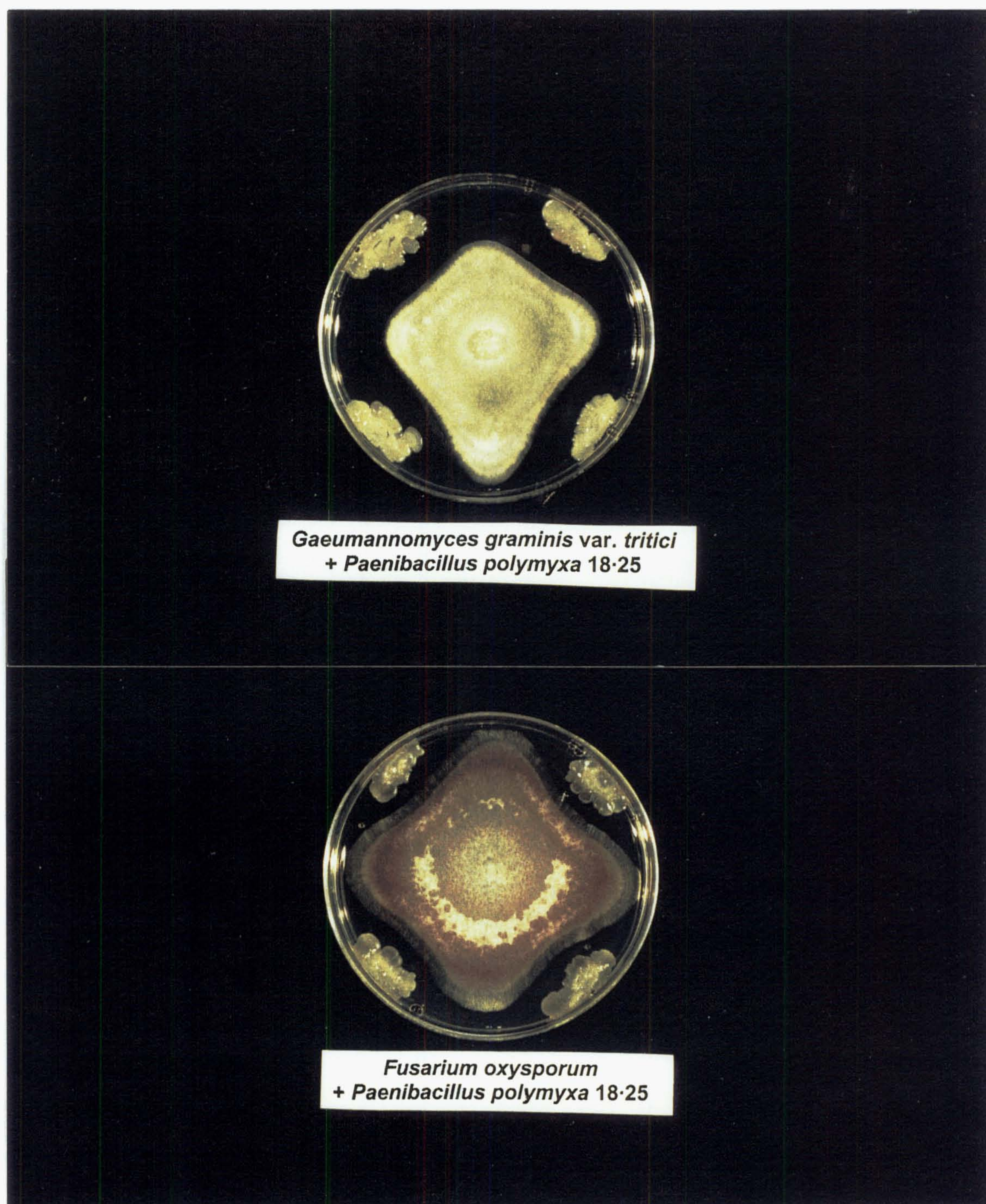

Figure 6.2: Anti-fungal activity by *P. polymyxa* 18·25 against *Gaeumannomyces graminis* var. *tritici* and *Fusarium oxysporum*.

6.4 Discussion

Paenibacillus polymyxa 18·25 inhibited a wide range of fungi *in vitro*. The spectrum of activity included fungi from the four subdivisions: Ascomycetes, Basidiomycetes, Deuteromycetes, and the Oomycetes. However, the bacterial isolate did not inhibit *Sclerotium rolfsii* or *Pythium ultimum*. Similar results have been reported before. Oedjijono *et al.* (1993), for example, found an isolate of *P. polymyxa* to suppress a wide range of fungi, including *Botrytis cinerea, Fusarium solani, Ggt, Penicillium echinulatum* Fassatiova, *Phytophthora cinnamomi*, *Phytophthora cactorum*, *Sclerotinia minor* and *S. sclerotiorum*, but the isolate was also not active against *Pythium ultimum*.

