Biological control of Rhizoctonia diseases of potato

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
by
Damian Bienkowski

Lincoln University
2012

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Potato (*Solanum tuberosum* L.) is one of the most important crops in New Zealand and internationally. Potato plants are susceptible to a range of pathogenic diseases, including the Rhizoctonia diseases, which are globally ubiquitous. Since synthetic fungicide control of the causal pathogen, *Rhizoctonia solani* Kühn, is not always efficacious, combined with a current drive to reduce synthetic agrichemical inputs in New Zealand agriculture, alternatives should be sought. The present study examined the potential for biological control (biocontrol) of Rhizoctonia diseases of potato from a New Zealand agricultural perspective. The study included investigations into inundative biocontrol (introduction of single/multiple strains of soil microorganisms) and conservation biocontrol, including organic matter amendments and crop rotation practice.

To investigate inundative biocontrol, isolations were conducted from New Zealand potato cropping soils for members of the genera *Trichoderma*, *Pseudomonas* and *Bacillus*, identified as strong potential biocontrol candidates from previous published studies. Comparisons between the bacterial isolates in dual culture with two *R. solani* strains suggested that one soil (from the Pukekohe region) harboured Pseudomonads with greater suppressive potential than those from three other soils. In greenhouse assays, selected isolates (89) were applied to plant growth medium as fungal spore or bacterial cell suspensions, to test for their capacity to suppress Rhizoctonia diseases of potato. Initially, 22 isolates demonstrated positive impacts on either Rhizoctonia canker symptoms and/or on plant parameters (e.g. tuber number or weight). However, no isolates gave strong and consistant suppression of Rhizoctonia diseases in repeated assays. The results indicated that inundative biocontrol was insufficient to achieve strong, consistent suppression of Rhizoctonia diseases of potato, when applied in this commercially realistic manner.

Investigations into conservation biocontrol included testing several organic matter (OM) amendments previously suggested as potential suppressors of Rhizoctonia diseases of potato. These OMs were based on pine bark compost, chitin or biochar. Very slight disease suppression was detected. Results from analysis of soil community substrate utilisation showed that increases in activity or diversity as a result of OM amendment, at commercially viable concentrations, were insufficient to suppress Rhizoctonia diseases. The soil population of *R. solani* AG 3 and AG 2-1, and fungal and bacterial soil community structure, as a result of four crop rotation treatments (including potato monoculture) from the Pukekohe region were assessed. No rotation treatment increased pathogen inoculum. Soil bacterial community was not affected by rotation treatment. Fungal community was affected by the most recent crop, but not crop sequence. The impact of the four rotation treatments on Rhizoctonia disease expression was also assessed. Soils from all four treatments gave similar disease expression. Culture dependent and independent techniques used to determine soil fungal and bacterial community richness, diversity or metabolic activity demonstrated that increases in these parameters did not correlate with decreases in Rhizoctonia disease measures.

The study concluded that consistent biocontrol of Rhizoctonia diseases of potato probably requires the presence and stimulation of a specific, small group of suppressive organisms. For biocontrol of these diseases to be a reliable option for potato growers, biocontrol agents will probably require appropriate and economically feasible nutritional support when introduced into crop disease management strategies.

Keywords: *Rhizoctonia solani*, biological control (biocontrol), potato (*Solanum tuberosum*), *Trichoderma, Pseudomonas, Bacillus*, organic matter amendment, crop rotation, soil community analysis.

Acknowledgements

I would like to thank the following people for their assistance and support throughout the course of my PhD studies:

My supervisors, Dist. Prof. Alison Stewart, Prof. Richard Falloon and Mark Braithwaite for their guidance and editorial prescision, and especially to Mark for his practical help with the experimental work. Dr. Andrew Holyoake for advice regarding molecular techniques. Assoc. Prof. Leandro Loguercio, Dr. Kirstin McLean and Emily Hicks for assistance with experimental work. Dr. Gavin Lear for the use of his bacterial ARISA protocol and reagents, and fellow student Jack Lee for showing me how to perform and analyse ARISA. Sandi Keenan (Plant and Food Research) for teaching me the soil DNA extraction and *R. solani* qPCR methods and analysis, and allowing me to use her reagents. Dave Saville (Saville Statistical Consulting Ltd.) for statistical advice and tuition.

Thank you to the friends I have made at Lincoln University for their personal support, especially my office mates, Natalia Cripps-Guazzone, Aimee McKinnon and Dr. Pierre Hohmann. Many thanks to Clair Dainter for housing Fiona and I for five months when we were displaced due to earthquake damage.

To my parents for supporting our move to New Zealand, and laying the foundations for all the successes I have enjoyed.

And to my fiancé Fiona MacNaught, for her patience, support and love, and general wonderfullness.

Final thanks to the New Zealand Ministry of Science and Inovation (MSI, formally FRST) for funding my studies.

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Chapter 1
Introduction

Potatoes are one of the most important food and commodity crops in New Zealand and internationally. Potato plants are susceptible to a wide variety of diseases, including Rhizoctonia diseases, which are globally ubiquitous. Synthetic pesticide control of the fungal pathogen *Rhizoctonia solani* (the causal agent of Rhizoctonia diseases) is not always efficacious, and there is a current trend to decrease the use of synthetic agrichemicals in crop production. If adequately understood, biological control of Rhizoctonia diseases of potato could become a significant component of an integrated pest management system for this valuable crop.

The aim of this project was to identify strains of beneficial microorganisms with the capacity to suppress Rhizoctonia diseases of potato, and generate information regarding their physical limitations and mode(s) of action. Failing the identification of such organisms, a second aim was to investigate if soil management practices, specifically organic matter amendments and different crop rotations, can reduce the severity of these diseases.

1.1 The host

Potato (*Solanum tuberosum* L.) is one of the most important crops globally. A recent special report by the Food and Agriculture Organisation of the United Nations highlighted the potato crop as "…a vital part of the global food system, and will play an ever greater role in strengthening world food security and alleviating poverty" (FAO, 2009). Worldwide, potato is the most cultivated non-grain crop, producing more starch per hectare than any other crop. Potatoes also have high nutritional value and a greater proportion of edible plant material compared to cereals. Because the majority of potato crops are produced for domestic rather than international trade, prices are determined by local production costs and not subject to fluctuations in the global market. This explains why potato is such an important crop for local food security.

Worldwide, potato crop loss due to pathogens ranges from 7 to 24%, with lower losses in regions with higher agrichemical inputs, such as New Zealand (Oerke, 2006). In 2011 in New Zealand, potatoes were grown on 10,600 ha of land, producing 522,000 tonnes with a combined domestic and export value of NZ$560.3 million (Aitken and Hewett, 2011). If properly understood and implemented, biological suppression of potato pathogens could

enable farmers in developed countries like New Zealand to reduce synthetic pesticide use without incurring loss of productivity or crop quality, and could offer farmers in developing countries a way of decreasing crop losses to pathogens without increasing synthetic pesticide use.

1.2 Rhizoctonia diseases of potato

1.2.1 The pathogen

The causal agent of the black scurf and stem/stolon/root canker diseases of potato is the soil-borne fungus *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) (Banville *et al.*, 1996). *Rhizoctonia solani* does not produce asexual spores, and exists as mycelium (hyphal growth form), sclerotia (dense asexual hyphal resting structures) or basidiospores (sexual spores) (Keijer, 1996). While basidiospores produced by some *R. solani* strains are inoculum for Rhizoctonia leaf diseases in other plant species, such as web blight on beans (Godoy-Lutz *et al.*, 1996), they are rarely produced by strains pathgenic to potato, and are not considered to be of economic importance with regards to Rhizoctonia diseases of potato (Ogoshi, 1987). Traditionally, *R. solani* has been subdivided into subsets based on grouping by anastamosis reactions; briefly, new isolates are co-plated with tester isolates and if there is fusion between hyphae of the two isolates, including a "killing zone" surrounding the fusion, they are grouped into the same Anastomosis Group (AG) (Anderson, 1982). To date 14 AGs have been described, and there are also many subsets within AGs based on a range of characteristics including host range, virulence, molecular and biochemical characteristics and morphology (Carling *et al.*, 2002). The introduction of Polymerase Chain Reaction and DNA sequencing combined with other molecular and biochemical tools has confirmed the AGs as genetically distinct groups (Sharon *et al.*, 2006).

Isolates from many *R. solani* AGs have been found associated with below-ground potato organs, but only strains of AGs 3, 5, 2-1 and possibly 8 are considered of economic significance on potato in temperate climates (Banville *et al.*, 1996; Campion *et al.*, 2003). AG 3 is the most frequently isolated AG from potato exhibiting Rhizoctonia disease symptoms in most areas of the world (Anderson, 1982; Justesen *et al.*, 2003; Lehtonen *et al.*, 2008b) including New Zealand (Farrokhi-Nejad *et al.*, 2007). However, within each AG pathogenic to potato, there exists a range of pathogenicity between isolates. Some consider AG 3 to be divisible into two sub-groups with non-overlapping host ranges, with AG 3 PT strains virulent to potato, and AG 3 TB virulent to tobacco (Ceresini *et al.*, 2002). Members of *R. solani* pathogenic to potato are non-obligate, and are saprophytes able to utilise a wide range of

substrates, although they are not usually considered to be strong competitors. This is one mechanism, however, which allows them to persist in the absence of host plants (Frank, 1981).

1.2.2 Pathogen life cycle and disease symptoms

Rhizoctonia diseases of potato separate into two phases: Rhizoctonia cankers, which are lesions on below-ground plant organs that can occur at any time during the growth of the plant; and the deposition of sclerotia (dense asexual fungal resting structures) on the surfaces of daughter tubers (Banville *et al.*, 1996). The life cycle of potato-pathogenic *R. solani* strains are probably asexual, and begins with inoculum sources, either mycelia or sclerotia, present in the soil or decaying plant matter in the field, or on the surfaces of seed tubers. During mycelial growth, the pathogen can exist saprophytically, or can infect potato roots, stems, stolons (below ground stems that produce daughter tubers at their tips) and developing tubers, as detailed in Figure 1.1. Hymenia of *T. cucumeris* (producing basidiopspores) are sometimes observed in field-grown potatoes, but these are considered unimportant as inoculum sources in the epidemiology of Rhizoctonia diseases.

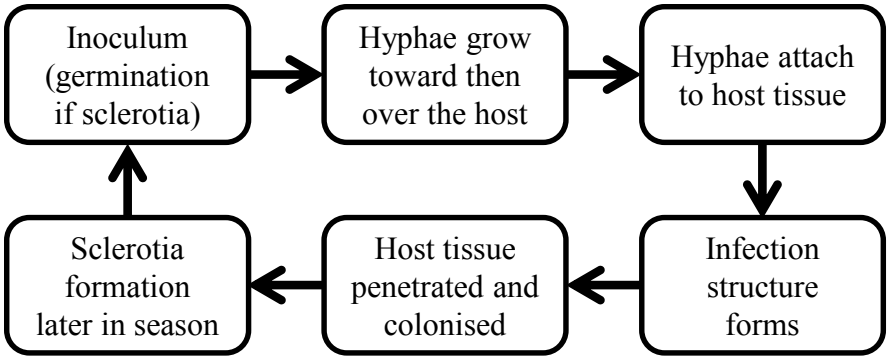

Figure 1.1 *R. solani* disease cycle of economic importance to potato cultivation and infection process, adapted from Keijer (1996).

Virulent *R. solani* forms infection cushions on the surfaces of host sprouts and roots, and penetration by the pathogen occurs only under these areas. Lesions develop under the infection cushions and reach the vascular bundles; these lesions develop into the 'canker' symptom. If lesions girdle and kill young sprouts, this is termed 'sprout-nipping' (Weinhold and Sinclair, 1996), which is often associated with poor plant emergence in affected crops (Figure 1.2). Above ground symptoms can include aerial tubers, upward leaf roll, chlorosis, purple leaf pigmentation, and stunting/rosetting of plant shoots and leaves (Frank, 1981).

However, there is often no effect of infection on the host haulms (Hartill, 1989). 'Nipping' of roots can lead to sparse root systems. Later in the growing season, this infection process can occur on newly forming stolons, killing them, or causing malformation and 'cracking' of tubers.

At the end of the growing season, sclerotia form on the daughter tubers (black scurf, Figure 1.2), but of the AGs most pathogenic to potato, only members of AG 3 are known to cause high incidences of black scurf on daughter tubers (Woodhall *et al.*, 2008). If the pathogen develops into the sexual stage then a 'white collar' (hymenium) producing basidiospores forms on stems just above the soil line, but only AG 3s have been shown to undergo this stage on potato (Woodhall *et al.*, 2008).

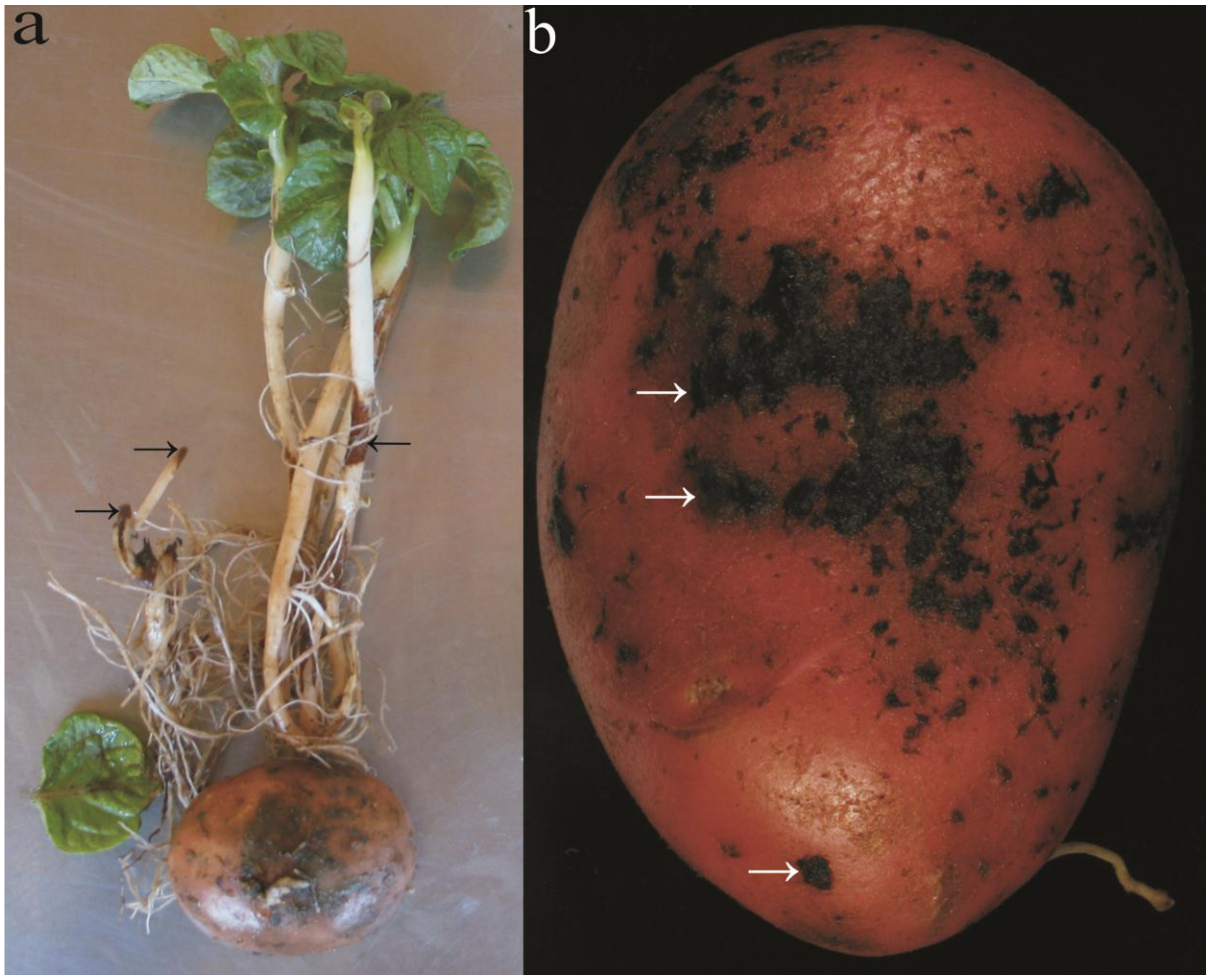

Figure 1.2 Examples of (a) Rhizoctonia stem canker as well as 'nipping' of emerging potato plant shoots, and (b) *R. solani* sclerotia (black scurf) on the surface of a potato tuber.

The yield impact of Rhizoctonia diseases of potato can vary depending on the *R. solani* strains present, potato cultivar and environmental conditions: In a four year study using ten potato

cultivars, Otrysko and Banville (1992) demonstrated that *R. solani* (AG 3) significantly decreased total and marketable crop yields and increased black scurf (not all cultivars were affected in all four years). Specific gravity of the tubers was significantly decreased in three of the four years, and colour of processed tubers (three months after harvest) was negatively affected in all four years. These authors highlighted the necessity of planting seed potatoes free of black scurf to avoid these symptoms. A study by Hide and Horrocks (1994) found that *R. solani* infection shifted tuber yield away from marketable size tubers to oversize, as well as darkening the colour of processed tubers. Stolon infection is responsible for shifts in marketable yield. For example, Hartill (1989) found that severe *R. solani* infection led to an increase in the number of tubers initiated, as well as tubers forming on leaf axils, suggesting that cankers on stolons block the transport of photosynthetic product (the production of which was unaffected by disease) to the usual sinks, forcing plants to create alternative sinks. A post-harvest Rhizoctonia disease symptom was recently described by Buskila *et al.* (2011). Infection of tubers by *R. solani* AG 3, before skin-set, was shown to be the cause of dark skin spots, likely due to an 'oversuberization' response of the tuber. This can reduce the value of potato varieties that are washed before sale.

1.2.3 Epidemiology

Soil-borne pathogens of potato plants, such as *R. solani*, are found in all potato producing regions of the world (Banville *et al.*, 1996), most likely because these pathogens have been dispersed on seed tubers, as potatoes are vegetatively propagated. Plant infection by *R. solani* can come from either tuber-borne or soil-borne inoculum (Tsror and Peretz-Alon, 2005), although in conventional potato production, seed dressing treatments with pesticides largely control tuber-borne inoculum (Weinhold *et al.*, 1982). In organic potato production, tuber-borne inoculum remains an important infection source. Therefore, if little or no crop rotation is practiced, then soil-borne inoculum is likely to increase. *Rhizoctonia solani* populations reduce over time in the absence of host plants, so in general, the more years between potato crops the lower the inoculum levels (Peters *et al.*, 2003). However, experiments by Carling *et al.* (1986) indicated that *R. solani* of AG 3 are able to form epiphytic relationships with crop species from a wide range of families (eggplant, tobacco, cauliflower, carrot, radish and oats), leading to extensive development of hyphae and sclerotia, making the selection of crops in rotation with potato important (see section 1.3.1). Disease is most prevalent under conditions which slow the growth of new shoots, particularly in cool, moist soils (Anderson, 1982). AG 3s have been shown to cause more damage to potato plants at 10°C than at higher temperatures (Carling and Leiner, 1990), and temperatures above 25°C have been shown to

inhibit canker severity (Anderson, 1982). Infection of potato plants by other pathogens has the potential to influence the expression of Rhizoctonia diseases of potato. *In vivo* experiments demonstrated that *R. solani* (AG 3) grew faster on medium amended with root exudates from potato infected with the potato cyst nematode (*Globodera rostochiensis*), than on media amended with root exudates from un-infected potato plants (Back *et al.*, 2010). The authors suggested this may explain a previously identified positive correlation between infections from these two pathogens in the field, with *R. solani* being stimulated to infect plants changes in root exudation. Organisms which compete with *R. solani* for root exudates may, therefore, have potential for reducing *R. solani* infections. Herbicide use may increase the incidence of Rhizoctonia diseases in potato fields, and various herbicides have been shown to stimulate *Rhizoctonia* spp. penetration of other crop species (Altman and Rovira, 1989).

1.3 Control strategies

1.3.1 Cultural control

Cultural controls for soil-borne diseases are very important in potato production, as other methods alone offer insufficient disease control (Sweetingham, 1996). The planting of certified seed potatoes is one of the most common cultural control methods for minimising potential disease. In New Zealand, the maximum level of black scurf for certified seed tubers is 5% of the individual tuber surface affected, on 5% of inspected samples (NZ Seed Potato Certification Authority, 2011). As black scurf generally begins to form several weeks after haulm kill, harvesting of the seed potatoes in the 3 week period after haulm kill is widely practiced in the seed potato industry to minimise black scurf on seed potatoes (R. Genet, personal communication).

Crop rotation can impact on soil inoculum levels, and is another widely practiced cultural control measure (Sweetingham, 1996). Long periods between potato crops generally favour decline in virulent *R. solani* inoculum levels. Certain crops also accelerate this decline in comparison with others. For instance, Garbeva *et al.* (2008) found lower percentages of AG 3 isolates present in maize and commercial grass mix rhizospheres than in those of barley or oat. The method of tillage can impact on the incidence and severity of Rhizoctonia diseases of potato: Peters *et al.* (2004) demonstrated that chisel ploughing reduced both stem canker and black scurf symptoms compared with mouldboard ploughing, in a three year rotation under minimum tillage. When Larkin *et al.* (2011) compared the expression of soil-borne diseases of potato between four crop rotation regimes and continual potato cultivation, it was found that Rhizoctonia stem canker was reduced in all rotations compared with monoculture, and that

black scurf was less in all systems compared to monoculture except one (an irrigated rotation which included yearly compost amendments). The disease was especially lessened in rotations which included 'disease-suppressive' crops: mustard/rapeseed followed by sudangrass/rye then potato. Overall irrigation increased black scurf, as well as yields. These authors used fatty-acid methyl ester (FAME) analysis and substrate utilisation (BIOLOG GN2) plates to analyse the soil microbial community, and they found that continual potato cropping resulted in the smallest microbial populations and least diversity, with least substrate utilisation richness and diversity (Larkin *et al.*, 2011).

Organic matter (OM) amendments are sometimes combined with crop rotations to reduce soil-borne diseases: Larkin (2008) used FAME to analyse the soil microbial community of three different crop rotations receiving an OM treatment, beneficial microbe treatment, combination of the two or control, and found that while OM treatments affected communities within rotation types, community structure was most closely defined by rotation. Hence rotation changed community structure to a greater extent than the treatments. Methods to analyse soil microbial communities in relation to disease suppression are covered in more detail in section 1.3.4.4.3.

Soil type has been shown to influence the development of Rhizoctonia diseases. For example, in the Netherlands, Jager and Velvis (1983) demonstrated that pleistocene, slightly acid sandy soil suppressed *R. solani*, compared with holocene marine soils; loamy sand, sandy loam, clay loam or clay. They attributed this suppression to an increase in *R. solani* sclerotia infected by fungal antagonists in the suppressive soil. As the likelihood of infection increases with decreased sprout emergence, cultural practices which accelerate sprout emergence, such as delaying planting until soil temperatures are warm, planting seed tubers only once correct physiological age is reached and planting in soils unlikely to become water-logged, can decrease the chances of sprout infection (Banville *et al.*, 1996).

1.3.2 Host resistance

While there is variation between potato varieties in resistance to *Rhizoctonia* diseases (Bains *et al.*, 2002), there are no varieties available which are completely resistant. Of the 86 potato varieties on The British Potato Varieties Database with data on resistance to black scurf, only four (Arrow, Blue Danube, Charlemont and Mayan Gold), have high resistance to *R. solani* (Potato Council, 2009). The majority of varieties, 43 of 86 listed, fall into the three (of nine) middle categories for resistance. Planting of resistant varieties can be a very cost-effective approach for controlling some plant diseases, as it reduces pesticide inputs. However, this

strategy often cannot be employed, because varieties more resistant to one pathogen may be less resistant to another which may have a greater impact on yield, or because resistant varieties have characteristics unacceptable for marketing or processing.

Genetic manipulations of disease response signalling and biochemical pathways, and the introduction of antimicrobial genes from non-plant sources, have achieved some success in controlling Rhizoctonia diseases of potato. For example; expression of an endochitinase from *Trichoderma harzianum* (Lorito *et al.*, 1998), as well as chitinases from a variety of plant sources (Punja, 2001) have been shown to suppress Rhizoctonia diseases. The use of transgenic crops remains a delicate topic, and their use is not an option available to all potato growers.

1.3.3 Synthetic pesticides

Synthetic chemicals for *R. solani* control can target either tuber-borne or soil-borne inoculum sources. Seed tuber dressings are effective and largely eliminate tuber-borne inoculum in conventional potato production. Effective fungicides are thiophanate-methyl, flutolanil, pencycuron and azoxystrobin (Campion *et al.*, 2003; Errampalli *et al.*, 2006; Wilson *et al.*, 2008b). Chemical disinfection of tubers has also been suggested. The choice of disinfectant is important, as some, like organic acids and reactive oxygen compounds, can damage sprouts, while others (e.g. ammonia) can both kill sclerotia and promote shoot growth (Zillger *et al.*, 2010). Variable control of soil-borne *R. solani* inoculum is achieved through fungicide applications, and these are less effective when initial inoculum levels are high (Brewer and Larkin, 2005; Tsror and Peretz-Alon, 2005). Fungicide control of *R. solani* is not available to growers of organic crops. Pencycuron, one of the common fungicides used for control of Rhizoctonia diseases of potato is very selective against the strains controlled, and while it was found to be effective at lower concentrations than another fungicide (PCNB), growth inhibition of a range of *R. solani* isolates in culture ranged from 0 to 100%. This selectivity was not related to anastomosis group (Roberts and Stephens, 1984). Azoxystrobin, a newer broad-range fungicide has been found to elicit stronger suppression of Rhizoctonia diseases of potato in the field then pencycuron (Djébali and Belhassen, 2010), although in a study by Brewer and Larkin (2005) it was not found to be efficacious in all experiments. A comparison between six fungicides found that captan, iprodione, mancozeb and fludioxonil were more effective in controlling Rhizocotnia stem canker and black scurf of potato than thiabendazole or thiophanate-methyl (Bains *et al.*, 2002). As stated earlier, there is a drive to decrease the application of synthetic agrichemicals in food production, so even where fungicides are effective, their use may become more restricted, and alternatives will have to be sought.

1.3.4 Biological control

In the case of diseases caused by soil-borne plant pathogens, biological control (biocontrol) refers to either the introduction of organisms that are antagonistic to the pathogen or reduce its effects, or to an increase in the density or activity of naturally occurring antagonistic organisms, resulting in a reduction of disease severity. Mechanisms of action which underpin biocontrol include either destruction of the pathogen directly (mechanisms include predation, mycoparasitism and/or production of antibiotic compounds), or excluding the pathogen through competition for resources, and the induction of host resistance (Bellows *et al.*, 1999).

In an effort to unify biocontrol terminology, Eilenberg *et al*. (2001) divided biological control into four strategies; classical, inoculation, inundation and conservation biological control. **Classical biocontrol** refers to the introduction of an agent into an environment hoping that it will permanently establish and give long-term control of the pest. This strategy is largely relevant to macroscopic pests and not to microbial pathogens. With **inoculation biocontrol**, agents are released into an environment with the expectation that control will be achieved once populations have increased sufficiently, often with the assistance of additional resources. Since this strategy has a lag period while the biocontrol agent multiplies to sufficient numbers, the timing of inoculations is critical. **Inundation biocontrol** is similar to inoculation biocontrol, only control is achieved by the introduced organisms themselves, rather than subsequent generations, and so the effect is more immediate. With the introduction of organisms to the soil for control of fungal crop pathogens it is difficult to separate if an organism is operating in an inoculative or inundative fashion until population studies are compared with disease severity over time, and so in section 1.3.4.2 they are considered together. **Conservation biocontrol** involves enhancing pre-existing natural enemies of the pest species, either in number or efficacy, by adopting specific management practices or modifying the environment. This strategy includes providing resources or habitat that natural enemies can exploit. In the case of soil-borne pathogens, examples of conservation biocontrol include growing crops in areas where soils are naturally suppressive to the pathogen, or encouraging development of suppressive-soils by controlling factors such as soil nutrient and organic matter content, or crop rotation practices. Understanding the mechanisms underpinning suppressive soils is essential to recreate their effect in different areas, and methods to achieve this are covered in section 1.3.4.4.

Whether biocontrol is achieved in an inundative/inoculative manner or through a conservation biocontrol approach, the mechanisms by which disease suppression is achieved traditionally fall into four general categories; competition, antibiosis, parasitism and stimulation of plant

resistance pathways, with growth promotion as a possible fifth indirect mechanism of biocontrol (Handelsman and Stabb, 1996; Hoitink and Boehm, 1999). All of these mechanisms of action have been implicated in control of Rhizoctonia diseases, as detailed in the following sub-sections:

1.3.4.1 Biocontrol mechanisms of action

1.3.4.1.1 Competition

Microorganisms in the soil or associated with plants constantly compete with each other for spatial and nutritional resources. Since *R. solani* is a saprophyte as well as a plant pathogen, organisms which suppress Rhizoctonia diseases may do so by preventing the pathogen from accessing nutrition in the soil or by blocking access to the plant infection courts (targets of infection). For example, a study by Weinhold *et al.* (1972) demonstrated that the virulence of *R. solani* to cotton seedlings was dependant on the nutritional status of the mycelium, with better nourished fungi being more pathogenic. In addition, Dijst (1988) found that *R. solani* AG 3 produced sclerotia with greater mass on nutrient rich media compared to nutrient poor media, and suggested that higher incidence of black scurf after haulm destruction may partially result from increased exudation of nutrients by host plants. Therefore it can be concluded that interactions which prevent the pathogen from accessing exogenous nutrition, from the soil or plant exudates, will decrease its pathogenic potential.

1.3.4.1.2 Antibiosis

Suppression of Rhizoctonia diseases by specific microorganisms is often attributed to the production of secondary metabolites which are directly toxic to the pathogen (El-Tarabily and Sivasithamparam, 2006; Homma, 1996; Whipps, 2001). Compounds implicated in biocontrol of *R. solani* are usually antibiotics or fungal cell-wall degrading enzymes. It has been demonstrated that the ability of many strains of rhizobacteria to suppress plant pathogens is dependent on their capacity to produce secondary metabolites which directly inhibit the pathogens, and these include antibiotics, siderophores, bacteriocins and cyanide (Homma, 1996). Strains of *Trichoderma* are often investigated for biocontrol of Rhizoctonia diseases, and many produce inhibitory compounds, such as 6-pently-α-pyrone, which inhibits the growth of *Rhizoctonia solani in vitro* (Scarselletti and Faull, 1994), trichodermin, an antimycotic compound (Bertagnolli *et al.*, 1998), and endochitinases and endoproteinases inhibitory to *R. solani* (Bertagnolli *et al.*, 1996). *Trichoderma* and rhizobacteria are discussed in more depth in section 1.3.4.3. Actinomyces are also often found to inhibit *R. solani* with secreted antifungal metabolites (El-Tarabily and Sivasithamparam, 2006). *In vitro* screening of suppression of pathogen colonies is often used to identify antagonist strains, but this

approach must be treated with caution, as *in vitro* antagonism often correlates poorly with *in vivo* disease suppression, if at all (Deacon, 1991; Fravel, 1988; Weller, 1988). Potentially this discrepancy occurs because the antifungal compounds are not produced by the organisms at the required physical locations at concentrations sufficient to prevent infection. However, there are many examples of biocontrol strains which lose disease suppression capacity when the production of specific antimicrobial compounds is disrupted by genetic modification (Homma, 1996; Mendes *et al.*, 2011).

1.3.4.1.3 Parasitism

As a strategy for biocontrol, mycoparasitism is appealing in principle, as it involves direct and targeted destruction of the pathogen by the biocontrol agent. There has been a large body of research exploring the potential of mycoparasites to control *R. solani*, and some of the more commonly investigated species include *Trichoderma harzianum*, *T. hamatum*, *T. viride*, *T. virens*, *Laetiaria arvalis*, *Pythium nunn*, *Gliocladium roseum*, *Verticillium biguttatum* and *Stachybotrys elegans* (Boogert, 1996). There are some pitfalls to mycoparasitism that limit the application of this mode of action, however: *Verticillium biguttatum* received a lot of attention as a potential biological control agent (BCA) of *R. solani* on potato, as it is dependent on *R. solani* for natural development (Boogert and Luttikholt, 2004; Boogert *et al.*, 1990; Morris *et al.*, 1995). However, the ecological range of *V. biguattatum* is more restricted than *R. solani*, so its host can escape infection. The biocontrol of Rhizoctonia damping-off of radish by a *Trichoderma hamatum* was reduced by the presence of thermophilic fungi in hardwood bark compost-amended container medium, potentially because the thermophyilic fungi provided an alternative target for parasitism (Chung and Hoitink, 1990). So if the environmental conditions are not conducive or if alternative hosts are present, mycoparasitism is unlikely to suppress the pathogen sufficiently.

1.3.4.1.4 Stimulated resistance

It has been demonstrated that potato plants respond to infection (nipping of emerging shoot tips) by virulent *R. solani* strains by systemic activation of an array of defence genes including chitin-hydrolysing enzymes, 1,3,-β-Glucanase, which are involved in hydrolysing fungal cell walls, and that this decreases the likelihood of infection of subsequent shoot tips (Lehtonen *et al.*, 2008a). Other non-pathogenic organisms which activate the same pathways may decrease Rhizoctonia diseases of potato. Cardinale *et al.* (2006) demonstrated that Rhizoctonia damping-off of radish was decreasead by a hypovirulent *R. solani* strain, which induced plant systemic resistance and also competed with a virulent *R. solani* strain for space

and nutrients. This approach was also demonstrated for control of the Rhizoctonia diseases banded leaf and sheath blight of maize (Pascual *et al.*, 2000).

1.3.4.1.5 Growth promotion

It is perhaps misleading to classify growth promotion as a mechanism of biocontrol activity, as a variety of factors may contribute. Some organisms promote growth by enhancing nutrient uptake (biofertilising) or abiotic/biotic stress tolerance, which may make host plants more able to cope with infection. Others synthesise phytohormones which directly stimulate plant growth (Diallo *et al.*, 2011). Examples of growth-promoting organisms with evidence of Rhizoctonia disease suppression include; root colonising growth-promoting yeasts which were shown to reduce *R. solani* diseases of sugar beet (El-Tarabily, 2004); many strains of growth promoting rhizobacteria are also suppressive to Rhizoctonia diseases (covered in more detail in section 1.3.4.3.1); and mycorrhizal fungi, which have been shown to promote plant growth, and reduce both stem canker and black scurf severity of potato in field trials (Larkin, 2008). One research group used production of the plant growth hormone indole-3-acetic acid followed by growth promotion assays as part of the selection criteria for potential BCAs of Rhizoctonia diseases (Faltin *et al.*, 2004).

1.3.4.2 Inundative and inoculative biocontrol

As this section will highlight, there has been a large amount of research into the inundative application of single (or combinations of) strains of microorganisms which can suppress Rhizoctonia diseases. However, conventional or organic agriculture have yet to integrate this approach into the cultivation of potatoes. This may be due to prohibitive cost, insufficient ecological range of the BCA or incompatibility with existing agrichemical practice (Tsror, 2010).

1.3.4.2.1 Organisms implicated in biocontrol of Rhizoctonia diseases

A diverse range of organisms has been implicated as suppressive agents of various Rhizoctonia diseases of plants, including potato. This includes several bacterial taxa of the γ-Proteobacteria. For example, *Lysobacter* spp. and members of Xanthamonadaceae, were implicated in suppression of *R. solani* AG 2 from soils suppressive to Rhizoctonia disease of sugar beet (Postma *et al.*, 2010b). Many members of Pseudomonadaceae, another γ-Proteobacteria family, have been highlighted as Rhizoctonia disease suppressors in several studies, including disease decline of Rhizoctonia disease of sugar beets (Mendes *et al.*, 2011), and reduction of rice sheath blight - where a combination of two isolates gave control equivalent to that achieved with the fungicide benomyl (Kazempour, 2004). Another bacterial group frequently associated with suppression of Rhizoctonia diseases are the Actinomycetes.

Most often it has been strains of the genus *Streptomyces* or their secondary metabolites, that are reported to inhibit *R. solani* or Rhizoctonia diseases (Kulik, 1996). Members of the genus *Bacillus* have been shown to suppress Rhizoctonia damping-off in pine (Ozdamar and Basim, 2002) and tomato (Szczech and Shoda, 2006), as well as stem canker of potato (Brewer and Larkin, 2005).

Fungal suppressors of Rhizoctonia diseases are just as diverse as the bacteria. These include arbuscular mycorrhyzae, which reduced both stem canker and black scurf severity of potato in the field (Larkin, 2008). Hypovirulent (non-pathogenic) *Rhizoctonia* strains have also been used to suppress Rhizoctonia diseases of potato (Escande and Echandi, 1991), sheath blight in maize (Pascual *et al.*, 2000) and radish seedling damping off (Sneh *et al.*, 2004). Many fungal mycoparasites have demonstrated suppression of Rhizoctonia diseases, including *Trichoderma harzianum*, *T. hamatum*, *T. viride*, *T. virens*, *Laetiaria arvalis*, *Pythium nunn*, *Gliocladium roseum*, *Verticillium biguttatum* and *Stachybotrys elegans* (Boogert, 1996).

Less commonly reported are mycophagous soil animals, such as *Folsomia fimetaria* (springtail) and *Aphelenchus avenae* (nematode). Lootsma and Scholte (1997) demonstrated reduced Rhizoctonia stem canker severity on potato by these organisms, over a range of soil moisture conditions with populations equivalent to those naturally occurring in the field.

Studies often compare disease suppression by a variety of biocontrol organisms. In a greenhouse screening of 28 biocontrol organisms by Brewer and Larkin (2005), the best control of stem canker was achieved by *Bacillus subtilis*, *Rhizoctonia zeae* and *Stilbella aciculosa*, while the best control of black scurf was by *Laetisaria arvalis* and *Rhizoctonia zeae*. These authors also demonstrated that a combination of *Bacillus subtilis* and a *Trichoderma virens* provided better control than either strain individually.