The lack of significant anti-fungal activity in dual culture by *B. mycoides* MW 27 was contrary to expectations. This bacterium was originally selected for glasshouse trials against Aphanomyces root-rot on peas because of its ability to reproducibly suppress the growth of *A. euteiches in vitro* (Wakelin *et al.*, 1998). It is possible that the bacterial isolate lost the ability to produce an anti-fungal agent after successive sub-culturing in the laboratory. Nevertheless, this isolate was found to be the most effective at reducing root rot disease on peas in the field. Production of antibiotic substances probably does not contribute to the disease suppression exhibited by this isolate.

Bacillus mycoides has been reported to be inhibitory to only a few plant pathogenic fungi. *In vitro*, *B. mycoides* completely inhibited the growth of *Pythium mamillatum* Meurs (Paul *et al.*, 1995) and *F. oxysporum* (Hammad and El-Mohandes, 1999). Pandey *et al.* (1997), reported that an isolate, tentatively identified as *B. mycoides*, was able to inhibit *Aspergillus nidulans* (Eidam) Winter, *A. parasiticus* Speare, *Cladosporium cladosporioides* (Fr.) de Vries, *Dicyma* sp., *F. oxysporum, Humicola fuscoatra* Traaen, *Mucor hiemalis* Wehmer but not *Penicillium raistrickii* Smith, *Sphaerostilbe repens* Berk. MA Curtis, or *Trichoderma pseudokoingii* Rifai. Unfortunately, in this study only one of these fungi, *F. oxysporum*, was common to the range of fungi tested and it was not inhibited.

Inhibition of fungi in *in vitro* bioassays involving a bacterial antagonist usually results from the production of cell-wall-degrading enzymes (lytic enzymes) or anti-fungal agents (antibiotics). *Bacillus mycoides* MW 27 did not produce any of the lytic enzymes that are

usually considered necessary for degradation of hyphal cell walls. Since lysis of *Ggt* hyphae in soil by an isolate of *B. mycoides* has been shown to be involved in the biological control of take-all disease (Campbell and Ephgrave, 1983), it seems likely that production of such enzymes may vary between strains.

Paenibacillus polymyxa 18·25 was found to produce chitinase and cellulase enzymes in this study, which may account for some of its anti-fungal activity. Strains of this bacterium have been shown to produce similar lytic enzymes in earlier studies, and this property was considered to cause inhibition of the growth of *Ggt* (Mavingui and Heulin, 1994). However, it is likely that the enzymes do not act alone in causing inhibition of fungal growth but, rather synergistically with other antibiotic compounds. Enzymatic activity on the cell walls may produce small pores which allow increased permeation of the anti-fungal compounds (Lorito *et al.*, 1994), thus providing a synergistic effect.

The anti-fungal compound(s) produced by *P. polymyxa* 18·25 were stable following autoclaving, freezing and electrophoresis, and were unchanged by attempts to extract them with ethanol, methanol and acetone solvents. On solid media, the anti-fungal activity produced by *P. polymyxa* 18·25 was found to migrate through the medium, indicating that it was polar, but could not be readily extracted from it despite using different solvent systems and electrophoresis. When grown in broth, however, no anti-fungal compound(s) were secreted into the liquid media; the only activity observed being that associated with, or attached to, the bacterial component. The activity, however, could not be separated directly from the bacterial matter using various solvents. Given the characteristics above, it is likely that the compound(s) is / are a small peptide, closely attached or bound to the bacterial spore or cell (pers. comm.; Dr Barry Seddon, University of Aberdeen). As such, future attempts at extraction of the active compound/s should focus on disruption of the cell, such as autoclaving cells in water, ultra-sonic destruction or enzymatic degradation.

Chapter 7 Summary and conclusions

7.1 Overview

Peas are an important agricultural species in New Zealand. They are valued for their high cash return, as a break crop between cereals, and for their ability to maintain soil fertility. Unfortunately, production of the crop is being limited due a root rot disease caused by the soil-borne Oomycete pathogen, *Aphanomyces euteiches*. The pathogen reduces crop yields and limits the planting of the crop to non- or slightly-infested soils. For various reasons, neither fungicides, crop rotation, nor pea genetic resistance provide acceptable levels of disease control. This means that disease avoidance is the only viable 'control' option.

Given the importance of peas as an agricultural crop species in New Zealand and the losses resulting from Aphanomyces root rot disease, The Foundation for Arable Research (FAR) and The Vegetable and Potato Growers Federation of New Zealand (VegFed) identified investigation into control methods for *A. euteiches* as a high priority. In recent years, conventional disease management practices have not been effective in control of this disease. This and the growing interest in organic and sustainable pea production indicated a need for a different approach to solving the problem of pea root rot in Canterbury. The potential for using naturally occurring antagonistic microorganisms had been identified in recent reports (Gritton *et al.*, 1995; King and Parke, 1993; Parke *et al.*, 1991; Wakelin *et al.*, 1998) and led to the development of a research programme whose aim was to "investigate the potential for biological control of *Aphanomyces euteiches* under local (Canterbury) conditions". To this end, six objectives were developed (Section 1.8.1), and are discussed in turn below.