1.3.4.2.2 Biocontrol products with reports of suppression of Rhizoctonia diseases of potato

Existing biocontrol or biofertiliser products have been tested for suppression of Rhizoctonia diseases of potato, and some have demonstrated strong suppression under the conditions examined. For example, field trials of three biocontrol products; Trianum-G® (*Trichoderma harzianum*, T-22), Mycostop® (*Streptomyces griseoviridis*) and Prestop® (*Gliocladium cantenulatum*) were tested in comparison with the fungicide flutolanil for control of Rhizoctonia diseases of potato by Wilson *et al*. (2008b), as well as T-22 + flutolanil as the BCA was resistant to the fungicide at field application rates. *Streptomyces* or *Gliocladium* did not consistently control Rhizoctonia diseases, and only treatments with flutolanil supressed early symptoms (sprout lesions), although the *Trichoderma* was required (alone or combined

with flutolanil) to increase the proportion of marketable tubers and decrease black scurf (not achieved by flutolanil alone). Another T-22 product RootShield® was tested along with SoilGard® *Trichoderma virens* (GL-21), Kodiak® (*Bacillus subtilis* GB03) and other isolates in a series of greenhouse assays (Brewer and Larkin, 2005). Overall, GB03 was among the best at reducing potato stem canker severity (40-49%), but this did not translate to black scurf control. While all products, in some trials, gave some reduction in stem canker symptoms or black scurf incidence or severity, control was variable and sometimes absent, but this was also true of the fungicide (azoxystrobin) treatment. The product Root-Pro® which contains two *Trichoderma harzianum* strains was tested for black scurf control over two experiments as in-furrow applications. Black scurf incidence was reduced by 68-97%, but black scurf severity (related to area of tuber surfaces covered in scurf) was only reduced in one of the two experiments (Tsror *et al.*, 2001).

Currently, none of these products (or different products with the same isolates as active ingredients) are registered for use in New Zealand, and their importation and use is heavily restricted.

1.3.4.3 Targeted genera

Identifying soil microbe taxa with a strong chance of yielding strains which possess the capacity to suppress Rhizoctonia diseases of potato when applied in an inundative manner allows targeted selection of organisms for screening. This should accelerate the screening process, allowing more resources to be committed to investigating the mechanism(s) of biocontrol activity.

1.3.4.3.1 Plant growth promoting rhizobacteria

Bacteria which live in close association with plant root systems and have net positive impacts on plant health are termed plant growth-promoting rhizobacteria (PGPR) (Compant *et al.*, 2005). These positive impacts have been attributed to production of microbial siderophores, antibiotics, surfactants (biofertilizing) and phytohormones, along with nutrient and spatial competition, induced systemic resistance and quorum quenching (Diallo *et al.*, 2011). In addition to direct pathogen inhibition, rhizobacteria may suppress Rhizoctonia diseases by disrupting growth of the pathogen on plant tissues, suppressing the formation of infection structures and stimulating plant defences. A review by Diallo *et al.* (2011), of the potential of potato rhizosphere/geocaulosphere (soil under influence of roots and stolons respectively) microbial communities highlighted the following points. First, the genera *Agrobacterium*, *Bacillus* and *Pseudomonas* were consistently identified from these environments. Of these, *Pseudomonas* extensively colonised potato rhizosphere, especially *P. fluorescens* and *P.*

putida, and they are also highly represented in the endorhiza (root internal microenvironment). Pseudomonads that are able to stimulate growth and that are also antagonistic to potato pathogens (including *R. solani*) have also been isolated from spores of mycorrhizal fungi which colonise potato. Many strains of *Pseudomonas* spp. are known to be PGPR, contributing to plant health either indirectly by suppressing growth and/or activity of organisms deleterious to plant health or sequestering heavy metals, or by directly promoting growth either by nutrient solubilisation or producing plant growth regulating compounds (Van Loon and Glick, 2004). This combination of appropriate physical niche and biocontrol potential makes members of the *Pseudomonas* genus good candidates for control of Rhizoctonia diseases.

There are many studies which observe strong biocontrol activity of Rhizoctonia diseases by *Bacillus* spp., most frequently *B. subtilis* (Brewer and Larkin, 2005; Kurzinger, 2007; Munoz Ruiz *et al.*, 2001; Schmiedeknecht, 1993; Schmiedeknecht *et al.*, 1997, 1998; Somani and Arora, 2010) but also *B. thuringiensis* (Abada and Abdel-Aziz, 2002), *B. cereus* (Somani and Arora, 2010) and *B. polymyxa* (Kurzawinska and Gajda, 2001). Strains from several *Bacillus* species have been shown to elicit induced systemic resistance in a range of plant species and offer protection against a variety of diseases as well as often promoting plant growth (Kloepper *et al.*, 2003), confirming their role as important PGPR.

For these reasons as well as previous reports of biocontrol strains in the literature (see section 1.3.4.2.1), the PGPR genera *Pseudomonas* and *Bacillus* were considered promising target groups as potential candidate biocontrol agents of Rhizoctonia diseases of potato in this project.

1.3.4.3.2 Trichoderma

The genus *Trichoderma* is among the most common groups of free-living fungi isolated from temperate soils, and it has been the target of a large body of biocontrol research. This is due to the capacity of strains to; 1) induce resistance in plants; 2) parasitise fungal plant pathogens; 3) produce a wide variety of extracellular compounds (including antibiotics and enzymes) which can suppress plant pathogens; 4) strongly compete with other soil organisms for resources and 5) improve plant growth (Harman *et al.*, 2004). A large number of *Trichoderma* isolates have been reported to suppress a wide range of Rhizoctonia diseases, often achieving strong biocontrol, including some commercial products (see section 1.3.4.2.2). For example, Grosch *et al.* (2006) screened 390 fungal isolates in a series of *in vitro* and *in vivo* assays, and the top six candidates for control of Rhizoctonia symptoms on potato sprouts in a pot trial and black scurf in a field were strains of *Trichoderma reesei* or *T. viride*. Another *T. viride* isolate

was shown by Coley-Smith *et al.* (1991) to be the best performing of nine *Trichoderma* isolates for control of *R. solani* bottom rot of lettuce, achieving control equivalent to the fungicide tolchlofos-methyl. Beagle-Ristaino and Papavizas (1985) found that field applications of a *Trichoderma viride* and a *Trichoderma* (*Gliocladium*) *virens* (which became the active ingredient in SoilGard®, see section 1.3.4.2.2) reduced severity of potato stem canker and the viability of sclerotia on tubers and in soil.

Strains of *Trichoderma harzianum* are frequently cited as suppressors of *R. solani* and Rhizoctonia diseases, including isolate T-22 (the active ingredient in several BCA products, see section 1.3.4.2.2). T-22 reduced early potato 'Rhizoctonia stem lesion index' in pot trials, although the antagonism diminished over time, and reduced black scurf on progeny tubers, as well as reducing the proportion of undersize, malformed or green tubers (Wilson *et al.*, 2008a). Other *T. harzianum* strains have reduced snap bean damping-off in the greenhouse (Marshall, 1982), and reduced black root rot of strawberry plants in nursery and field applications, resulting in increased yield (Elad *et al.*, 1981). A *T. harzianum* isolate reduced the proportion of bean plants with Rhizoctonia disease in greenhouse and field experiments, but control decreased as soil temperature increased (Elad *et al.*, 1980). Another *T. harzianum* strain reduced field damping-off of cotton seeds as effectively as the fungicide pentachloronitrobenzene, as did a strain of *T. hamatum* (Elad *et al.*, 1982). Mihuta-Grimm and Rowe (1986) demonstrated that another *T. hamatum* provided better field control of radish damping-off than pentachloronitrobenzene.

Since strains of *Trichoderma* potentially offer the major mechanisms of biocontrol (section 1.3.4.1), combined with the multiple reports of biocontrol of Rhizoctonia diseases (above) and their prevalence in commercial biocontrol products (section 1.3.4.2.2), they are excellent targets as candidate biocontrol isolates for Rhizoctonia diseases of potato.

1.3.4.4 Conservation biocontrol

In contrast to the inundative/inoculative approach for control of soil-borne plant pathogens outlined above (1.3.4.2), conservation biocontrol largely refers to the cultivation of crops in soils suppressive to pathogens, or the creation of suppressive conditions, often through the application of organic matter amendments to enhance the activity of natural antagonists (Bonanomi *et al.*, 2010). The techniques commonly used to investigate how disease control is achieved (section 1.3.4.4.3) are applicable to both naturally occurring and induced suppressive soils.

1.3.4.4.1 Suppressive soils and disease decline

As previously detailed, some soils are naturally suppressive to Rhizoctonia diseases (Jager and Velvis, 1983). The phenomenon of 'disease decline' has also been described, where continuous cultivation of the same crops leads to initial increase in pathogen and disease incidence, followed by decline in disease levels, usually attributed to the build-up of soil microbial communities antagonistic to the pathogen (Borneman and Becker, 2007; Garbeva *et al.*, 2004; Hyakumachi, 1996; Weller *et al.*, 2002). While there are many reports of disease decline relating to soil-borne plant pathogens in the field, the mechanisms are rarely fully investigated, as such studies may have to span many years to give definitive answers. Suppression is classically subdivided into 'general suppression' - where the total microbial biomass confers suppression and is non-transferrable between soils, and 'specific suppression' - due to individual or several groups of microorganisms and is transferrable (Weller *et al.*, 2002). Transferrable suppression is of great interest for the inundative/inoculative biocontrol approach (section 1.3.4.2) as a source of antagonist isolates. General suppression is often linked to addition of organic matter amendments (covered in section 1.3.4.4.2), specific crop practices or increased soil fertility. One of the more studied examples of induction of disease suppressive soils is decline in take-all of wheat ("take-all decline"), where monoculture of wheat leads to spontaneous decline in disease severity caused by *Gaeumannomyces graminis* var. *tritici*. This phenomenon was shown to correlate with increases in populations of Pseudomonads antagonistic to the pathogen, demonstrated both *in vitro* and *in vivo* (Borneman and Becker, 2007). There are a few published reports of in field Rhizoctonia disease decline, and some studies have attempted to decipher the underlying mechanisms. For example, Rhizoctonia disease (root rot, AG2-2IV) decline has been reported due to monoculture of sugar beet in Japan, as reviewed by Hyakumachi (1996), and was reported to be linked to *Bacillus* species. Mendes *et al.* (2011) reported that decline of Rhizoctonia disease of sugar beet in the Netherlands was strongly linked to members of the Pseudomonadaceae. Decline of wheat root rot (AG 8) due to monoculture has also been reported in Australia (Roget, 1995). Suppression of pre- and post-emergence cauliflower damping-off (AG 2-1) in a 40-year monoculture field in the Netherlands was demonstrated to be as a result of the monoculture, and was linked to populations of *Lysobacter*, *Streptomyces* and *Pseudomonas* spp. (Postma *et al.*, 2010a). Continual cropping (5 years of cultivation) of potato at two sites resulted in heterogeneous decline of black scurf and AG 3 populations, associated with an increase in population of AG 5 in one site but not the second (Jager and Velvis, 1995). In greenhouse studies of disease decline, many researchers have created Rhizoctonia disease decline through monoculture, where successive plantings are not limited

to one per year. However, it is advisable that conclusions should be drawn from field studies, as greenhouse conditions and mechanisms of decline may not reflect those of the field (Hyakumachi, 1996).

Suppressive soils and disease decline offer valuable tools in the search for conditions and organisms which could be exploited in biocontrol of Rhizoctonia diseases. Understanding how suppression is achieved is challenging, and approaches regarding this are covered in section 1.3.4.4.3.

1.3.4.4.2 Organic matter amendments

There have been many studies of the potential of a wide variety of organic matter (OM) amendments for control of a range of soil-borne pathogens, with *Rhizoctonia* spp. being one of the most commonly investigated (Bonanomi *et al.*, 2007). Meta-analyses by Bonanomi *et al.* (2007; 2010) found that there are contrasting trends regarding the suppression of Rhizoctonia diseases with organic amendments. For certain OMs, increases in overall soil microbial activity correlated with increased disease suppression (indicating general suppression), but the converse of this was true for other OM amendments. Overall, the factors which positively correlated with disease suppression most often were populations of fluorescent Pseudomonads (73% of cases), endospore forming bacteria (60% of cases) and *Trichoderma* spp. (56% of cases), while none of these measures ever negatively correlated with disease suppressiveness. There have been several reports that the addition of selected OM amendments to inundative biocontrol treatments enhanced the suppressive capacity of either component alone (Krause *et al.*, 2001; Kwok *et al.*, 1987; Pugliese *et al.*, 2011; Scheuerell *et al.*, 2005). This supports the hypothesis that Rhizoctonia diseases are controlled by a narrow spectrum of soil microorganisms (Hoitink and Boehm, 1999), and therefore OM amendments will only suppress these diseases if they can support populations of suppressive organisms already present in soil or strains which are introduced along with the amendment. Research by Larkin (2008) suggested that some crop rotations supported introduced microorganisms suppressive to Rhizoctonia (and other) diseases of potato better than others. For example, 'aerobic compost tea' alone or combined with a commercial mix of beneficial microorganisms plus organic nutrients (Complete Plus, Plant Health Care Inc., PA) reduced black scurf and stem canker of potato in a barley/rye rotation, but not in a barley/cover or continual potato rotation. The beneficial microbe mix alone reduced black scurf in the barley/rye rotation but not the other two rotations.

Chapter 5 contains more detailed discussion of specific amendments which have been demonstrated to suppress Rhizoctonia diseases.

1.3.4.4.3 Techniques for investigating the biological nature of disease suppression in soil

After the identification of a soil which suppresses disease caused by selected soil-borne pathogens (either naturally occurring or created through certain cropping/amendment practices), methods of determining whether suppression is biological in nature and which organisms are responsible have been summarised by Borenman and Becker (2007). First, to determine if suppression is biological, soil samples can be sterilised (steam treatment, gamma-irradiation, fumigation) and they should lose suppressive capacity if suppression is biotic. Soil treatments such as heating to a range of temperatures, selective antibiotics or fungicides and alteration of environmental parameters (e.g. pH, water potential, temperature) can also be used to indicate which component of the soil microflora is responsible for suppression. For example, if heating to 80°C does not destroy suppression but heating to 100°C does, suppression is likely to be linked to organisms which can persist at high temperatures, such as endospore-forming bacteria. From this juncture, a 'population-based' approach can be employed, where involvement of populations of microbial taxa in disease suppression is inferred by correlation with soil suppressiveness, then this association is validated with selective quantitative PCR (qPCR) and then by testing if transfer of these groups to a disease-conducive soil induces suppressiveness. A range of soil suppression levels can be created by combining increasing proportions of suppressive soil with disease-conducive soil, or in the case of amendment-linked suppression, creating soils with a range of amendment concentrations. There are many approaches to the analysis of soil microbial community structure for this population-based method.

Many studies rely on culture-dependant techniques, such as enumeration on taxa-selective media. For example, in pot experiments, monoculture of radish and cucumber (successive weekly plantings) resulted in disease decline, correlating with increased populations of *Trichoderma*, with *T. harzianum* found as the most effective at inducing suppressiveness when inoculated into soil (Liu and Baker, 1980). Baker (1980) also reported that monoculture of radish induced Rhizoctonia suppressive soils, and this was correlated with high population densities of *Trichoderma* spp. Culture-dependant substrate utilisation techniques have also been employed to determine if differences in the nutrient sources used by the (culturable) soil microbial community have links to disease suppressiveness, often using commercial multi-substrate plates like BIOLOG® EcoPlates™ or GN™ (Larkin *et al.*, 2011; Mazzola, 2004; Pane *et al.*, 2011; Stefanowicz, 2006).

Since only a small proportion of soil microorganisms are amenable to culture in this manner, approx. 17% for known fungi (Bridge and Spooner, 2001) and 1% for known bacteria (Kirk *et*

al., 2004), there exist many culture-independent techniques for analysis of soil microbial communities. Culture-independent techniques carry their own disadvantages. For example, with PCR-based methods, if some taxa have cells which are more resistant to lysis, then they will be under-represented in an analysis. Amplification of DNA or RNA may also occur with varying efficiency between different taxa, potentially biasing results. Specific methods for culture independent analysis of soil communities include fatty acid methyl esters analysis (FAME), DNA microarrays and hybridisation (including fluorescent *in situ* hybridisation, FISH), denaturing and temperature gradient gel electrophoresis (DGGE and TGGE), single strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), restriction and terminal restriction fragment length polymorphism (RFLP and T-RFLP) and ribosomal (and automated ribosomal) intergenic spacer analysis (RISA and ARISA), each with its own advantages and disadvantages (Anderson and Cairney, 2004; Garbeva *et al.*, 2004; Kirk *et al.*, 2004; Saito *et al.*, 2007). More recent next-generation sequencing (e.g. pyrosequencing) can allow much deeper analysis of soil community metagenomes, metatranscriptomes and metaproteomes and will probably become popular in studies of suppressive soil communities (Su *et al.*, 2012). Specific enzymatic analysis of soils, such as gluconase, NAGase and chitobiosidase (Pane *et al.*, 2011), have also been used to indicate whether differences in levels of particular enzymes can be linked to soil suppressiveness.

Using a population-based approach, similar to that described above with culture-dependant techniques (agar plating and dual culture analysis with *R. solani*), Postma *et al.* (2010a) inferred the role of *Lysobacter* spp. along with *Streptomyces* and *Pseudomonas* spp. in disease suppressiveness of cauliflower monoculture to *R. solani* AG 2-1. These authors could not identify an exact mechanism of disease control, however. Mendes *et al.* (2011) conducted one of the most comprehensive investigations to date into the soil microbial populations underpinning suppressiveness in a soil suppressive to Rhizoctonia disease of sugar beet. Using a bacterial/archaeal 16S rDNA oligonucleotide microarray to identify rhizosphere community members associated with disease suppressive soils (different degrees of suppressiveness were created by combining suppressive and conducive soils as well as various heat treatments), they showed that Pseudomonadaceae, Burkholderiaceae, Xanthomonadales, Lactobacillaceae and Actinobacteria were the taxa most dynamically associated with disease suppression. Isolation of members of the Pseudomonadaceae confirmed that three haplotypes were more often suppressive to *R. solani in vitro* than the other seven, and they protected sugar beet seedlings from *R. solani* infection. Random transposon mutagenesis created strains which no longer protected the seedlings from

infection, and analysis revealed these strains lacked functionality because of disrupted production of a chlorinated lipopeptide (antifungal compound).

Gaining a fuller understanding of the mechanisms underpinning suppression of Rhizoctonia diseases under natural conditions will help to inform the direction of applied biocontrol of these diseases. Thus, using the most appropriate techniques to elucidate these mechanisms is of considerable importance.