7.2 Thesis objectives

7.2.1 Objective 1

The first objective was to develop a model for biological control of Aphanomyces root rot disease and to identify a class of microorganism to suit the model. As the disease occurs on the root tissue of pea plants, the model for biological control was based on the treatment of pea seed with a biological control agent, resulting in the colonisation of the root zone and disruption of one or more of the life-cycle phases of the pathogen in the rhizosphere / rhizoplane environments. Spore-forming bacteria were selected as the preferred biological control agent for a number of reasons: (a) their ubiquitous distribution in soils and rhizosphere environments (including that of pea; Patwari, 1956); (b) production of a wide range of anti-fungal metabolites, including antibiotics and cell wall degrading enzymes; (c) numerous reports in the scientific literature of their suppression of soil-borne plant diseases whilst not inciting disease themselves; (d) the relative ease with which they can be isolated, cultured, and formulated and; (e) examples of their successful commercialisation into biological control products (Brannen and Kenney, 1997), which ultimately stem from the properties listed above.

7.2.2 Objective 2

The second objective was to determine whether spore-forming bacteria were present in Canterbury soils and, if so, whether isolates could be found which exhibited antagonism towards *A. euteiches*. To achieve this, 704 bacterial isolates were obtained from a number of soil samples. These included samples taken from the rhizosphere region of pea plants since bacteria present in this environment may have adaptations which would enable them to better establish and colonise when returned as a biological control agent back to that environment. Each isolate was assayed against *A. euteiches in vitro* and 31 suppressed the mycelial growth of the fungus. Each mycelial-suppressive bacterium was additionally screened for suppression of zoospore germination and germtube growth. On the basis of these assays, seven bacterial isolates were selected for ongoing testing. They were identified as strains of *B. cereus*, *B. mycoides*, *B. pumilus*, *B. subtilis*, *P. macerans* and *P. polymyxa* based on multiple carbohydrate fermentation profiles (API 50 CHB microtubule system). A higher proportion of bacterial isolates from the rhizosphere were antagonistic

towards *A. euteiches* than isolates taken from open field soil (19% compared with 0.5%). A similar effect has been observed before (Berg, 1996; Pandey *et al.*, 1997), and is probably a reflection of the highly competitive nature of the rhizosphere environment (Lynch, 1990). There have also been a number of examples published in the scientific literature of the suppression of Oomycetous plant pathogens by isolates of *Bacillus* and *Paenibacillus*. For example, *B. cereus* was shown to produce the antibiotic compounds zwittermycin and kanosamine which inhibit the growth of *Phytophthora* sp. and *A. euteiches* (Milner *et al.*, 1996; Silo-Suh, 1994). Oedjijono *et al.* (1993) demonstrated suppression of a *Phytophthora* sp. with *P. polymyxa*, and McKeen *et al.* (1996) found similar results with *B. subtilis*.

The results from these trials clearly showed that spore-forming bacteria were abundant in Canterbury soils, were naturally present in the soil surrounding pea roots, and a proportion were antagonistic to *A. euteiches*. It also showed that the rhizosphere was an excellent reservoir of bacteria with antagonistic traits towards *A. euteiches*.

7.2.3 Objective 3

The third objective was to determine which of the bacterial isolates which inhibited the pathogen *in vitro* could suppress disease in a glasshouse assay. In order to fulfill this, a suitable glasshouse assay, able to give reproducible levels of disease was developed. Similar glasshouse trials reported in the scientific literature have described the use of artificially-produced inoculum, such as oospores or zoospores, as a source of the pathogen. However, it was considered that naturally-infested soil might be a better option when testing for biological control activity, as it has a mixture of pathogenic types of *A. euteiches,* other root rot causing fungi, and soil microorganisms. This was expected to provide a more stringent test of the biological control agents than would be achieved by challenging them against *A. euteiches* alone under less competitive conditions. Chapter 3, described the development of a glasshouse-based assay for generating Aphanomyces root rot disease on pea. A local soil was used as a source of pathogen inoculum. The glasshouse trial was then used to screen 12 suppressive bacterial isolates for biological control activity when applied to peas as a seed coat dressing. In the first trial, plants were assessed for disease, based on their visual root rot symptoms. It was subsequently found, however, that a proportion of the disease pressure was incited by root rot pathogens other

than *A. euteiches*. This was consistent with reports of *A. euteiches* being only one part of a root disease complex which exists in many soils (Oyarzun and Van Loon, 1989; Tu, 1987). On repetition of the trial, assessments were not only made on the level of visual disease symptoms, but also on the weight of the pea roots and the numbers of oospores within the root tissue. The visual disease scores gave an indication of the overall root rot disease pressure, and the oospores counts indicated the proportion of root tissue with *A. euteiches* infection. The trials demonstrated that the fungicide Apron, although very good at reducing visual disease symptoms, was ineffective at reducing oospore numbers in pea roots. The best treatments for reducing the build up of *A. euteiches* inoculums were bacterial. These results justified the preliminary selection of spore-forming bacteria as potential biological agents of Aphanomyces root rot. *Bacillus* spp. have been found to reduce Aphanomyces root-rot in glasshouse trials previously (Parke *et al.*, 1991), however, the identity of the isolates and the magnitude of disease reduction were not given.