1.4 Summary

Potato is an important crop to New Zealand and internationally. Strains of the fungus *R. solani* pathogenic to potato are found in cropping soils around the world (including New Zealand) and Rhizoctonia diseases of potato can lead to economic yield losses. Pesticide control of these diseases is not always effective, and biological control has the potential to augment existing crop management practices for control of Rhizoctonia diseases. In spite of its importance as a crop, and the prevalence of *R. solani* as a pathogen, biocontrol of Rhizoctonia diseases is not practiced commercially. Many organisms have been implicated in biological suppression of Rhizoctonia diseases, and of these it is often members of the genera *Trichoderma*, *Pseudomonas* and *Bacillus* which have demonstrated most promise in terms of commercial application, but even the most promising isolates of these organisms are very variable in the degree of disease suppression they achieve. Identifying biocontrol isolates for suppression of Rhizoctonia diseases and generating detailed knowledge of their mechanisms of action and limitation of biocontrol is a key component for successfully incorporating biocontrol into existing commercial potato production. Crop rotations and soil amendments with organic matter are others. The influence of the soil microbial community on both *R. solani* and potential biocontrol isolates also needs to be understood so that cropping practices, such as the selection of crops to include in rotations and the most suitable type of organic matter amendments, can be modified to influence community structure to maximise biocontrol potential.

1.5 Research aims and objectives

1.5.1 Aims

The aim of this research was to identify a microbial isolate (or isolates) with the capacity to provide strong *in vivo* suppression of Rhizoctonia diseases of potato when applied in an inundative manner, from targeted microbial genera. Following this, the study aimed to generate detailed information regarding the mechanisms underpinning the identified

biocontrol activity and the limitations to its disease control activity. In the event that no such isolate was identified, a secondary aim was to investigate the potential for suppression of Rhizoctonia diseases of potato with selected organic amendments and crop rotation practices, in relation to their effects on soil microbial communities.

1.5.2 Objectives

1.5.2.1 Inundative biocontrol

In order to stand a reasonable chance of identifying a strong biocontrol candidate from *in vivo* (rather than *in vitro*) screens to take forward for further investigation, the objectives were to:

- Isolate members of the targeted genera *Trichoderma*, *Pseudomonas* and *Bacillus* from soil and the below-ground organs of potatoes grown in agricultural soils from the main cropping regions of New Zealand.
- Design an appropriate *in vivo* greenhouse Rhizoctonia disease assay to be used for screening of potential biocontrol candidate isolates.
- Select isolates representing a range of isolation locations, and dual agar plate interaction types (with *R. solani*), and screen them from suppression of Rhizoctonia diseases of potato using the designed assay.
- If an isolate was identified with strong biocontrol activity, carry out experiments to examine mechanisms underpinning biocontrol activity and its physical limitations (not conducted due to insufficient control from previous objective).

1.5.2.2 Organic matter amendments

To investigate suppression of Rhizoctonia diseases of potato using organic matter (OM) amendments, the objectives were to:

- Identify from the literature OM amendments which previous researchers had highlighted for suppression of Rhizoctonia diseases, or which are of agricultural interest.
- Use an *in vivo* greenhouse assay to test for suppression of Rhizoctonia diseases of potato at a variety of OM amendment rates, and select one which was likely to produce incremental suppression over several rates.
- If effective disease suppression was identified, investigate the biological nature of OM-related disease suppression by comparing differences in soil microbial

communities between treatments of increasing OM amendment rate in a further assay (not conducted due to lack of disease suppression in amendment rate assay).

1.5.2.3 Crop rotation practices

In order to compare the impact of different crop rotations on the expression of Rhizoctonia diseases of potato, the objectives were to:

- Sample soils from four different rotations at the long-term crop rotation experiment at Pukekohe, New Zealand, and characterise those soils in terms of their culture-dependant (bacterial and fungal enumeration and substrate utilisation) and culture independent (bacterial and fungal ARISA, qPCR of *R. solani* AG 3 and 2-1) soil communities.
- Examine the expression of Rhizoctonia diseases in the soils of the different crop rotations in a glasshouse assay (with or without added *R. solani* inoculum), and examine the effect of crop rotation on *R. solani* soil populations (qPCR).
- Correlate soil community factors with levels of Rhizoctonia disease expression to indicate what might underpin differences in levels of disease.

1.5.3 Thesis format

This thesis consists of seven chapters. Five chapters outline experiments, and each of these chapters includes sections of an abstract, introduction, materials and methods, results, discussion and summary. Literature cited in the thesis is presented at the end.

1.6 Research outputs (as at October 2012)

Research from Chapter 3 was reported, in part, as a refereed, published paper and oral conference presentation.

Bienkowski, D., Stewart, A., Falloon, R. E., Braithwaite, M., and Loguercio, L. L. (2010). A disease assay for *Rhizoctonia solani* on potato (*Solanum tuberosum*). *New Zealand Plant Protection, 63*, 133-137.

Research from Chapter 4 was reported, in part, as an oral conference presentation.

Bienkowski, D., Hicks, E., Braithwaite, M., Falloon, R. E., McLean, K. L. and Stewart, A. (2012, August) Improved potato yields from in-furrow application of Trichoderma. *The 12th International Trichoderma and Gliocladium Workshop, Lincoln University, NZ*. Proceedings and Abstracts p.59. http://bioprotection.org.nz/tg2012

Chapter 2
Isolation, characterisation and selection of microorganisms for inundative biocontrol

2.1 Abstract

Members of the fungal genus *Trichoderma*, fluorescent Pseudomonads and endospore-forming bacteria were selectively isolated from soil samples from the three main potato-producing regions in New Zealand, as well as from below-ground tissues from potato plants grown in soil samples from the same regions. Categorisation of these isolates with regard to their *in vitro* interactions with two *Rhizoctonia solani* isolates known to be pathogenic to potato allowed selection of isolates which were strong physical competitors of the pathogen or produced compounds which inhibited its growth. Sixty-seven isolates possessing these traits and/or potential for plant growth promotion and potato plant endophytism were selected, along with 22 isolates from an existing culture collection, to progress to *in vivo* screening for suppression of Rhizoctonia diseases of potato. Statistical analysis of the bacterial *in vitro* interactions with *R. solani* allowed comparisons to be made between groups of isolates from the different soil locations, plant tissues and microbe groups.

2.2 Introduction

To investigate the potential of inundative biological control of Rhizoctonia diseases of potato, a collection of soil microorganisms was generated from which a sub-set was selected for *in vivo* screening. Since Rhizoctonia diseases can affect potato plants at any point in their life cycle (Banville *et al.*, 1996), a successful biological control agent (BCA) will have to form a close relationship with the plant to provide protection of shoots and roots as they grow through the soil. Also, the BCA will have to be adapted to the common potato cropping soil types in New Zealand. As outlined in Chapter 1, this study focuses on members of the genera *Trichoderma*, *Pseudomonas* and *Bacillus*, as potential BCAs. To obtain suitable microorganisms with potential for disease control and adapted to New Zealand potato cropping soils, including strains with a close relationship with potato plants, the following methods were used: Field soils from the main potato cropping regions were collected and selective isolations for *Trichoderma*, *Pseudomonas* and *Bacillus* spp. were made from the soils, as well as from below-ground organs of potato plants grown in the collected soils.

Selection of isolates to progress to *in vivo* screening was based on the evidence of possession of the following characteristics: production of compounds inhibitory to *R. solani*, physical competition with *R. solani*, endophytic capability (able to internally colonise potato plant tissues) and/or plant growth promotion (which was determined in experiments which ran parallel to the present study). The rationale behind these selection criteria is explained: The production of compounds inhibitory to plant pathogens (e.g. antibiotics) and to a lesser extent competition for space and nutrients are often indicated as the mechanism(s) by which microbes achieve biological control (Handelsman and Stabb, 1996; Whipps, 2001). Physical competition can prevent the pathogen from accessing the infection court, or the nutrient resources required to support infection. Endophytism indicates close association with host plants and may offer a mechanism by which BCAs can escape damaging environmental conditions (Liu *et al.*, 2009). Direct plant growth promotion, while possibly not directly suppressing the pathogen, can suppress disease symptoms by producing compensatory organs and would also be a favourable trait even in the absence of the pathogen. Screening isolates which possess one or several of these characteristics for *in vivo* disease suppression will provide indications as to which are the most useful predictors of potential suppression of Rhizoctonia diseases of potato.

Isolates from the Bio-Protection Centre Culture Collection, Lincoln University, with previously identified capabilities for biocontrol of fungal pathogens or plant growth promotion, were also selected for *in vivo* screening. Suppression scores generated from bacterial *in vitro* interactions with two *R. solani* strains allowed quantitative comparison between isolates from the different cropping regions, selective procedures, sampling times and plant tissue types (both non-sterile and surface sterilised).

2.3 Methods

2.3.1 Sample collection

Soils were collected in July 2009 from fields in the three main potato producing regions of New Zealand, two from Canterbury (research field, Lincoln and commercial field, Southbridge), one from Auckland (research field, Pukekohe) and one from Manawatu (research field). Details of the four soils collected are presented in Table 2.1. Soil was stored at 8°C in plastic bins prior to experiments. Tubers were also collected from the Lincoln and Southbridge sites, as well as potato plants from a Christchurch home garden, for isolations. Soil pH (Table 2.1) was determined by suspending 10 g of air-dried soil in 25 mL of

Nanopure® water (10 min on an orbital shaker at 225 rpm, room temperature) then left to stand overnight before pH was measured (pH Benchtop Meter 2210, Hanna® Instruments).

Table 2.1 Details of soil sample collected for isolation of microorganisms

Soil location	pH	Local classification	Soil taxonomy[+]	Field cultivation history
Lincoln	6.15	Whakanui silt loam	Aquic Haplustepts	3×Potato>Oat
Southbridge	5.25	Waimakariri sandy loam	Typic Ustorthents	Pumpkin>Potato
Pukekohe	6.28	Patumahoe mottled clay loam	Typic Haplohumuls	6×Potato
Manawatu	5.70	Kairanga silt loam	Humic Endoaquepts	2×Potato>Pasture

[+]Classified according to the USDA Soil Survey Staff (2010).

2.3.2 Selective Isolations of candidate microorganisms

Selected microbes were obtained from soil samples and plant tissues as detailed in the following sections.

2.3.2.1 Soil dilutions

Soil samples were mixed thoroughly, then 10 g sub-samples were each combined with 90 mL sterile water agar (0.01% agar) and shaken for 5 min on an arm shaker (500 osc/min) to create a 0.1 g mL^{-1} soil suspension. To recover endospore-forming bacteria (of which *Bacillus* spp. are members), 5 mL aliquots of soil suspensions were transferred to sterile 18 mL capacity universal bottles and placed in an 80°C water bath for 20 min (Priest, 1989). Aliquots (50 µL) of heat-treated samples were spread onto nutrient agar (Appendix A.1.2) and incubated (25°C, darkness) until discrete colonies formed. Colonies were purified by repeated sub-culturing. Suspended soil samples were also each serially diluted to 1×10^{-5} g/mL in water agar. Aliquots (200 µL) of 1×10^{-4} and 1×10^{-5} dilutions were spread onto Kings Medium 'B' (Appendix A.1.3) and *Trichoderma* selective medium (TSM, Appendix A.1.4) in Petri plates, and incubated at 25°C in darkness until discrete colonies formed. Colonies on Kings Medium 'B' fluorescing under UV light (366 nm) were sub-cultured to purify fluorescent Pseudomonads (Braun-Kiewnick and Sands, 2001). Fungal colonies on TSM with *Trichoderma* spp. morphology (Barnett and Hunter, 1998, p. 92) were sub-cultured onto Petri plates containing potato dextrose agar (PDA, Appendix A.1.1). These plates were sealed with plastic film (GLAD®) and left on a benchtop at ambient temperature and light conditions to sporulate. Isolates were purified by plating diluted spore suspensions (from sporulating colonies, suspended and diluted in sterile tap water) onto PDA and sub-culturing single-spore colonies.

2.3.2.2 Potato plant tissues

Isolations were carried out from potato tubers and plants collected from field sites (Lincoln, Southbridge and a Christchurch home garden) and from the microbe 'baiting trial' (section

2.3.2.3). Roots, below-ground stems, stolons and tubers were first washed free of excess soil in tap water. A portion of each tissue was transferred to an 18 mL capacity universal bottle with 10 mL of sterile tap water, and placed in an 80°C water bath for 20 min. Both heat-treated and non-treated tissues were divided into two, with one half receiving surface sterilisation (1 min in 2% sodium hypochlorite solution then rinsed twice in sterile water). All tissue samples were each separately macerated in a few drops of sterile water and portions of non-heat treated macerated tissue were transferred to TSM for selection of *Trichoderma* spp. (as in 2.3.2.1). A sterile loop was used to spread macerated tissue fluid from each heat-treated sample onto nutrient agar, and from each non-heat treated sample onto Kings Medium 'B', with endospore-forming bacteria and fluorescent Pseudomonads selected and purified as detailed in section 2.3.2.1.

2.3.2.3 'Baiting' isolates from soil

An experiment was established to 'bait' organisms from collected soils. Soils were mixed 1:1 (v/v) with quartz sand and 4 L planter bags filled with this plant growth medium. Certified seed potato tubers (cv. 'Ilam Hardy') were planted one per bag, and bags arranged in a greenhouse in a randomised block experimental design and watered as required. At experiment set-up, selective isolations as detailed above were made from each collected soil (detailed in Table 2.1). Three harvests were conducted on the resulting potato plants, at 29, 39 and 58 days after planting. At each harvest, one plant was taken per soil type and excess soil shaken free from roots, which were then rinsed with tap water. Isolations (as detailed above) were made from roots, stems and stolons/tuber initials of each plant, both untreated and surface sterilised. Up to four isolates for each tissue sample were sub-cultured and purified from each selective medium.

2.3.2.4 Long-term storage of isolates

Bacterial stock cultures were created by culturing isolates in Luria Bertani Broth (LB, Appendix A.1.6, 5 mL in 18 mL capacity universal bottles, each loop-inoculated from a pure agar culture, incubated overnight at 25°C in an orbital shaker at 180 rpm), and then combining 500 µL of culture with the same volume of a sterile 50% glycerol solution in a cryotube. *Trichoderma* sp. isolates were maintained on minimal agar (Appendix A.1.7) slopes at 4°C, each inoculated with a plug of mycelium from the growing edge of a pure colony on agar, and incubated at 25°C for 2 days prior to refrigeration. *Trichoderma* sp. isolates were also stored as conidia in 25% glycerol by harvesting spores in sterile water from pure colonies grown on PDA for 10 days, and combining 500 µL of the spore suspension with 500 µL of sterile 50% glycerol solution in a cryotube. All cultures in cryotubes were stored at -80°C.

2.3.2.5 Additional Trichoderma sp. isolates

The total number of *Trichoderma* sp. isolates recovered from samples was low (Table 2.2), so their numbers were supplemented with 104 previously isolated *Trichoderma* spp. isolates from the Pukekohe field soil sample site (Bio-Protection Culture Collection, Lincoln University). These *Trichoderma* spp. were isolated from potato plant rhizosophere soil dilutions, from a mixed crop rotation (potato-onion-oats-brassica-potato) of five growing seasons.

2.3.3 Dual plate assays

Each isolate from the soil dilutions and bait trial was tested *in vitro* for interactions with two *R. solani* isolates pathogenic to potato, R73-13b (Anastimosis Group (AG) 3, Bio-Protection Culture Collection, Lincoln (Sneh *et al.*, 2004)) and Rs043-2 (AG 2-1, received from Dr Farhat Shah, Plant and Food Research Ltd., Lincoln). The AGs were determined by ITS sequence analysis performed by Plant and Food Research Ltd., Lincoln. *Rhizoctonia solani* isolates were maintained as refrigerated cultures on minimal agar, as detailed in section 2.3.2.4. Bacterial isolates were each inoculated from -80°C stock cultures onto nutrient agar and incubated for 2 days (25°C, darkness), then point inoculated four times around the margin of a Petri plate containing ¼ strength PDA (Appendix A.1.5). Plates were incubated for 24 h (25°C, darkness). Full strength PDA plates were inoculated with agar plugs from refrigerated *R. solani* or *Trichoderma* sp. cultures and incubated (25°C, darkness) for 5 days prior to dual plate assay initiation. For bacterial assays, agar plugs (7 mm diam.) from *R. solani* hyphal colony margins were placed (mycelia side to agar) at the plate centre, as in Figure 2.1. Agar plugs (7 mm diam.) of fungal test isolates from the growing margins of a mycelial colonies were placed (mycelia side to agar) 70 mm from an *R. solani* mycelia plug as detailed in Figure 2.1. Duplicate plates were inoculated for each interaction, and plates were sealed with plastic film (GLAD®) and incubated (25°C, darkness). Dual plate interactions were scored between 3 to 7 days after inoculation, once colony interactions were observed. The interactions between *R. solani* and test isolate colonies were characterised using the method described by Ghaffar (1969), into four categories; A, the microbe colonies intermingle but remain clearly distinguishable (due to morphological differences); B, the growing margins of the colonies meet, and *R. solani* is inhibited and becomes overgrown by the test microbe; C, the colony margins of both organisms come close and then both stop growing; D, the growth of one of the organisms is inhibited at a distance, leaving a clear inhibition zone. Where inhibition zones are observed, the distance between organisms was recorded. Examples of the four dual agar plate interaction types are shown in Figure 2.2.

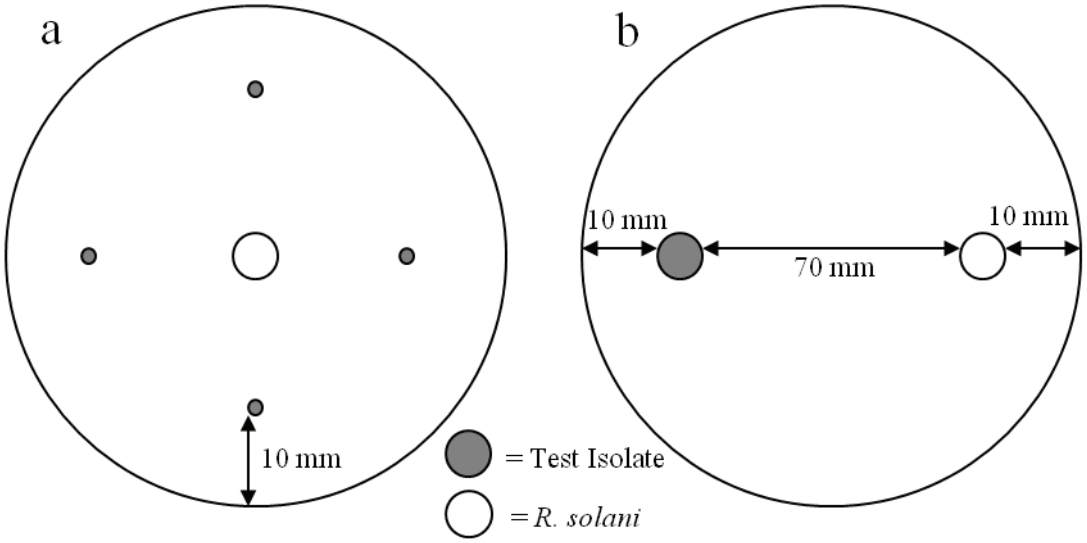

Figure 2.1 Diagram of inoculation positions for dual agar plate assay of *Rhizoctonia solani* against (a) bacterial and (b) fungal isolates

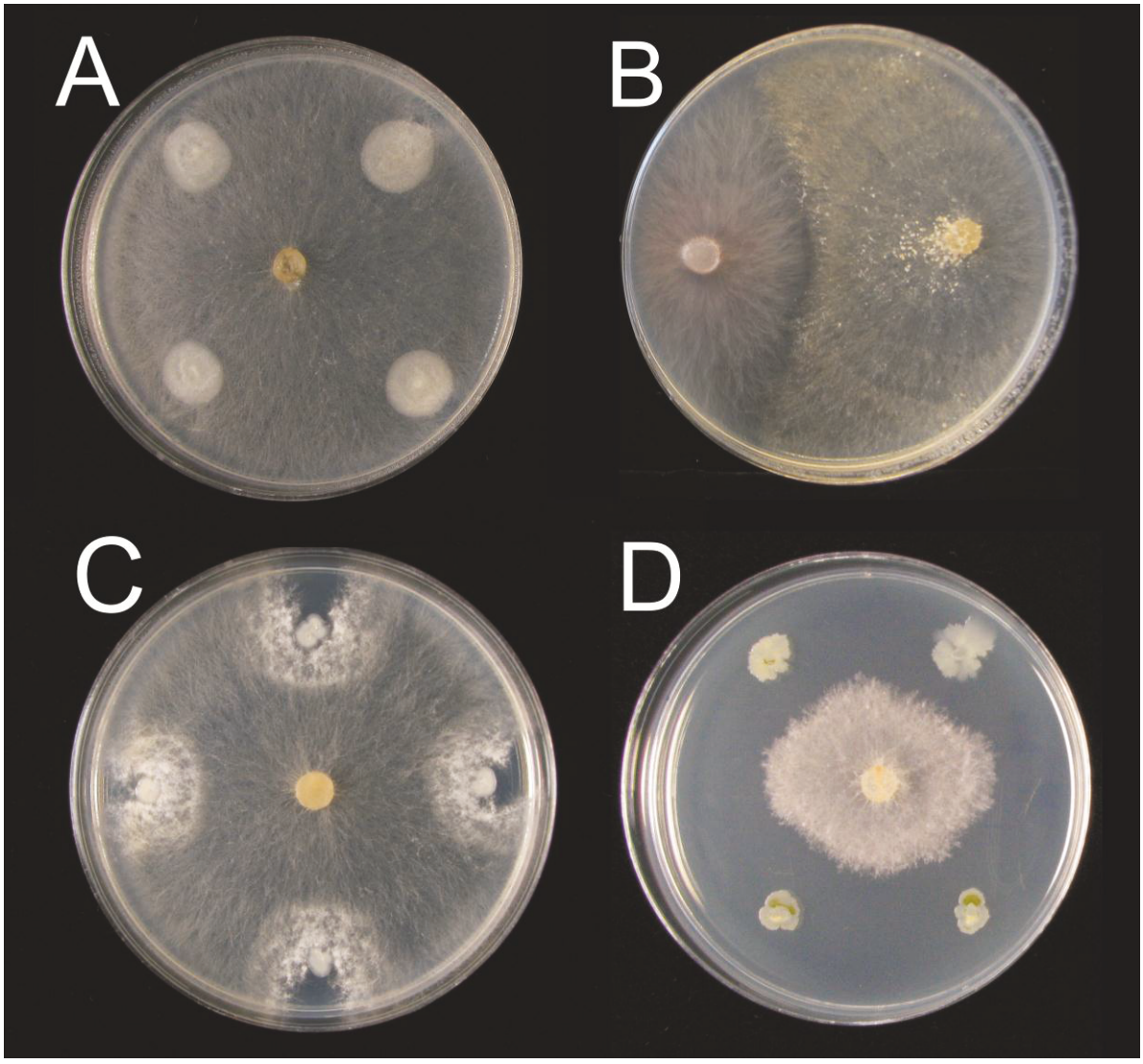

Figure 2.2 Examples of the four dual agar plate interaction categories (A, B, C and D) as detailed in section 2.3.3

Since *in vitro* production of antibiotics and other inhibitory compounds by bacteria correlates (within limits) with *in vivo* suppression of plant pathogenic *Rhizoconia* spp. (Homma, 1996), an *in vitro* suppression scale was created so that the bacterial dual plate interaction data could be statistically analysed for the soil dilution and bait trial isolation samples (for which far more fluorescent Pseudomonads and endospore-forming bacteria were isolated than *Trichoderma* spp. (Table 2.2)). The 0 to 5 *in vitro* suppression scale was based on the assigned interaction categories: Interactions of type A = score 0, B = score 2, C = score 1, D with inhibition zone < 1 mm = score 3, D with inhibition zone > 1 but < 3 mm = score 4 and D with inhibition zone > 3 mm = score 5. An average dual plate score was generated for each *R. solani* isolate/test bacterium interaction, and these data were subjected to unbalanced analysis of variance, with soil location, harvest time, plant tissue type, surface sterilisation, selective procedure and *R. solani* isolate as factors.

2.3.4 Identification of bacteria

DNA was extracted from each bacterial isolate cultured on nutrient agar and amplified using the REDExtract-N-AmpTM Plant PCR kit (Sigma-Aldrich®) as per manufacturer's instructions. Primers targeted the variable portion of the 16S rRNA gene (F27 5' AGAGTTTGATCCTGGCTCAG 3', R1494 5' CTACGGTTACCTTGTTACGAC 3'). PCR parameters were: 94°C for 3 min followed by 30 cycles of; 94°C for 1 min; 57°C for 1 min; 72°C for 2 min then 10 min at 72°C. PCR was performed using an Icycler (Bio-Rad Laboratories, Inc.) and products were visualised by agarose gel (1%, 1 x TAE) electrophoresis. Sequencing was performed using Big Dye Terminator v3.1 (Applied Biosystems™) and PCR cleanup with CleanSEQ® (Agencourt Bioscience Co.). An ABI Prism 3130xl Genetic Analyzer (Applied Biosystems™) was used to generate sequences. Sequences were analysed using Sequencher 4.9 (Gene Codes Co.) software and identifications made using EzTaxon2.1 (Chun *et al.*, 2007).

2.4 Results

2.4.1 Isolations

A total of 345 microbe isolates were obtained from selective isolations conducted on soil samples or potato plant tissues. A summary of the isolates recovered is presented in Table 2.2.

Table 2.2 Total numbers of isolates obtained from three selective procedures; fluorescent on Kings medium 'B' (KB), heat treatment (80°C) and *Trichoderma* selective media (TSM), from soil dilutions, non-sterile (N.S.) and surface-sterilised (S.S.) potato plant tissues.

		Potato plant tissue							
Soil	**Selective**	Root		Stem		Stolon/Tuber			
location	**procedure**	N.S.	S.S.	N.S.	S.S.	N.S.	S.S.	Soil	**Total**
Lincoln	KB	10	4	10	0	8	1	8	41
	80°C	18	5	8	3	16	2	9	61
	TSM	0	1	0	0	4	0	0	5
Southbridge	KB	9	0	6	1	4	1	2	23
	80°C	9	11	8	3	4	2	10	47
	TSM	2	0	0	0	4	0	0	6
Pukekohe	KB	10	0	3	0	10	3	2	28
	80°C	9	0	6	0	5	0	11	31
	TSM	0	0	0	0	1	0	0	1
Manawatu	KB	10	0	2	2	4	0	2	20
	80°C	10	1	6	2	7	0	9	35
	TSM	0	2	0	0	4	0	0	6
Christchurch Garden	KB	1	4	2	4	6	2	-	19
	80°C	1	5	1	1	7	0	-	15
	TSM	3	1	2	0	1	0	-	7
Total		92	34	54	16	85	11	53	345

2.4.2 Selection of isolates for *in vivo* assays

Isolates for *in vivo* screening for suppression of Rhizoctonia diseases of potato were selected on (potential) possession of four traits: Production of antibiotics or other compounds inhibitory to *R. solani*, strong competition with *R. solani*, colonisation of internal plant tissues (endophytic habit) or growth promotion. A parallel study into growth promotion of potato with bio-inoculants screened many of the isolates from this study, and those results determined the selection of potential growth promoters. The dual plate assay categories allowed selection of antibiotic producers, of interaction type D, and strong competitors of *R. solani*, of interaction type B (or C if colonies grew quickly), while those isolates from surface sterilised tissues were regarded as potential endophytes. The selection results are presented in Table 2.3. Isolates from the Bio-Protection Research Centre Culture Collection, Lincoln, were also selected for *in vivo* screening based on evidence of previous plant growth promotion (five isolates) or biocontrol of fungal plant pathogens (17 isolates), all selection rationales are presented in Appendix B, Table 7.1.

Table 2.3 Microbial isolates from potato plant tissues and soils selected for *in vivo* screening against *Rhizoctonia solani*

Selection details[+]	Isolated from	Identity[‡]	Location	Project Identifier
A, E	Root	*Bacillus soli*	Lincoln	RSP2027
		Flavobacterium sp.		RSP2026
				RSP2193
			Southbridge	RSP2094
	Shoot	*Pseudomonas mohnii*	Manawatu	RSP2089
	Stolon/tuber	*Pseudomonas jessenii*	Southbridge	RSP2042
		Pseudomonas sp.		RSP2057
B	Rhizosphere	*Trichoderma* sp.	Mixed crop rotation	RSPT003
				RSPT007
				RSPT018
				RSPT028
				RSPT029
				RSPT031
				RSPT036
				RSPT060
				RSPT079
				RSPT093
				RSPT105
				RSPT106
				RSPT107
				RSPT110
				RSPT113
				RSPT116
	Root	*Trichoderma* sp.	Southbridge	RSPT124
	Shoot		Christchurch garden	RSPT5080
	Stolon/tuber			RSPT5030
			Lincoln	RSPT5163
			Pukekohe	RSPT125
B, E	Root	*Trichoderma* sp.	Manawatu	RSPT122
B, GP	Rhizosphere	*Trichoderma* sp.	Mixed crop rotation	RSPT001
				RSPT005
				RSPT084
				RSPT085
				RSPT097
B, GP, E	Root	*Trichoderma* sp.	Christchurch garden	RSPT5075
C	Root	*Bacillus mycoides*	Southbridge	RSP2072
	Shoot		Southbridge	RSP2013
		Flavobacterium sp.	Lincoln	RSP2053
	Stolon/tuber	*Bacillus mycoides*	Lincoln	RSP0170
		Pseudomonas brassicacearum	Lincoln	RSP2083
		Pseudomonas sp.	Lincoln	RSP2086
C, E	Root	*Bacillus thuringiensis*	Southbridge	RSP2016
		Endospore bacterium	Manawatu	RSP2090
C, GP	Shoot	*Bacillus mycoides*	Southbridge	RSP2009
C, GP, E	Root	*Bacillus mycoides*	Southbridge	RSP2002
		Flavobacterium sp.	Southbridge	RSP2004

Selection details[+]	Isolated from	Identity[‡]	Location	Project Identifier
D	Cauliflower	*Gliocladium* sp.	Southbridge	RSPT5182
	Root	*Bacillus* sp.	Manawatu	RSP2067
		Pseudomonas reinekei	Pukekohe	RSP2183
		Pseudomonas sp.	Southbridge	RSP2110
	Shoot	*Bacillus methylotrophicus*	Pukekohe	RSP2071
		Bacillus thuringiensis	Christchurch garden	RSP0017
			Manawatu	RSP2158
	Soil		Manawatu	RSP0268
			Southbridge	RSP0144
		Paenibacillus sp.	Lincoln	RSP0362
	Stolon/tuber	*Brevibacillus laterosporus*	Southbridge	RSP2024
		Pseudomonas koreensis	Manawatu	RSP2125
		Paenibacillus peoriae	Pukekohe	RSP2139
D, E	Root	*Bacillus thuringiensis*	Christchurch garden	RSP0002
	Shoot	*Pseudomonas jessenii*	Manawatu	RSP2023
	Stolon/tuber	*Pseudomonas lurida*	Pukekohe	RSP2116
			Pukekohe	RSP2120
D, GP	Rhizosphere	*Trichoderma* sp.	Mixed crop rotation	RSPT037
	Root	*Pseudomonas koreensis*	Manawatu	RSP2014
	Shoot	*Bacillus* sp.	Lincoln	RSP2015
D, GP, E	Root	*Flavobacterium* sp.	Lincoln	RSP2020

[+]First letter is dual agar plate interaction type as detailed previously, GP is potential growth promoter and E is potential endophyte.
[‡]The identity of the bacterial isolates was based on the closest sequence match using the BLASTN and megaBLAST programmes to search the EzTaxon database. While genus identification will be accurate, the species identification may not be.

2.4.3 *In vitro* suppression of *Rhizoctonia solani* by bacteria

When the data set for dual plate suppression scores (excluding *Trichoderma* isolates as detailed previously) was analysed with *R. solani* isolate, soil location and selective procedure as factors (2×4×2 factorial), *R. solani* isolate, *R. solani* isolate × selective procedure and soil location × selective procedure were each statistically significant (Table 2.4).

Table 2.4 Unbalanced factorial ANOVA of dual plate suppression data *P* values

Factor/interaction	*P* value
R. solani isolate	0.001
Soil location	0.728
Selective procedure	0.198
R. solani isolate × soil location	0.744
R. solani isolate × selective procedure	0.001
Soil location × selective procedure	0.002
R. solani isolate × soil location × selective procedure	0.780

Overall, *R. solani* isolate Rs043-2 was more strongly suppressed *in vitro* by the test isolates than isolate R73-13b, with mean suppression values of 0.877 and 0.572, respectively.

Mean suppression scores for isolates from both selective procedures against each *R. solani* isolate (Figure 2.3 a) showed that fluorescent Pseudomonads were, overall, less suppressive to *R. solani* isolate R73-13b than the endospore-forming bacteria. There were no significant differences in mean suppression scores of fluorescent Pseudomonads and endospore-forming bacteria for isolate Rs043-2, although both of these means were greater than the mean suppression score of the fluorescent Pseudomonads for R73-13b.

Analysis of soil locations with the selective isolation procedure (Figure 2.3 b) showed that for test isolates from Pukekohe, fluorescent Pseudomonads gave greater mean suppression scores *in vitro* to the *R. solani* isolates than the endospore-forming bacteria, but there were no significant differences between fluorescent Pseudomonads and endospore-forming bacteria for the other locations. The fluorescent Pseudomonads from Pukekohe, overall, gave greater mean suppression scores than those from the other three locations, but there were no differences between mean suppression scores of endospore-forming bacteria groups across the four locations.

To analyse the effects of harvest time, plant tissue identity and surface sterilisation on test isolate suppression scores, the data for isolates from soil dilutions was omitted and the data was re-analysed. Plant tissue, harvest time, soil location, selective procedure and surface sterilisation were analysed as factors in various combinations. The significance of the analyses is presented in Table 2.5. For those analyses which were significant ($P < 0.05$), the data are presented graphically (Figures 2.4, 2.5 and 2.6).

Table 2.5 Unbalanced factorial ANOVA of dual plate suppression data *P* values (omitting soil dilution isolates)

Factor/interaction	*P* value
Harvest time	0.768
Plant tissue type	0.234
Selective procedure	0.511
Plant tissue type x harvest time	< 0.001
Harvest time x selective procedure	< 0.001
Plant tissue type x selective procedure	0.582
Harvest time x plant tissue type x selective procedure	0.850
Surface sterilisation	0.106
Plant tissue type x surface sterilisation	0.010
Surface sterilisation x selective procedure	0.004
Plant tissue type x surface sterilisation x selective procedure	0.144
Soil location x harvest time	0.010
Soil location x plant tissue type	0.098
Soil location x surface sterilisation	< 0.001

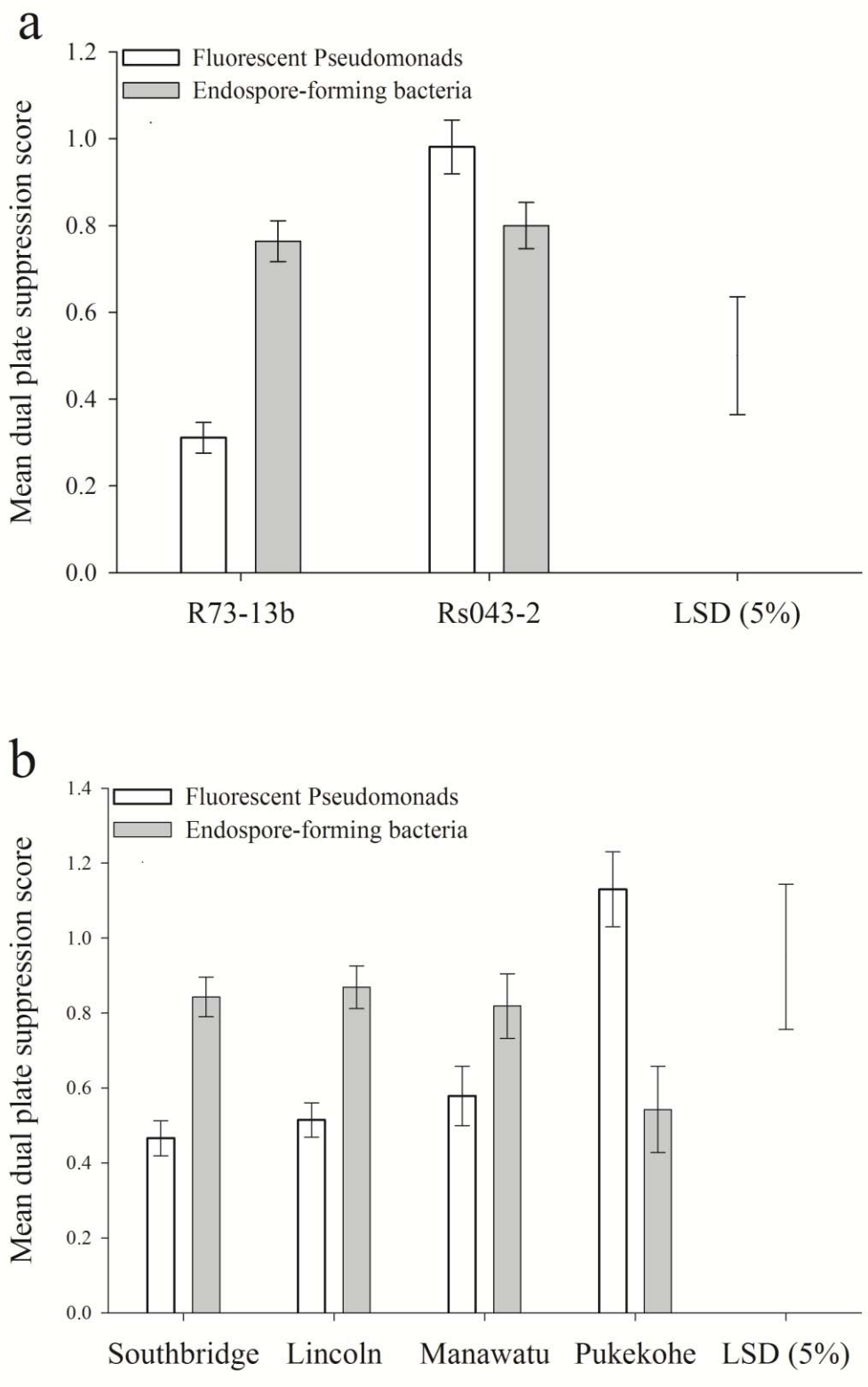

Figure 2.3 Mean dual plate suppression scores of test isolates from two selective procedures for (a) each *Rhizoctonia solani* isolate and (b) each soil location. SEM bars are shown on group means and floating bars are average LSDs (5%) from unbalanced ANOVAs.