7.2.4 Objective 4

The fourth objective was to evaluate the putative biological agents for suppression of the disease under field conditions. Initially, a field site area was set up at the Field Services Centre at Lincoln University by excavating an area of soil and replacing it with soil infested with *A. euteiches* (as used in the glasshouse trials, Chapter 3). In the first trial at the site, all seven bacterial isolates tested reduced root rot disease relative to the nil-control. However, significant levels of control (α=0.05) were not demonstrated. This was partially a result of the scoring method used to assess visual root disease. Isolations of fungi from diseased pea roots also pointed to a relatively low incidence of *A. euteiches* and a high incidence of *Fusarium* spp. To address these problems, a second field trial site was selected at a farm in Punawai, Mid-Canterbury, containing a predominantly *A. euteiches*-based root rot disease complex. In addition, assessments were taken of other plant parameters including root weight, plant height and yield-based parameters. The results of four subsequent field trials, three at the Punawai site and one at the Lincoln site, showed that *B. mycoides* MW 27 had the greatest potential for biological control of Aphanomyces root rot. The bacterium significantly (α=0.05) increased yield of peas planted in high Aphanomyces disease-indexed soils (field trial 2), yet had no deleterious effect on peas planted in low Aphanomyces disease-index soil (field trial 3). No significant (α=0.05) benefits were observed by combining the bacterium with the fungicide Apron. This was

understandable, however, since Apron was not effective alone indicating that the non-Aphanomyces disease pressure in the trial was low and no synergism between the two treatments could be expected (field trial 4). In field trial 5, *B. mycoides* MW 27 and *P. polymyxa* 18·25 were applied to pea seed both individually and as a combined treatment. The most effective treatment effect occurred when *B. mycoides* MW 27 was applied alone.

Although *B. mycoides* MW 27 was identified as the most promising bacterial isolate for control of Aphanomyces root rot after the first five field trials, it was felt that its true potential had not fully determined. This was due to various reasons or limitations in the trials, such as reduction of replication to accommodate more treatments within trials (resulting in limited capacity to detect statistical significance), out-of-season planting of some trials and a lack of disease pressure in other trials. Therefore, a final trial (trial 6) was carried out. This was planted at the traditional Canterbury pea planting time (early October) and ran through until full maturity. Commercial-quality prill, granule, and seed coat formulations of *B. mycoides* MW 27 were generated at AgResearch and tested for their effect on plant stand (emergence), and plant yield parameters. As a result of significant spring rains, both damping-off and root rot disease pressures were generated. In a seed coat formulation, *Bacillus mycoides* MW 27 controlled damping-off disease at a significant level (α=0.05); treatments receiving the bacterial formulation had 10% higher plant stand than the nil-treated control. The effects of the same bacterial isolate in the prill formulation were, however, quite different. This treatment had no significant (α=0.05) effect on plot stand but did significantly (α=0.05) increase both the number and weight of pods per plant. Overall, the yield following treatment with *B. mycoides* MW 27 in the prill formulation was 482.2 g $plot^{-1}$, which was significantly (α=0.05), greater than that of the nil-control (399.62 g $plot^{-1}$).

In summary, six field-based trials were conducted over the course of this work to determine the efficacy of spore-forming bacteria for the control of pea root rot disease. One bacterial isolate, *B. mycoides* MW 27, was identified as being very promising. In addition to increasing plant yield and reducing root-rot disease, the bacterium also exhibited efficacy against pea damping-off disease. Since it was a spore-forming bacterium, existing formulation technology was able to be applied to the bacterium to produce commercial-grade prill, granule, and seed coat formulations within a very short

time frame. Furthermore, all formulations were found to have a shelf-life of at least 70 d when stored under ambient conditions (Section 4.3.5).

7.2.5 Objective 5

Objective 5 was to determine whether biological control agents successful against Aphanomyces root-rot had efficacy against other soil borne plant diseases. Following control of damping-off of peas in the field, glasshouse trials were set up to determine if the bacterial isolates *B. mycoides* MW 27, *B. subtilis* PT 69, *B. pumilus* PT 10, *P. macerans* PT 1 or *P. polymyxa* 18·25 had any effect on *Pythium ultimum* damping-off of lettuce. Unfortunately, conditions in the trials were too conducive towards disease development and no effects could be detected. As there are several examples in the scientific literature of the control of Oomycetous plant diseases with spore-forming bacteria (Hwang *et al.*, 1996; Paul *et al.*, 1995), further testing should be carried out before these isolates are considered ineffective. Suggestions were made (Chapter 5) as to how the trial design could be improved to better determine their potential.

In addition to testing for control of *P. ultimum* damping-off of lettuce, a trial was conducted in the glasshouse to determine the potential for biological control of wheat take-all disease (*Ggt*; Chapter 5). This disease was chosen because of its importance to the grain industry, the lack of conventional control methods, and the number of reports in the scientific literature describing the use of biologically-based control methods (Capper and Campbell, 1986; Faull and Capper, 1979; Mathre, 1992; Ryder and Rovira, 1993; Simon, 1989; Sivasithamparam, 1998; Weller *et al.*, 1988). As there have been reports of strains of *B. mycoides, B. pumilus* and *P. polymyxa* exhibiting antagonism towards *Ggt* in the scientific literature, *B. mycoides* MW 27, *B. pumilus* PT 10 and *P. polymyxa* 18·25 were selected for evaluation. *Bacillus pumilus* PT 10 was found to significantly (α=0.05) increase the yield of plants grown in take-all amended potting mixture. In fact, the yield of *B. pumilus* PT 10 treated plants was not significantly different to that of control plants which were grown in the absence of the pathogen (Figure 5.1). *Bacillus pumilus* PT 10 was, therefore, identified as a potential biological control agent for wheat take-all disease and is currently being integrated into a larger take-all control project in association with Crop and Food Research, HortResearch, and Lincoln University.

7.2.6 Objective 6

The final objective was to determine the spectrum of *in vitro* activity of *B. mycoides* MW 27 and *P. polymyxa* 18·25, the two most widely used bacterial isolates in this work, and to investigate their mode-of-action (production of antibiotics, cell-wall-degrading enzymes etc). The bacterial isolates were challenged *in vitro* against a range of plant pathogenic fungi from a number of different taxonomic groups (Chapter 6). *Paenibacillus polymyxa* 18·25 inhibited the growth of nearly all fungi tested, whilst *B. mycoides* MW 27 was only slightly suppressive to *A. euteiches* and *Fusarium culmorum*. *Paenibacillus polymyxa* 18·25 secreted enzymes capable of degrading cellulose and chitin, the major cell wall components in the fungi, whilst *B. mycoides* MW 27 did not. Furthermore, *P. polymyxa* 18·25 secreted a broad-spectrum antibiotic compound which was found to be closely associated with the cell or spore wall. Evidence in the literature suggests that the compound is likely to be a small peptide (Katz and Demain, 1977). Other isolates of *Paenibacillus polymyxa* have been shown to suppress a wide range of plant pathogenic fungi *in vitro* (Oedjijono *et al.*, 1993).

7.3 *Bacillus mycoides* MW 27 as a biological control agent of *Aphanomyces euteiches* – advantages, limitations and conclusions

As far as can be ascertained, this work is the first to describe the use of *Bacillus mycoides* as a biological control agent of Aphanomyces root rot disease on pea. For such purposes, this species of bacterium can be viewed as being particularly valuable as it is not a recognised plant, animal, or human pathogen. This aspect has been a major problem for the development of some species of biological control agents, such as *Burkholderia cepacia* which can be infectious to human cystic-fibrosis patients (Govan and Deretic, 1996). This may, in fact, have led to the discontinuation of its use for biological control of *A. euteiches* (J. Kraft; Pers comm.).

The major limitations to the use of *B. mycoides* are, in essence, the same as those for any microorganism which is to be used for the control of soil-borne plant diseases. Primarily, the major limitation would be the ability of the bacterium to control disease under a range of soil and environmental conditions. This, in turn, would be limited by the range of environmental conditions under which *B. mycoides* has become adapted to grow and live. To fully determine the breadth of its ecological niche, fundamental studies relating to the

ecology of *B. mycoides* MW 27 would need to be undertaken. However, evidence in the literature suggests that for some strains of *Bacillus*, the most critical factors affecting their growth in soil are pH levels and nutrient supply (Heulin *et al.*, 1994; West *et al.*, 1985). To a large extent, these may be buffered by or supplied through the formulation process (e.g. incorporation of specific nutrients or pH buffers incorporated into the prill). Another major limitation is acceptance of the technology by growers. Unlike many chemical pesticides which can dramatically improve plant health when applied to a diseased crop, *B. mycoides* MW 27 would probably only limit the impact of the disease on crop yield. However, with increasing grower awareness of soil health practices, sustainable production systems, and an increase in organic pea production in New Zealand, this problem may be resolved by effective marketing of the bacterial product. For example, it could be marketed on the basis of its ability to increase yields under root rot conditions and to improve crop stand, but also its ability to reduce the build-up of the pathogen in the soil, thereby reducing the requirement for exceedingly long crop rotation cycles. In any case, until a fungicide which is effective against Aphanomyces root rot is registered in New Zealand, a product based on the bacterium would enjoy market dominance!

In conclusion, work described in this thesis has shown *Bacillus mycoides* MW 27 has clear potential for the control of *Aphanomyces euteiches* under local (Canterbury) conditions. In addition, a local strain of *Bacillus pumilus* was identified as a potential biological control agent of *Gaeumannomyces graminis*.