The analysis of mean suppression scores of isolates from the three plant tissue types over the three harvest times (Figure 2.4 a) demonstrated no difference between isolates from roots, stems or stolons at harvests 1 or 3. At harvest 2 test isolates from stolons gave greater mean suppression scores than those from roots or stems (with no difference between roots and stems).

The analysis of mean suppression scores of isolates from the two analysed selective procedures over the three harvest times (Figure 2.4 b) indicated that at harvest 1, endospore-forming bacteria from plant tissues were more suppressive than the fluorescent Pseudomonads, but there were no significant differences between mean suppression scores at harvests 2 and 3.

The analysis of mean suppression scores of isolates from the two analysed selective procedures over the three potato plant tissues types (Figure 2.5 a) demonstrated that the isolates from surface-sterilised stolons had a greater mean suppression score than those from non-sterile stolons. There were no significant differences between suppression score means of isolates from non-sterile and surface-sterilised roots or stems.

The analysis of selective procedure with surface sterilisation of tissue (Figure 2.5 b) showed that for the fluorescent Pseudomonads, mean suppression score for isolates from surface sterilised tissues was greater than from non-sterile tissues, and greater than both group means of the endospore-forming bacteria. There was no significant difference between mean suppression scores of the endospore-forming bacteria between surface sterilised and non-sterile tissue.

The analysis of isolates mean suppression scores from the four soil locations over the three harvest times (Figure 2.6 a) showed that for isolates from Southbridge soil, mean suppression score harvest 2 was less than at harvest 1, with no significant difference between harvests 2 and 3 or between harvests 1 and 3. For isolates from Lincoln and Manawatu soils there were no differences in mean suppression scores over the three harvest times. For isolates from Pukekohe soils, mean suppression scores of isolates from harvests 2 and 3 were both greater than from harvest 1, with no significant difference between harvests 2 and 3.

In the analysis of isolates from non-sterile and surface sterilised tissues across the four soil locations, the only difference was that mean suppression score of isolates from sterilised potato plant tissues grown in Pukekohe soil were greater than all other isolate groups, with no significant differences between the means of the other groups (Figure 2.6 b).

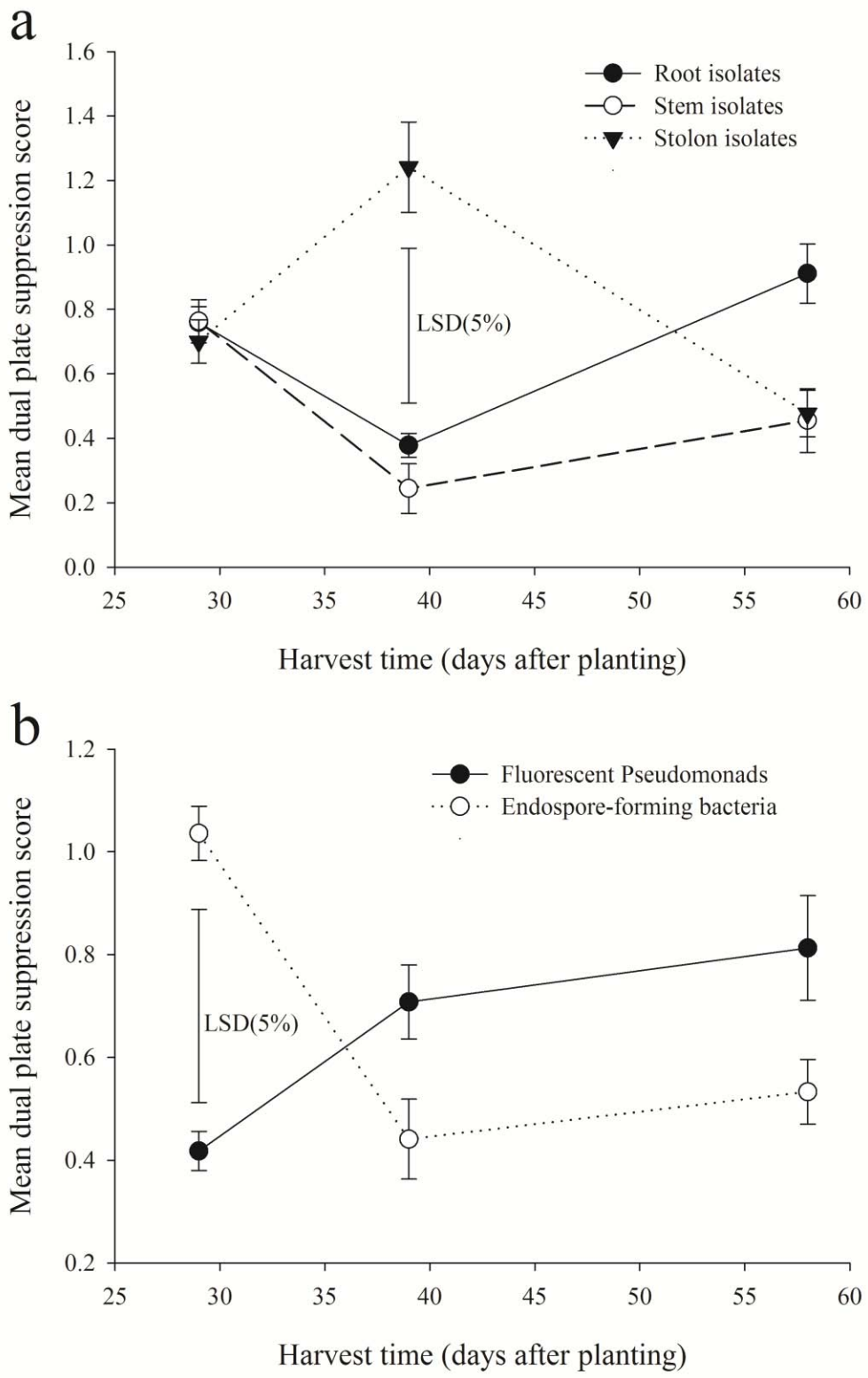

Figure 2.4 Mean dual plate suppression scores of test isolates from three harvests from (a) three different plant tissues and (b) two selection procedures. SEM bars are shown on group means and floating bars are average LSDs (5%) from unbalanced ANOVAs.

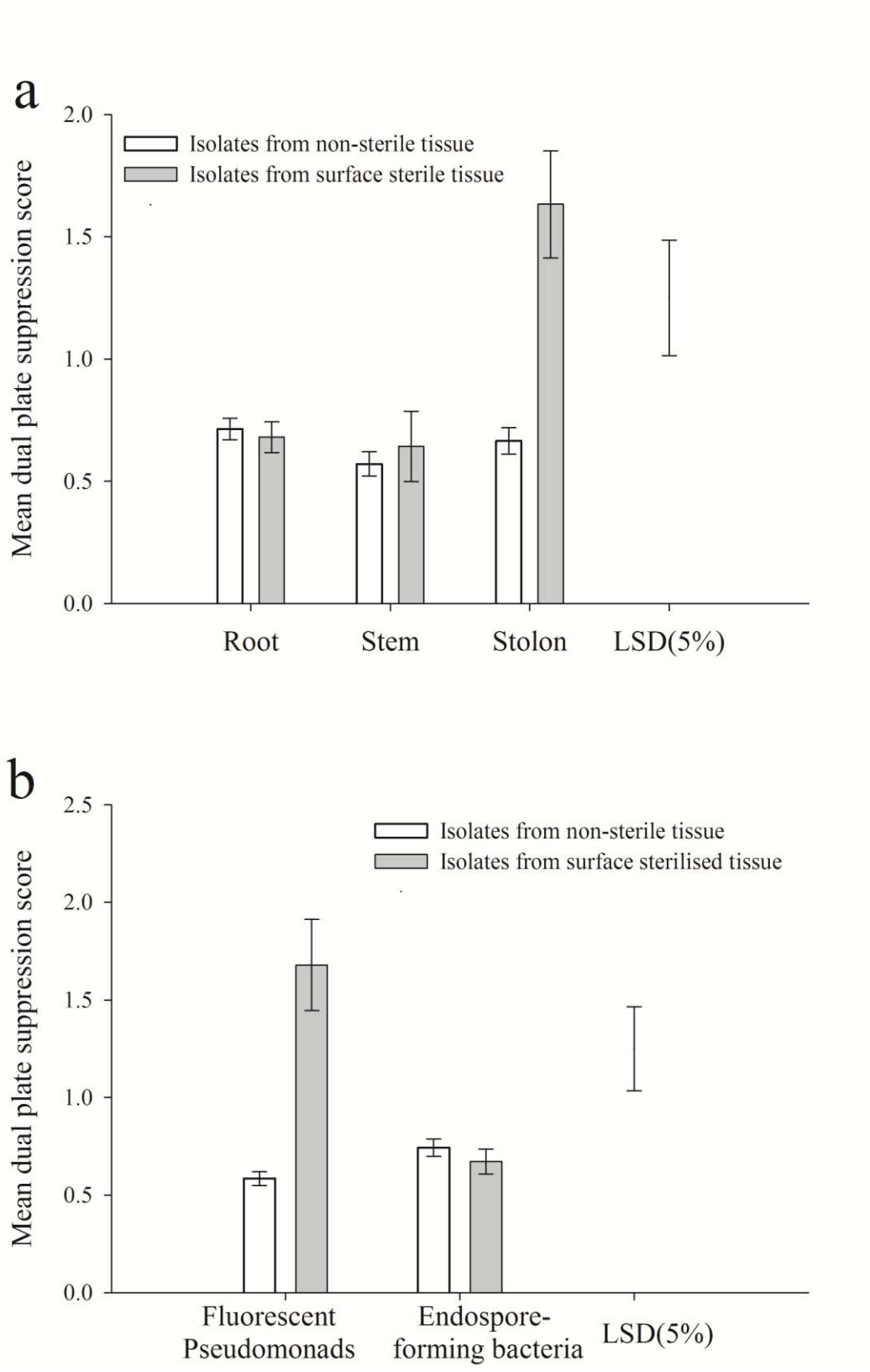

Figure 2.5 Mean dual plate suppression scores of test isolates from non-sterile or surfaced sterlised potato tissues averaged over (a) three plant tissue types and (b) two selection procedures. SEM bars are shown on group means and floating bars are average LSDs (5%) from unbalanced ANOVAs.

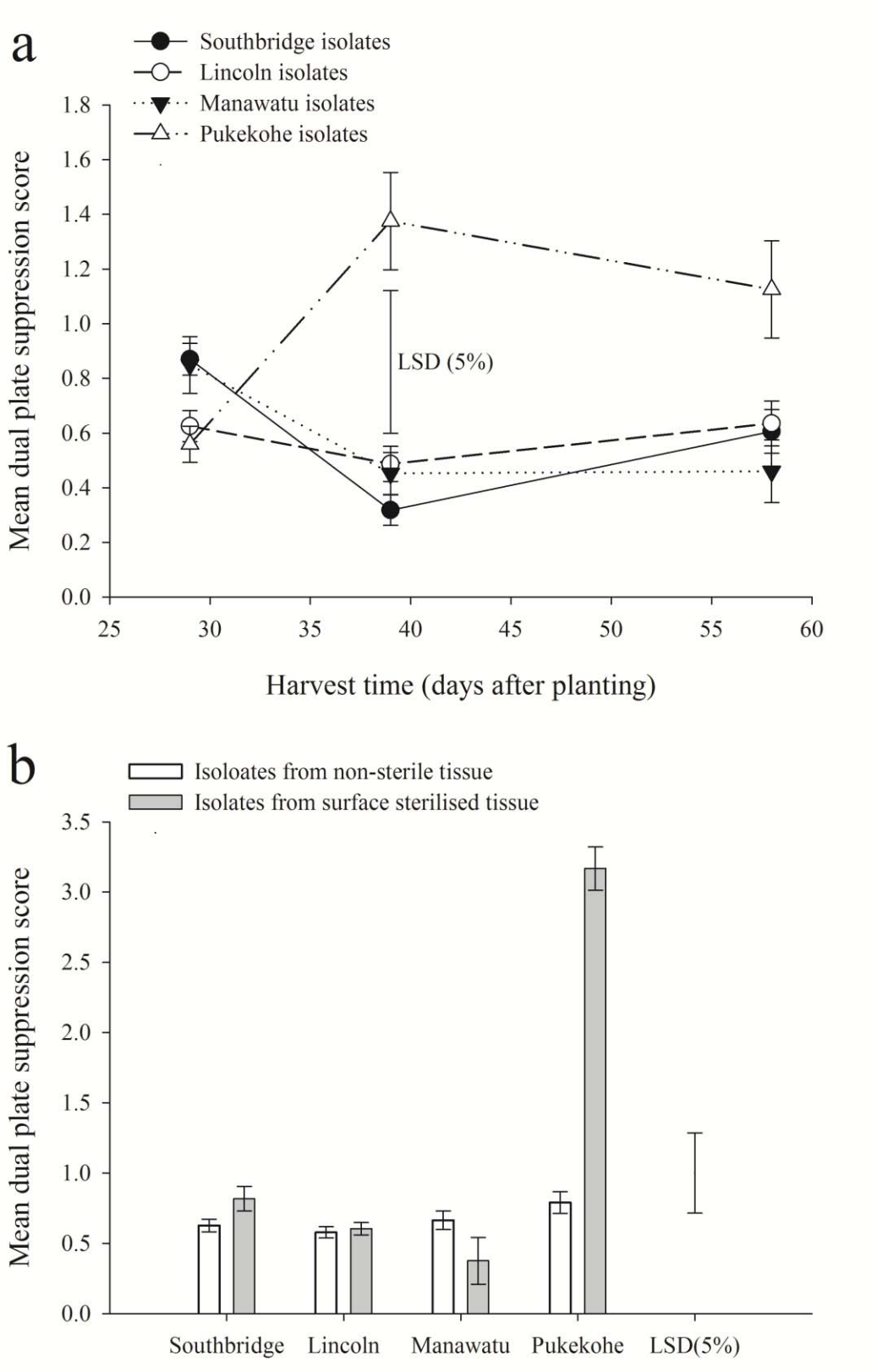

Figure 2.6 Mean dual plate suppression scores of test isolates from (a) each soil location for each harvest time and (b) from non-sterile or surfaced sterlised potato tissues from each soil location. SEM bars are shown on group means and floating bars are average LSDs (5%) from unbalanced ANOVAs.

2.5 Discussion

In vitro screening of microbes as candidates for biological control of plant pathogenic *Rhizoctonia* spp. is commonly performed in a hierarchical manner. For example Faltin *et al.* (2004), subjected 434 test isolates to dual plate assays with *Rhizoctonia* spp. and selected 20 isolates with the greatest antifungal activity to then be screened for seedling growth promotion. Only 17 isolates progressed to *in vitro* suppression of Rhizoctonia diseases, and because each isolate possessed both growth promotion and anti-fungal characteristics, it was difficult to determine which mechanism was the more important for biological control. The present selection of isolates for *in vivo* screening based on potential plant growth promotion, antibiotic/inhibitory compound production, physical competition with *Rhizoctonia* spp. or endophytism, with some overlap between these categories, allows for future analysis of which of these factors, or combination of factors, is the most reliable indicator of an isolate's capacity to reduce the severity of Rhizoctonia diseases of potato.

In the present study, the dual plate suppression score results for the bacterial isolates showed that the two *R. solani* isolates were suppressed to different degrees by the same isolates, with Rs043-2 more strongly suppressed than R73-13b. It would therefore be prudent to test any potential biocontrol isolate against a variety of potato pathogenic *R. solani* isolates *in vivo* to ensure that their range of control is broad enough to make commercial development economically worthwhile. The fact that fluorescent *Pseudomonas* isolates were, overall, less suppressive to R73-13b than were the endospore forming bacteria, while there was no difference between the two groups on Rs043-2 (Figure 2.3 a), suggests that some taxonomic groups may suppress a range of *R. solani* isolates more consistently than others.

There were no significant differences between the mean suppression scores for endospore forming bacteria across the four soil locations (Figure 2.3 b). This suggests that soil physical parameters, cropping history or geographical location have little effect on the overall suppressive capacity of these populations. The fluorescent Pseudomonads isolated from the Pukekohe soil were more suppressive to the *R. solani* isolates than those from the other three soil locations or the endospore forming bacteria from Pukekohe. This suggests that there is something about the Pukekohe soil which supports a fluorescent Pseudomonad community with higher suppressive activity towards *R. solani*, potentially related to the physical nature of the soil. However, Jager and Velvis (1983) found clay loam soil to be no more suppressive to *R. solani* than loamy sand, sandy loam or clay soils, and less suppressive than slightly acid sandy soil, so physical soil characteristics may be less important than biological ones. Also the pH was probably not a dominant factor influencing the proportion of suppressive

fluorescent Pseudomonads from the Pukekohe soil, as the pH of that soil was similar to the Lincoln soil. The Pukekohe soil sampled had a cropping history atypical in agriculture, six consecutive potato crops. The higher proportion of suppressive fluorescent Pseudomonads from this site could be related to the continuous potato cropping sequence. This difference may result from a more complex interplay between crop history and soil type. A review by Garbeva *et al*. (2004) noted that such complex interactions may influence plant-pathogen antagonist populations. Rhizoctonia disease decline as a result of monoculture (and subsequent build-up of pathogen antagonists) has been reported previously for sugarbeet, and the mechanism was ultimately linked to increases in the relative abundance of Pseudomonadaceae antagonistic to *R. solani*, as well as other bacterial taxa, including Burkholderiaceae, Xanthomonadales and Actinobacteria (Mendes *et al.*, 2011). A similar process could be happening at the Pukekohe research site, although comparisons between the Pseudomonads and other bacterial populations between continual potato and non-potato fields of the same soil type would need to be conducted to determine if this is the case.

There were fluctuations in the (*in vitro*) mean suppressive capacity of selected microbial populations associated with below-ground plant tissues over time. Overall, populations of fluorescent Pseudomonads tended to be more suppressive to the *R. solani* isolates tested over time, while populations of endospore forming bacteria showed the reverse trend (Figure 2.4 b). Also there were differences in the suppressive capacity of populations isolated from different plant tissues at some time points, and for populations of isolates from the same plant tissues over time (Figure 2.4 a). These results suggest a dynamic relationship between potato plants and potentially protective soil microbial populations that could complicate biological control strategies, for to be successful this may have to incorporate multiple antagonists which complement plant age. The pattern of these fluctuations of suppression score with time were also different between some of the four soil locations (Figure 2.6 a). Plants grown in the Pukekohe soil developed and maintained a more suppressive population than plants grown in the other three soils, although whether this is related to soil type, soil history or a combination of both would, as previously mentioned, require further elucidation. What is clear from Figure 2.6 b is that it was the isolates from surface-sterilised tissues, which presumably have a close interaction with host plants, that made populations from the Pukekohe soils more suppressive on average than those from the other soil locations.

The data presented in Figure 2.5 a indicate that organisms recovered from surface-sterilised stolons (which are presumably endophytic or at least live in very close association with the stolons) were more suppressive than those from non-sterilised stolons, but for roots and stems

there was no difference between the two groups. This is of interest, as stolons are the target of the canker phase of Rhizoctonia disease of potato (Frank, 1981). Furthermore, the fact that isolates living in close association with stolons demonstrate greater suppression of *R. solani* indicates that potato plants may recruit microbial partners antagonistic to pathogens of the specific organs at risk of infection. To support this hypothesis correlation between the *in vitro* suppressive capacity of the isolates and *in vivo* disease suppression would have to be demonstrated.

Overall, the fluorescent Pseudomonad isolates from surface-sterilised tissues were more suppressive than those from non-sterile tissues and both endospore forming bacterial isolate groups (Figure 2.5 b). If potato plants do form close partnerships with microorganisms to enhance protection against *R. solani* infection, these results indicate that the partnerships they form with fluorescent Pseudomonads offer greater potential of suppression of *R. solani* than endospore forming bacteria.

Caution is advisable when considering the implications of the suppression score results with regards to biological control, for two reasons. First, the categorical scale used to create the suppression scores may not reflect the true *in vivo* suppression of *R. solani*, as the *in vitro* suppressive capacity of an isolate may not be expressed at the same level in a more complex soil-plant environment. Second, the actual range of levels of Rhizoctonia disease expression on plants grown in the soil samples from the four locations was not investigated, and so the mean suppression scores for the soils cannot be linked to actual disease levels, leaving the conclusions regarding comparisons between isolate groups as speculative. However, even with these caveats, the differences between mean suppression scores of isolate groups are still useful in forming hypotheses regarding how the origin of each test isolate may inform as to its biological control potential.

2.6 Conclusions

Isolation and categorisation of members of *Bacillus*, *Pseudomonas* and *Trichoderma* genera from soil samples and below ground tissues from plants, from the three main cropping regions of New Zealand, has enabled selection of a range of isolates to progress on to *in vivo* screens for suppression of Rhizoctonia diseases of potato, as well as comparisons between *in vitro* suppression of *R. solani* of groups of isolates from different isolation locations. This study has indicated that differences exist between the *in vitro* antagonism towards *R. solani* by endospore forming bacteria and fluorescent Pseudomonads from some soil types and potato

plant tissues, but not all, along with some differences in the *in vitro* antagonism of bacteria isolated from different plant growing stages and surface sterilisation procedures.

2.7 Acknowledgement

Extractions and sequence analyses of bacterial isolates was conducted by Emily Hicks, Bio-Protection Research Centre.

Chapter 3
Optimisation of a greenhouse bioassay for Rhizoctonia diseases of potato

3.1 Abstract

A greenhouse bioassay was developed to enable screening of potential biological control agents of Rhizoctonia diseases of potato. Different experimental conditions for the bioassay were optimised, including plant propagule types, pathogen isolates, inoculum culture media and rates of inoculation. Two isolates of *R. solani* pathogenic to potato were cultured on two types of media. The disease expression of each pathogen/inoculum combination was analysed at five potting mix inoculation rates, on plants grown either from minitubers or tissue-cultured plantlets. Differences between pathogen isolates over most disease and plant physical parameters were found. The inoculum medium type often made a difference to disease parameters in plants grown from minitubers, but rarely affected disease expression in plants from tissue culture. Polynomial trends with inoculation rate were significant for many parameters and allowed comparison of patterns of disease expression between pathogen isolates and plant propagule types. Based on the results, a set of experimental conditions which satisfied the requirements for a routine greenhouse screening bioassay were selected. Pathogen isolate Rs043-2 (Anastamosis Group 2-1) cultured on barley + V8® juice medium and an inoculation rate of 0.1 or 0.3% (w/w) was selected as the most practically appropriate inoculum option for future disease suppression studies, based on severity of disease and impact on plant physical parameters. Other conditions for the assay included 0.9 L plant pots with a peat:pumice based plant growth medium, and a growth period of 7 weeks to allow sufficient time for expression of canker symptoms on stolons as well as early tuber formation to allow assessment of impact on yield-related parameters.

3.2 Introduction

3.2.1 Requirement of bioassay

For a disease bioassay to be of practical use for reasonably high-throughput screening, the assay needs to satisfy some general requirements. The assay must make efficient use of space where this is limited, allowing high numbers of treatments with appropriate experimental replication to be tested, while still providing plants with adequate growth conditions. Plant growing time must allow sufficient expression of the disease for analysis, balanced with the

need to run multiple assays in short time periods. To allow comparisons to be made between assays, environmental conditions must be within specified parameters, with little deviation. The purpose of experiments described in this chapter was to select experimental conditions which produced disease levels most appropriate to a high-throughput bioassay system which could be applied to evaluate potential biocontrol agents for control of Rhizoctonia diseases of potato. The experiments also aimed to verify that details of the experimental set-up, including pot size, plant growing medium, experimental run time, greenhouse conditions and disease assessment method, were suitable for the requirements of a bioassay.

3.2.2 Greenhouse bioassays for Rhizoctonia diseases of potato in the literature

Several greenhouse bioassay methods for assessing the severity of Rhizoctonia diseases of potato have been previously described (Table 3.1). These have differed considerably in the methods of inoculum production, rates of inoculation, plant growing substrates, container sizes, experiment durations and disease assessment methods. The scale on which these bioassays have been conducted is much smaller than that which is required for screening, so most used much longer experimental run-times and more labour intensive scoring methods (for example, assessing the percent lesion coverage of every stolon and assigning them into severity categories) than are practical for screening. Assessment of these methods indicates that, in general, *R. solani* isolates pathogenic to potato can produce levels of disease suitable for comparisons under a wide range of experimental conditions, and at varied initial inoculum rates. Because the majority of the studies used one *R. solani* isolate, and there are likely to be differences between isolates in their requirements for expression of pathogenicity, it is not possible to determine if the experimental conditions used in those studies would be suitable for other isolates, such as those available for the present study, without testing them first. An appropriate existing experimental set-up cannot, therefore, be directly duplicated from the literature, and it was necessary to test a range of experimental conditions, with the isolates available, to determine which are the most suitable for the purposes of a greenhouse screening bioassay.

Table 3.1 Details of greenhouse bioassays from the published literature, used to assess Rhizoctonia diseases of potato

R. solani culture medium and conditions	Plant growing substrate	Container size	Pathogen inoculum rate	Plant propagule type	Harvest time(s) after planting	Disease scoring method(s)	Publication
Wheat grain + water, 8 days at 21-25°C, dried	Soil:sand (3:1, w/w)	Pot holding 1.2 kg mix	0.4% w/w	Seed pieces, three per pot	3 to 4 weeks	Stem canker (0-5 scale), black scurf on seed pieces (0-5 scale)	Brewer and Larkin (2005)
Quinoa seed + sand + water, 3 weeks at 22°C, mixed 1:1 with sand	Sand	2.5 L pot	1.4 to 17.8 g per pot	Minituber, one per pot	Multiple time points	Stem canker severity assigned to 6 classes	Wilson *et al.* (2008a)
Barley + water, 21 days at ambient	Sterile potting mix	1 L pot	10 mL layer at bottom of pot	Cultured stem cutting, one per pot	11 weeks	Presence/absence of lesions, malformations and sclerotia on tubers	Campion *et al.* (2003)
Potato dextrose broth, 10 days at 25°C	Soil	Pot holding 12 kg	50 mL of a 10-fold diluted PDB culture	Seed potato, one per pot	110 days	Yield and number of sclerotia on tuber	Tariq *et al.* (2010)
Malt-peptone agar, 4 weeks	Soil	15 L buckets	3 sclerotia, placed near potato bud	Seed potato, one per bucket	4 weeks after haulm destruction	Tubers rated for sclerotial density	Boogert and Jager (1984)
Malt extract peptone drenched perlite, 4 weeks at 21°C	Soil + 1% (w/w) potato stem/stolon pieces	1.24 L pots with 250 g dry soil equivalent	125 sclerotia per pot	Minituber, one per pot	-	Sclerotial formation on minituber assessed	Boogert and Luttikholt (2004)
Wheat grain + water, 8 days at 21-25°C, dried	Soil:sand (3:1, w/w)	1.6 L trays	1.2% w/w	Four seed pieces per tray	4 weeks	Root and stem canker (0-5 scale) and shoot height	Larkin and Griffin (2007)

R. solani culture medium and conditions	Plant growing substrate	Container size	Pathogen inoculum rate	Plant propagule type	Harvest time(s) after planting	Disease scoring method(s)	Publication
PDA, 5 day old	Heat treated sand:soil 2:1	235 mL tubes	Five mycelial agar plus per tube	One cut potato seed piece per tube	23 days	Lesion damage on shoots and roots (0-4 scale)	Carling and Leiner (1990)
Oats+water, 3 weeks at ambient, dried	Soil:peat-based grow mix:ceramic soil conditioner (3:1:1, v/v/v)	18 cm diam. pots	Five oat grains per plant	Plant from tissue culture	Multiple time points	Crown/shoot rot (0-5 scale), tuber, root and shoot fresh weights	Yao *et al.* (2002)
Barley kernels, 3 weeks at 20°C	Sand:soil (1:1, v/v)	2.88 L pot	Six kernels per pot	Seed potato	3 weeks	Stem canker severity (1-5 scale)	Grosh *et al.* (2005) and (2006)
Quinoa seed + sand + water, 14 days at ambient	Washed sand	10 L pot	10 g in layer 1 cm above tuber	Minituber, one per pot	48 and 120 days	Lesion coverage on organs (6 categories). Black scurf severity (0-4 scale)	Lehtonen *et al.* (2009)
Barley:wheat 2:1 + water, 18 days at 25°C	Sterilised clay:sand:farm-yard manure 1:1:1	2.88 L pot	10/15/12 g mixed into top 5 cm per pot	Seed potato, one per pot	30 and 90 days	Eye germination and sprout killing. Black scurf severity (0-5 scale) and yield	Farah *et al.* (2008)
Wheat grain + water, 10-14 days at 27°C, dried	Fumigated peat:vermiculite: perlite 5:3:2	600 L containers	275 grains/m2	Minitubers or seed potatoes with slight sclerotial load, 15 per container	Maturity	Black scurf (4 categories), disease incidence and severity (0-5 scale) on stem/stolons, sclerotial density on roots (0-3 scale)	Tsror and Peretz-Alon (2005)
Rye, 30 days	Sterilised commercial potting mix	Pot size not detailed	5% w/w	Seed potato, one per pot	10 weeks and 3 months	Stem lesion (1-5 scale), yield	Lahlali and Hijri (2010)

3.2.3 Experimental conditions

A pot size (0.9 L) was selected to balance maximising replicate numbers in a greenhouse cell with providing adequate plant growth over the time-course of the experiment. A seed raising mix (Appendix A.3.1), rather than a soil-based plant growth medium, was selected as the plant growth medium to avoid interference from naturally occurring *Rhizoctonia* populations. Furthermore a peat-based plant growth medium was selected over others based on different organic components as peat usually has either no effect, or is conducive to Rhizoctonia diseases (Bonanomi *et al.*, 2007).

For the initial experimental conditions examined, the inoculum culture media represent greater (barley + V8® juice) and lower (bran + water) sources of nutrients. The two *R. solani* isolates used represented different Anastomisis Groups (AGs) and both isolates were known to be pathogenic to potato. The five inoculation rates covered a 100-fold difference in inoculum concentration. Of the potato host propagule types commonly used for screens, tissue-cultured plantlets and minitubers were selected, as their production ensures that they are disease-free (Pruski, 2007; Struik, 2007), and their small size compared with seed potatoes makes them more appropriate for an assay where space is limited.

Both host propagule types have benefits and drawbacks: Plants grown from tissue-cultured plants do not have the 'emerging shoot' stage (that is susceptible to Rhizoctonia disease) while minitubers do, potentially altering the other symptoms of disease expression. However, tissue-cultured plantlets are available from commercial producers throughout the year, while minitubers are produced annually in advance of spring planting in the field. The choice of cultivars for both propagule types was restricted at the time of experiment initiation, so the tissue-cultured plantlets were cv. 'Gladiator', and the minitubers were cv. 'Desiree' (Alex McDonald Merchants, Lincoln). While the cultivars were different, which may impact comparisons drawn between them, both are susceptible to Rhizoctonia diseases ('Gladiator' known to be susceptible from pilot studies, and 'Desiree' from the literature, e.g. Farah *et al.*, (2008)).

The method for disease assessment needed to be suitable for processing samples in large numbers, as a requirement for the assay was that all plants had to be harvested and scored within a short timeframe. For this reason, disease incidence and presence or absence of lesions on shoots/stolons, rather than severity (e.g. percent total area covered in lesions) was used to assess disease levels. Plant parameters were also determined at harvest (tuber fresh weight and number, stolon number, plant dry weights) as adjuncts to disease impact assessments.

3.3 Methods

3.3.1 Pathogen isolates and production of inoculum

The *R. solani* isolates selected for this experiment were Rs043-2 and R73-13b, which at the time of the experiments were thought to belong to AG 3. Genetic analysis not conducted as part of this study demonstrated that Rs043-2, obtained from a surface-sterilised tuber-borne black scurf sclerotium, is an AG 2-1 isolate (provided by Dr Farhat Shah, Plant and Food Research Ltd), and R73-13b, isolated from New Zealand field soils, is an AG 3 (held in the Bio-Protection Research Centre Culture Collection, Lincoln University (Sneh *et al.*, 2004)). Both isolates were maintained on barley grain stored at 4°C.

Isolates were cultured on potato dextrose agar (Difco, Sparks, USA, Appendix A.1.1) for 5 to 7 days at 25°C in darkness. Erlenmeyer flasks containing either barley grain + V8 juice® (Campbell's Soups Australia, Australia) (1:1 w/v, Appendix A.2.1) or wheat bran + RO water (1:2 w/v, Appendix A.2.2) were each inoculated with five 7 mm diameter agar plugs from the growing edge of a *R. solani* colony. The flasks were then plugged with cotton wool, capped with tinfoil and incubated at 25°C in darkness for 13 days. Uninoculated media were incubated in the same way, for use as experimental controls.

3.3.2 Experimental Design

Two experiments investigated effects of different inoculum rates, inoculum media and *R. solani* isolates on disease incidence on potato, the first using tissue-cultured plantlets ('Gladiator') and the second using minitubers ('Desiree'). Inoculum was added to the potting mix (Appendix A.3.1) at the following five rates (treatments); 0.03, 0.1, 0.3, 1 and 3% w/w, for both isolates and inoculum media. Three experimental controls were used in each experiment: no inoculum, uninoculated bran + water or uninoculated barley + V8®, both at 3% w/w. The required potting mix and inoculum were weighed and thoroughly mixed by hand then divided between ten 0.9 L capacity plastic pots with saucer. This procedure was used for all treatments, except 0.03% barley + V8®. For this, the required weight of inoculum was distributed evenly amongst the pots, with barley kernels placed at mid-depth in each pot.

Tissue-cultured plantlets (Figure 3.1 a) were planted singly into pots. Minitubers (Figure 3.1 b) were pre-sprouted (25:15°C, 16:8 h light:dark, lighting: two 30W/860 Lumilux® Daylight lamps - Osram, Germany) for 3 days then planted one per pot. Each experiment was laid out in a randomised block design with ten replicates, with two pots of each of the three experimental controls per replicate. The experiments were conducted in a greenhouse unit (Figure 3.1 c), with temperature control, and supplementary lighting (16:8 h light:dark, 7 h

total supplement from ten SON-T AGRO 400 lamps - Philips, Belgium). Temperature and relative humidity were recorded with a HOBO® Pro v2 logger enclosed in a solar radiation shield (both: Onset Computer Corporation, MA, USA) suspended at pot height.

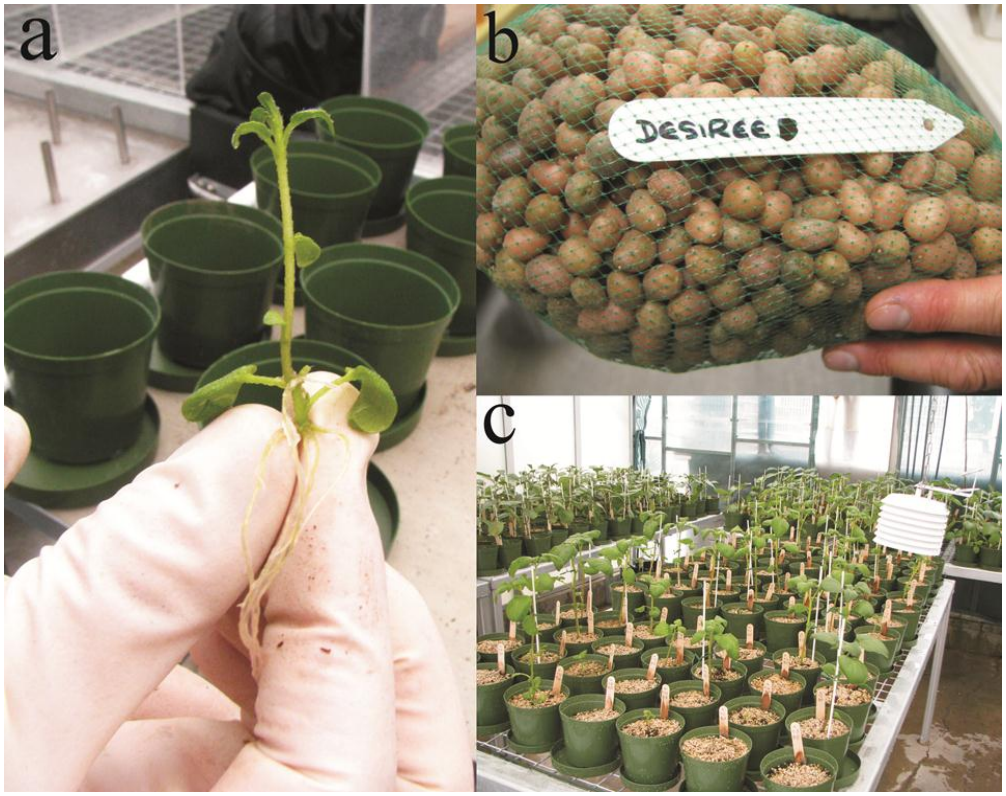

Figure 3.1 Photographs of (a) a tissue-cultured plantlet, (b) minitubers and (c) greenhouse assays used to optimise pathogen inoculum medium and initial concentration.