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Appendix 1 – Origin of soils

Soil Sample	Origin
18 (*A. euteiches* suppressive)	D. Lemon, Killinchy, RD 2, Leeston.
15 (*A. euteiches* suppressive)	S. Lemon, Willis Rd, Southbridge.
9 (*A. euteiches* suppressive)	Kimihia Research Station, Lincoln. F Block.
3 (*A. euteiches* conducive)	Crop & Food Research Farm, Lincoln. Chapman's Block, Paddock A3.
2 (*A. euteiches* conducive)	Crop & Food Research Farm, Lincoln. Block C6 (paddocks 5 + 6).
A. euteiches indexing soil	MAF, Lincoln.
Pea rhizosphere (top)	Crop & Food Research Farm, Lincoln. Duncan's Block, Wakanui soil.
Pea rhizosphere (bottom)	Crop & Food Research Farm, Lincoln. Duncan's Block, Wakanui soil.

Appendix 2 – Bacterial Identification

P. polymyxa 18·25 nucleotide sequence from the SP 6 primer

```
1     CGTGCCAGCA GCCGCGGTAA TACGTAGGGG GCAAGCGTTG TCCGGAATTA TTGGGCGTAA
61    AGCGCGCGCA GGCGGCTCTT TAAGTCTGGT GTTTAATCCC GAGGCTCAAC TTCGGGTCGC
121   ACTGGAAACT GGGGAGCTTG AGTGCAGAAG AGGAGAGTGG AATTCCACGT GTAGCGGTGA
181   AATGCGTAGA GATGTGGAGG AACACCAGTG GCGAAGGCGA CTCTCTGGGC TGTAACTGAC
241   GCTGAGGCGC GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
301   AACGATGAAT GCTAGGTGTT AGGGGTTTCG ATACCCTTGG TGCCGAAGTT AACACATTAA
361   GCATTCCGCC TGGGGAGTAC GGTCGCAAGA CTGAAACTCA AAGGAATTGA CGGGGACCCG
421   CACAAGCAGT GGAGTATGTG GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG
481   ACATCCCTCT GACCGGTCTA GAGATAGGCC TTTCCTTCGG GACAGAGGAG ACAGGTGGTG
541   CATGGTTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
601   CTTATGCTTA GTTGCCAGCA GGTCAAAGCT GGGCACTCTA AGCAGACTGC CGGTGACAAA
661   CCGAGGAANG TGGGGATGAC GTCAAATCAT CATTGCCCCC TTATTAACTG GGCTACACAC
721   GTACTACAAT GGCCGGTACA ACCGGGAAGC GAAAATCGCG AGGTGGAGCC CATCCTANAA
781   AAGCCGGGTC TCAGTTCGGA TTGGTAGGCT GGCAACTCGC CTACATTAAA TCGGAATNGC
841   TTAGTATCGC AGATCACATG CCCCGGTGAA TACTTNCCCG GTTTTGNCAC ACCGNCCGTC
901   ACACCCGAGA GTTNCAACAC CNAAAT
```

P. polymyxa 18·25 nucleotide sequence from the T7 primer

```
1     AAGGAGGTGA TCCAGCCGCA CCTTCCGATA CGGCTACCTT GTTACGACTT CACCCCAATC
61    ATCTACCCCA CCTTCGGCGG CTGGCTCCCT TGCGGGTTAC CCCACCGACT TCGGGTGTTG
121   TAAACTCTCG TGGTGTGACG GGCGGTGTGT ACAAGACCCG GGAACGTATT CACCGCGGCA
181   TGCTGATCTG CGATTACTAG CAATTCCGAC TTCATGTAGG CGAGTTGCAG CCTACAATCC
241   GAACTGAGAC CGGCTTTTCT AGGATTGGCT CCACCTCGCG ATTTCGCTTC CCGTTGTACC
301   GGCCATTGTA GTACGTGTGT AGCCCAGGTC ATAAGGGGCA TGATGATTTG ACGTCATCCC
361   CACCTTCCTC CGGTTTGTCA CCGGCAGTCT GCTTAGAGTG CCCAGCTTGA CCTGCTGGCA
421   ACTAAGCATA AGGGTTGCGC TCGTTGCGGG ACTTAACCCA ACATCTCACG ACACGAGCTG
481   ACGACAACCA TGCACCACCT GTCTCCTCTG TCCCGAAGGA AAGGCCTATC TCTAGACCGG
541   TCAGAGGGAT GTCAAGACCT GGTAAGGTTC TTCGCGTTGC TTCGAATTAA ACCACATACT
601   CCACTGCTTG TGCGGGTCCC CGTCAATTCC TTTGAGTTTC AGTCTTGCGA NCGTACTCCC
```

```
661     CAGGCGGAAT GCTTAATGTG TTAACTTCGG CACCAAGGGT ATCGAAACCC TAACACCTAG
721     CATTCATCGT TTACNGCGTG GACTACCAGG GTATCTAATC CTGTTTGGTC CCCACGCTTT
781     CGCGCCTCAG CGTCAGTTAC AGCCCAAAAG AGTCGCCTTC GCCACTGGGT GTTCCCTCAC
841     ATTCTCTTAC GCATTTCACC GCTTACACGT GGGAATTCCA CTCTCCTCTT CTGNACTCAA
901     AGCTCCCCAG TTTCCAGTGC GAACCGNANT TTANCCTCCG GAT
```

B. mycoides MW 27 nucleotide sequence from the SP 6 primer

```
1       CGTGCCAGCA GCCGCGGTAA TACGTAGGTG GCAAGCGTTA TCCGGAATTA TTGGGCGTAA
61      AGCGCGCGCA GGTGGTTTCT TAAGTCTGAT GTGAAAGCCC ACGGCTCAAC CGTGGAGGGT
121     CATTGGAAAC TGGGAGACTT GAGTGCAGAA GAGGAAAGTG GAATTCCATG TGTAGCGGTG
181     AAATGCGTAG AGATATGGAG GAACACCAGT GGCGAAGGCG ACTTTCTGGT CTGTAACTGA
241     CACTGAGGCG CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
301     AAACGATGAG TGCTAAGTGT TAGAGGGTTT CCGCCCTTTA GTGCTGAAGT TAACGCATTA
361     AGCACTCCGC CTGGGGAGTA CGGCCGCAAG GCTGAAACTC AAAGGAATTG ACGGGGGCCC
421     GCACAAGCGG TGGAGCATGT GGTTTAATTC CGAAGCAACG CGAAGAACCT TACCAGGTCT
481     TGACATCCTC TGAAAACTCT AGAGATAGAG CTTCTCCTTC GGGAGCAGAG TGACAGGTGG
541     TGCATGGTTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
601     CCTTGATCTT AGTTGCCATC ATTAAGTTGG GCACTCTAAG GTGACTGGCC GGTGACAAAC
661     CGGAGGAANG TTGGGGATGA CGTCAAATCA TCATGCCCCT TATTANCTGG GCTACACAC
721     GTTGCTACAA TGGGACNGTA CAAAGAAGCT TGCAAGACCN CGAAGGTGGA GCTTAATCTC
781     ATAAAAACCC GTTCTCCAGT TCGGGANTGG TANGGCTTGC AAACNCCCCC TTACATTGAA
841     AGCTTGGGAA TTCNCTAAGT AAATCCCCNG GATTCAAACA ATTGNCCCCC NGGGTGNAAA
901     TAACGTTTNC CCCGGGGCCT TTGTTANAAN ANCCGGNCCG GTTTAAAACC CACGNAGAAG
961     NTTTTNGGTA ACANCCCCGA AAGTNCG
```

B. mycoides MW 27 nucleotide sequence from the T7 primer

```
1       AAGGAGGTGA TCCAGCCGCA CCTTCCGATA CGGCTACCTT GGTTACGACT TCACCCCAAT
61      CATCTGTCCC ACCTTAGGCG GCTGGCTCCA TAAAGGTTAC CCCACCGACT TCGGGTGTTA
121     CAAACTCTCG TGGTGTGACG GGCGGTGTGT ACAAGGCCCG GGAACGTATT CACCGCGGCA
181     TGCTGATCCG CGATTACTAG CGATTCCAGC TTCATGTAGG CGAGTTGCAG CCTACAATCC
241     GAACTGAGAA CGGTTTTATG AGATTAGCTC CACCTCGCGG TCTTGCAGCT CTTTGTACCG
301     TCCATTGTAG CACGTGTGTA GCCCAGGTCA TAAGGGGCAT GATGATTTGA CGTCATCCCC
361     ACCTTCCTCC GGTTTGTCAC CGGCAGTCAC CTTAGAGTGC CCAACTTAAT GATGGCAACT
421     AAGATCAAGG GTTGCGCTCG TTGCGGGACT TAACCCAACA TCTCACGACA CGAGCTGACG
481     ACAACCATGC ACCACCTGTC ACTCTGCTCC CGAAGGAGAA GCTCTATCTC TAGAGTTTTC
541     AGAGGATGTC AAGACCTGGT AAGGTTCTTC GCGTTGCTTC GAATTAAACC ACATGCTCCA
601     CCGCTTGTGC GGGCCCCCGT CAATTCCTTT GAGTTTCAGC CTTGCGGCCG TACTCCCCAG
661     GCGGAGTGCT TAATGCGTTA ACTTCAGCAC TAAAGGGCGG AAACCCTCTA ACACTTAAGC
```

721 ACTCATCGTT TACGGGGTGG ACTAACAAGG GTATCTAAAT NCTGTTTGNT CCCCACGCCT
781 TTCGCGCCTC AGTGGTCAGT TACAGAACCA GAAAAGTCGC CTTCGCCCAC TGGGTGGTTN
841 CCTCCCATAA TTTTCTTACC GCATTTTTCA ACCCGCTNAC ACATTGGGGA AATTCCACTT
901 TTTCCCTCTT TCTGGGAACT TCAAAGTTTN TCCCCAAGTT TTTCCAAATT GAACCCCTCC
961 CACCGGGTTG GANNCCCGNG GGGGGCTTTT AAC

Bacterial carbohydrate fermentation profiles (API 50 CHB).

Substrate	Bacterial isolate PT 1	PT 10	PT 69	15·80
Control				
Glycerol	■	■	■	
Erythritol				
D-Arabinose				
L-Arabinose	■	■	■	
Ribose	■	■		■
D-Xylose	■	■		
L-Xylose				
Adonitol				
β-Methyl-D-Xyloside				
Galactose	■			
D-Glucose	■	■	■	■
D-Fructose	■	■	■	■
D-Mannose	■	■	■	
L-Sorbose				
Rhamnose				
Dulcitol				
Inositol			■	
Mannitol	■	■	■	
Sorbitol			■	
α-Methyl-D-Mannoside				
α-Methyl-D-Glucoside			■	
N-Acetyl-Glucosamine				■
Amylgdaline	■	■	■	
Arbutine	■	■	■	■
Esculine	■	■	■	■
Salicine	■	■	■	■
Celobiose	■	■	■	
Maltose	■		■	■
Lactose	■			
Melibiose	■		■	
Saccharose	■	■	■	
Trehalose	■	■	■	■
Inulin	■		■	
Melezitose				
D-Raffinose	■		■	
Starch	■		■	■
Glycogen	■		■	■
Xylitol				
Gentiobiose	■	■	■	
D-Turanose	■		■	
D-Lyxose				
D-Tagatose		■		
D-Fucose				
L-Fucose				
D-Arabitol				
L-Arabitol				
Gluconate	■			
2-Keto-Gluconate				
5-Keto-Gluconate				

Shading within cells indicates positive substrate utilisation.

Substrate	Bacterial isolate 18·25	18·94	MW 12	MW 18	MW 27
Control					
Glycerol	■	■	■	■	
Erythritol					
D-Arabinose					
L-Arabinose	■	■	■	■	
Ribose	■	■	■	■	■
D-Xylose	■	■		■	
L-Xylose					
Adonitol					
β-Methyl-D-Xyloside	■	■			
Galactose	■	■		■	■
D-Glucose	■	■	■	■	■
D-Fructose	■	■	■	■	■
D-Mannose	■	■	■	■	
L-Sorbose					
Rhamnose					
Dulcitol					
Inositol					
Mannitol	■	■	■	■	
Sorbitol					
α-Methyl-D-Mannoside					
α-Methyl-D-Glucoside	■	■			
N-Acetyl-Glucosamine			■		■
Amylgdaline	■	■	■		■
Arbutine	■	■	■	■	■
Esculine	■	■	■	■	■
Salicine	■	■	■	■	■
Celobiose	■	■	■	■	■
Maltose	■	■			■
Lactose	■	■			■
Melibiose	■	■			
Saccharose	■	■	■	■	■
Trehalose	■	■	■	■	■
Inulin	■	■			
Melezitose					
D-Raffinose	■	■			
Starch	■	■			■
Glycogen	■	■			■
Xylitol					
Gentiobiose	■	■			
D-Turanose	■	■			
D-Lyxose					
D-Tagatose					
D-Fucose					
L-Fucose					
D-Arabitol					
L-Arabitol					
Gluconate					
2-Keto-Gluconate					
5-Keto-Gluconate					

Shading within cells indicates positive substrate utilisation.

Appendix 3 - Special agars and broths

Bacillus fermentation broth (BfB)

Glucose	10 g
peptone	7 g
KH_2PO_4	6.8 g
$MgSO_4.7H_2O$	0.3 g
$ZnSO_4.7H_2O$	0.02 g
$FeSO_4.7H_2O$	0.02 g
$MnSO_4.H_2O$	0.02 g
$CaCl_2$	0.1 g
H_2O	1000 mL

Basal medium (assay for lytic enzymes, Section 6.2.1)

agar	15 g
yeast extract	1.0 g
KH_2PO_4	1.25 g
$MgSO_4.7H_2O$	0.625 g
peptone	0.625 g
H_2O	1000 mL

Cabbage broth (CB10)

cabbage leaves (homogenized)	100 g
H_2O	900 mL

Campbell's half strength V8-Juice broth

Campbell's V8 Juice	100 mL
H_2O	900 mL
$CaCO_3$	3g
cholesterol (BDH Ltd.) (in ethanol)	30 mg

LB broth / agar

bacto tryptone	3 g
yeast extract	1.5 g
NaCl	1.5 g
H_2O	300 mL
agar	4.5 g

Ampicillin (Amp 100): added at 100 μL 100 ml^{-1} of agar or broth

Overlay medium (assay for lytic enzymes)

agar	15 g
malt extract	5 g
yeast extract	1 g
cellulose, laminarin or chitin substrates	20 g
H_2O	1000 mL

Tryptose glucose yeast extract broth (TGYB)

peptone 140	5 g
yeast extract	2.5 g
dextrose	1 g
H_2O	1000 mL

Tryptose soy broth TSB

Peptone 140	17.0 g
Peptone 110	3.0 g
dextrose	2.5 g
NaCl	5.0 g
Potassium phosphate, dibasic	2.5 g
H_2O	1000 mL