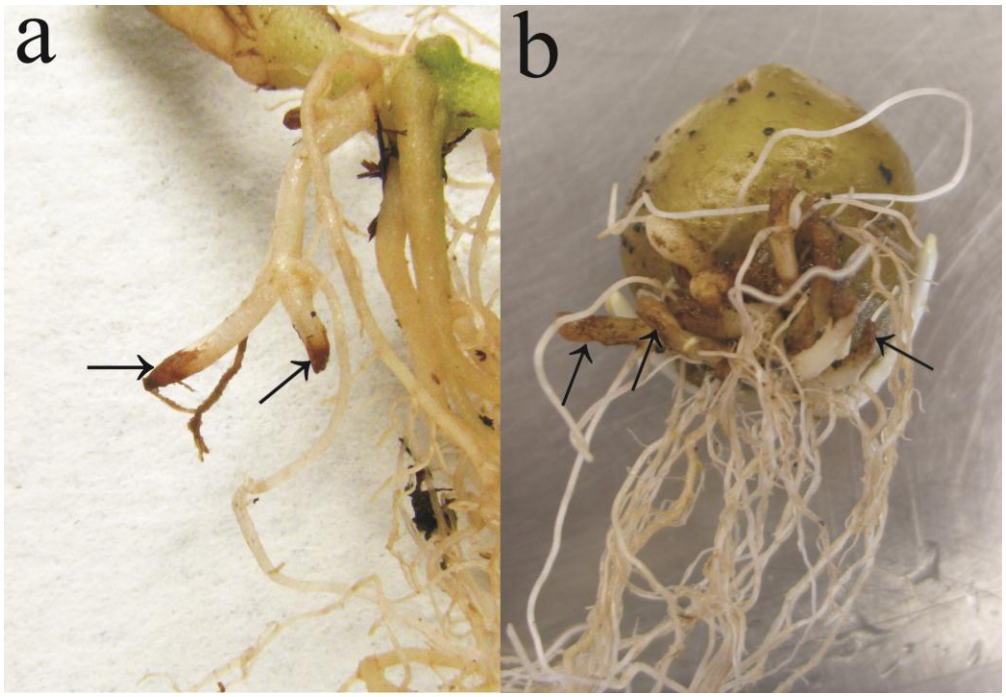

Figure 3.2 Black arrows indicate examples of Rhizoctonia cankers 'nipping' (a) a stolon (below-ground stem) and (b) emerging shoots of potato plants.

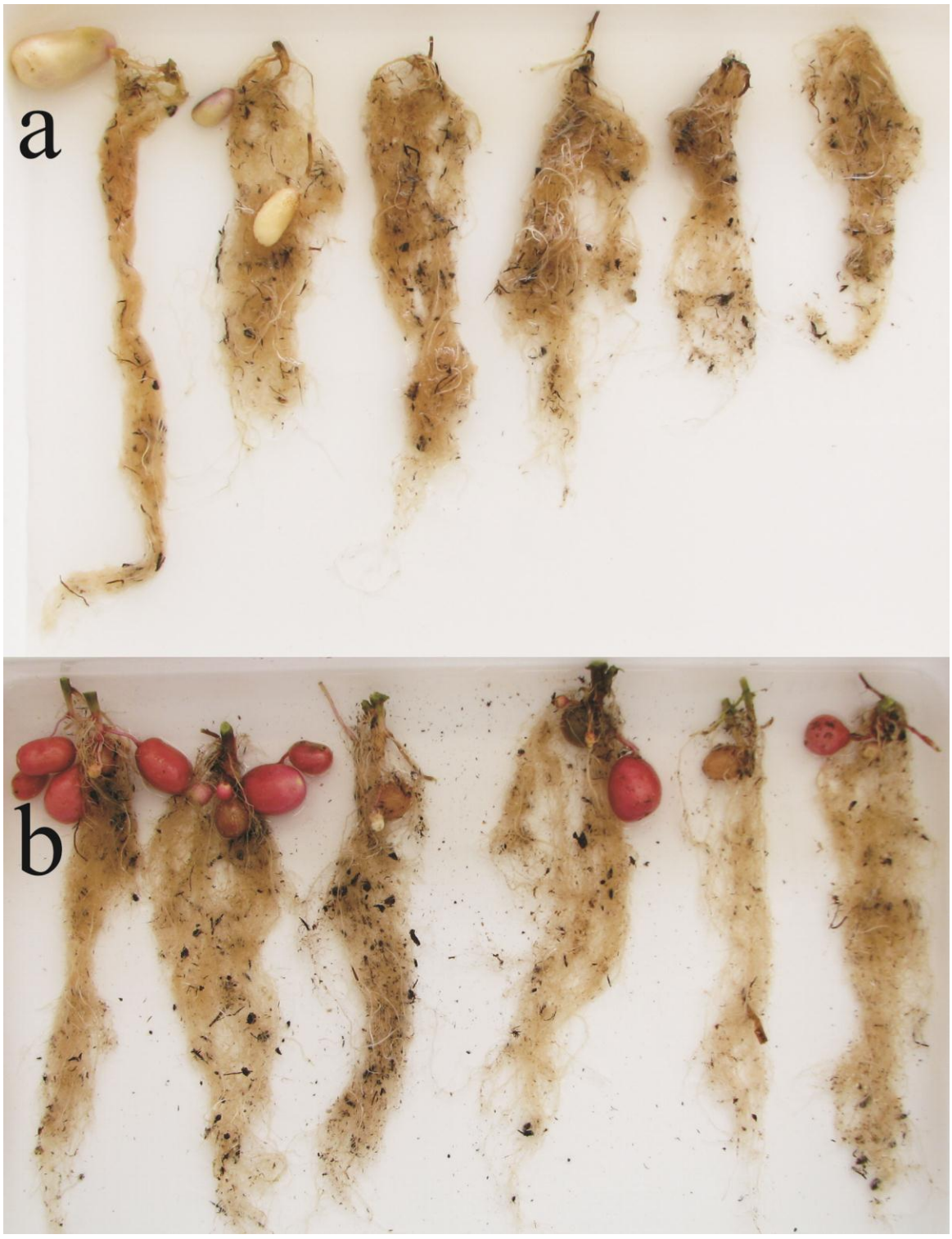

Figure 3.3 Examples of below-ground portion of potato plants, grown from (a) tissue-cultured plantlet or (b) minituber and harvested after 7 weeks, from assay used to optimise pathogen inoculum medium and initial concentration.

3.3.3 Disease assessments and plant parameters

After 7 weeks, all plants were washed free of plant growth medium (Figure 3.3) and the total number of stolons and the number of stolons with visible lesions were recorded for each plant

(Figure 3.2 a). For the minituber experiment, the number of emerged shoots and number and proportion of non-emerged shoots with rotted or dead apices ('nipped', Figure 3.2 b) were also determined for each plant. The number and fresh weight of tubers, and root and shoot dry weights, were also recorded for each plant.

3.3.4 Statistical analyses

All data were subjected to analysis of variance for randomised block designs with treatment structures of 2 × 2 × 5 factorial + three controls. The factors were isolates, inoculum media and $\log_{10}$-transformed initial inoculum concentrations, for which linear and quadratic polynomial components were included in the analyses. The statistical significance of these polynomial components was used to provide an appropriate order of approximating polynomial to fit to each data set for graphing trends. Only effects and polynomial components significant at $P \leq 0.05$ are presented.

3.4 Results

3.4.1 Temperature and relative humidity

During both experiments, the mean temperature was 17.6°C (maximum 23.2°C, minimum 15.4°C) and the mean relative humidity was 70.5% (maximum 93.9%, minimum 41.7%).

3.4.2 Proportion of diseased stolons

In the tissue-cultured plantlet experiment, the mean proportions of diseased stolons per plant (Figure 3.4 a) were affected by isolate and inoculum rate but not inoculum medium. For isolate Rs043-2, both linear and quadratic components were significant. Isolate R73-13b produced no significant trend across the inoculum rates.

In the minituber experiment, the mean proportions of diseased stolons (Figure 3.4 b) were affected by isolate, inoculum rate, and inoculum medium. Isolate Rs043-2 cultured on bran + water produced a significant linear trend with inoculum rate, but no significant trend occurred from barley + V8® inoculum. No significant trends with inoculum rate were produced by isolate R73-13b cultured on either media.

For isolate R73-13b, the proportion of diseased stolons was unaffected by different inoculum rates, while isolate Rs043-2 at higher rates produced proportionally fewer diseased stolons. This pattern was reasonably consistent between the two host propagule types, although for minitubers the Rs043-2, barley + V8®, 0.10% inoculum rate data point ran contrary to this pattern.

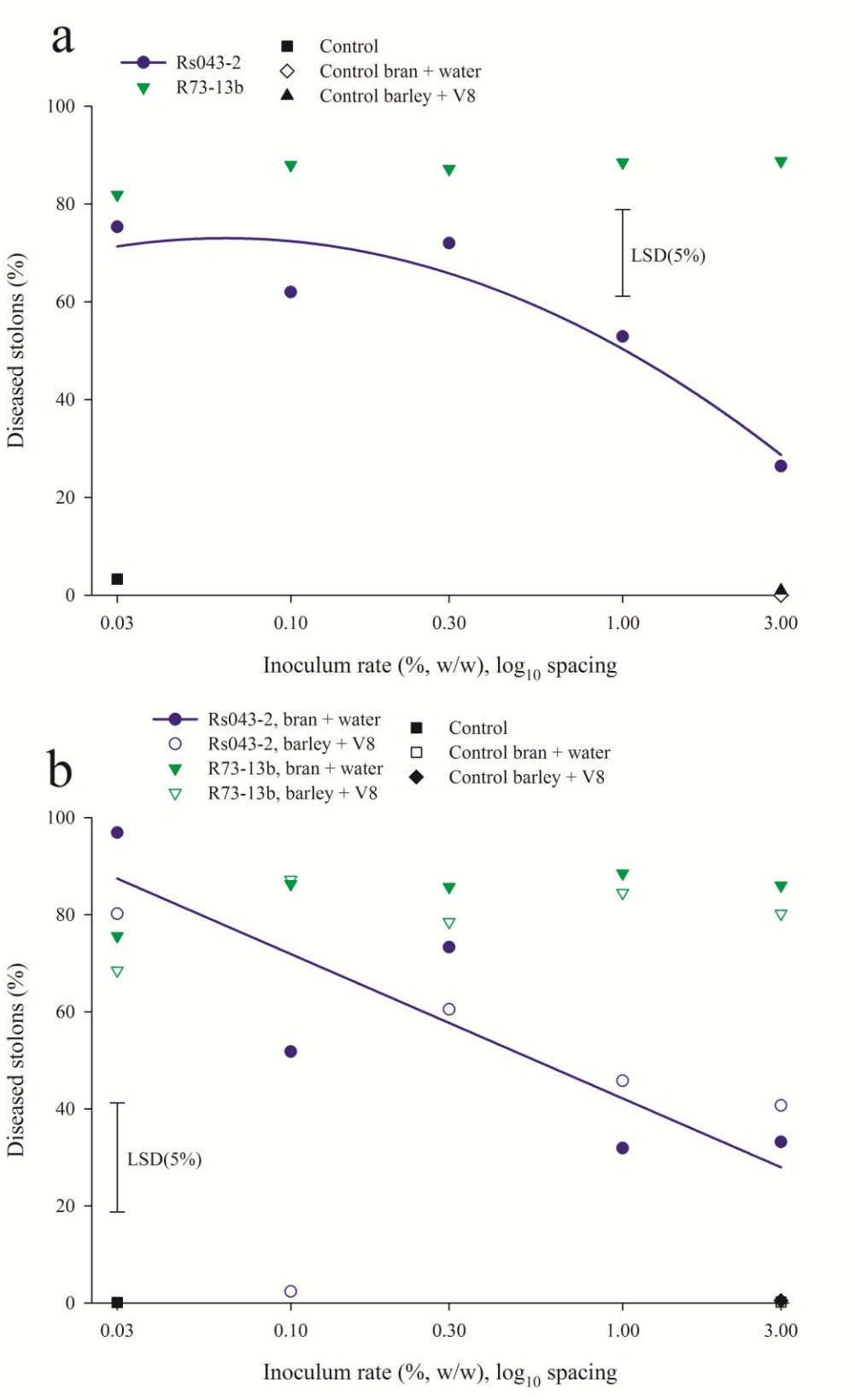

Figure 3.4 Mean percentage of diseased stolons on potato plants grown from (a) tissue-cultured plantlets (averaged over two inoculum media) and (b) minitubers. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.4.3 Number of symptomless stolons

In both experiments, the data of number of stolons without cankers (symptomless) per plant were square root transformed to satisfy the ANOVA requirement of normal distribution.

In the tissue-cultured plant experiment the (transformed) mean number of symptomless stolons per plant (Figure 3.5 a) was affected by isolate but not by inoculum rate or type. The interaction between pathogen isolate and inoculum rate was significant, which justified separate analyses of pathogen isolates with inoculum rate. There was a significant linear trend across inoculum rates for isolate Rs043-2, but no significant trend for isolate R73-13b.

In the minituber experiment the (transformed) mean number of symptomless stolons (Figure 3.5 b) was affected by inoculum rate but not inoculum type or isolate. The interaction between pathogen isolate and inoculum rate was significant, which justified separate analyses of pathogen isolates with inoculum rate. Isolate Rs043-2 produced significant linear and quadratic trends with inoculum rate, while the linear trend of isolate R73-13b across inoculum rates was significant.

For the plants from the tissue-cultured plantlets, the pattern of disease expression was consistent between the data of proportion of diseased stolons and number of symptomless stolons. For the minituber experiment, the pattern of disease expression for number of symptomless stolons was slightly altered compared with the proportion of diseased stolons, with isolate R73-13b producing a significant but slight trend to fewer symptomless stolons at higher inoculum rates, and isolate Rs043-2 again producing more symptomless stolons at the highest inoculum rate, but without significant differences between means at the lower rates.

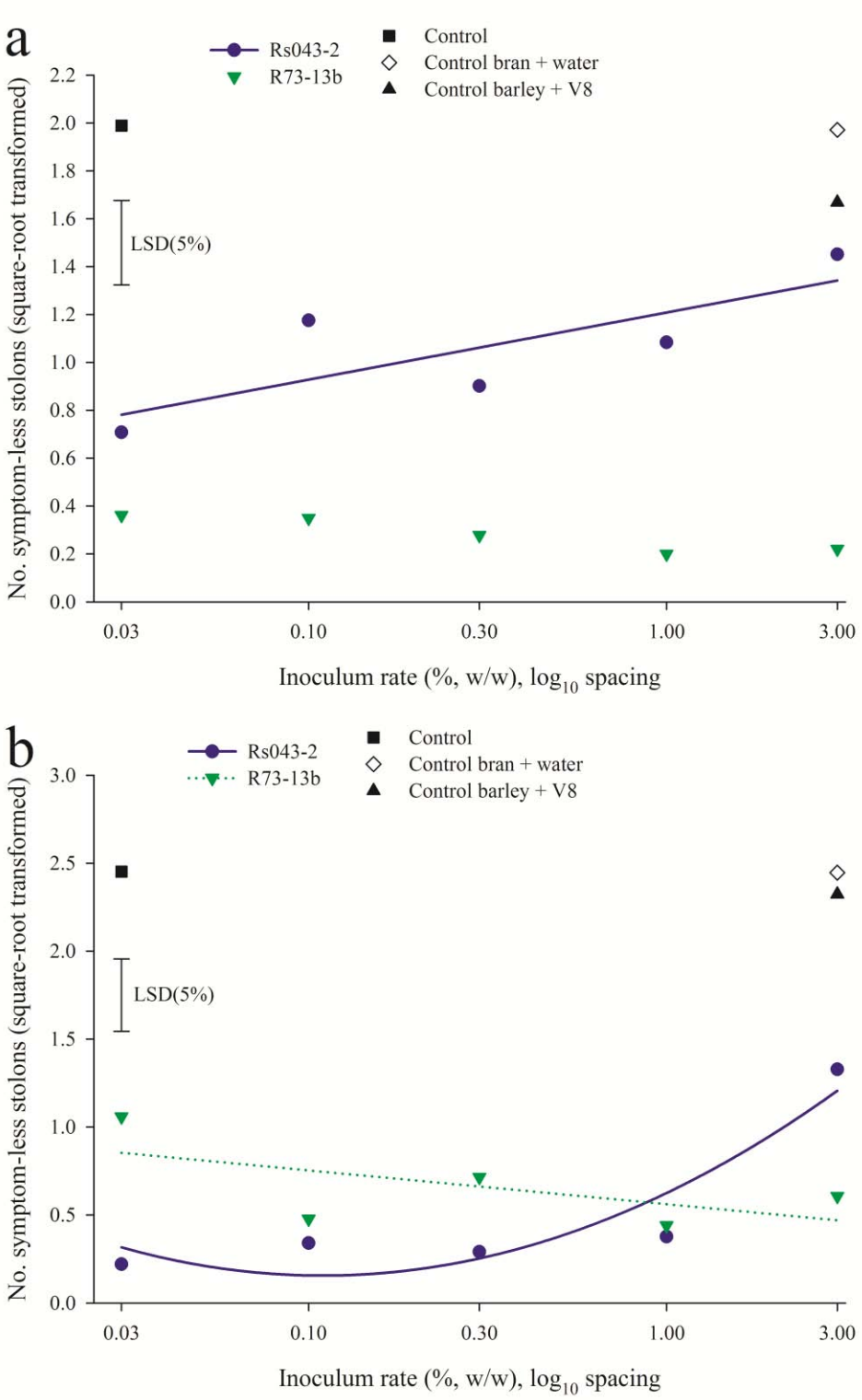

Figure 3.5 Mean number of symptomless stolons on potato plants grown from (a) tissue-cultured plantlets and (b) minitubers, both averaged over two inoculum media. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.4.4 Proportion of 'nipped' shoots and number of tubers

In both experiments, the data of numbers of progeny tubers was square root transformed to satisfy the ANOVA requirement of normal distribution.

In the tissue-cultured plant experiment, the mean number of tubers was affected by pathogen isolate but not by inoculum type or rate. No polynomial components of the interactions with rate were significant. The mean number of progeny tubers for isolate Rs043-2 was 1.99, and for isolate R73-13b was 1.32. The mean number of tubers for the uninoculated control was 3.02, for control bran + water was 3.01 and for control barley + V8® was 2.03, with no significant difference between these means.

In the minituber experiment, the mean number of tubers (Figure 3.6 b) was affected by pathogen and inoculum rate but not by inoculum type. For isolate Rs043-2, the quadratic trend across inoculum rates was significant, but the linear trend was not, while the converse was true for isolate R73-13b.

The proportion of shoots 'nipped' applies only to the minituber experiment. The mean percentage of shoots nipped (Figure 3.6 a) was affected by pathogen isolate, inoculum type and rate. The linear trend across inoculum rates was significant for isolate Rs043-2 cultured on both media, and the quadratic trend was also significant for barley + V8®. The only significant polynomial trend produced by isolate R73-13b was the quadratic trend for bran + water inoculum.

The pattern of disease expression for the mean number of nipped shoots is consistent with those seen in the proportion of diseased stolons (Figure 3.4). As with the percent diseased stolons, there was no significant trend connecting increasing concentrations of the isolate R73-13b barley + V8® inoculum and the mean percent nipped shoots, and although these data showed a significant n-shaped trend for bran + water inoculum, this did not reflect a large spread of treatment means. Mean percent nipped shoots from the isolate Rs043-2 treatments also followed the general pattern found in the percent diseased stolon data, with trends that linked greatest levels of inoculum to less disease expression. The main difference between the results for proportion of diseased stolons and nipped shoots was that isolate R73-13b generally caused consistently greater stolon disease than isolate Rs043-2, whereas the converse was true for shoot nipping.

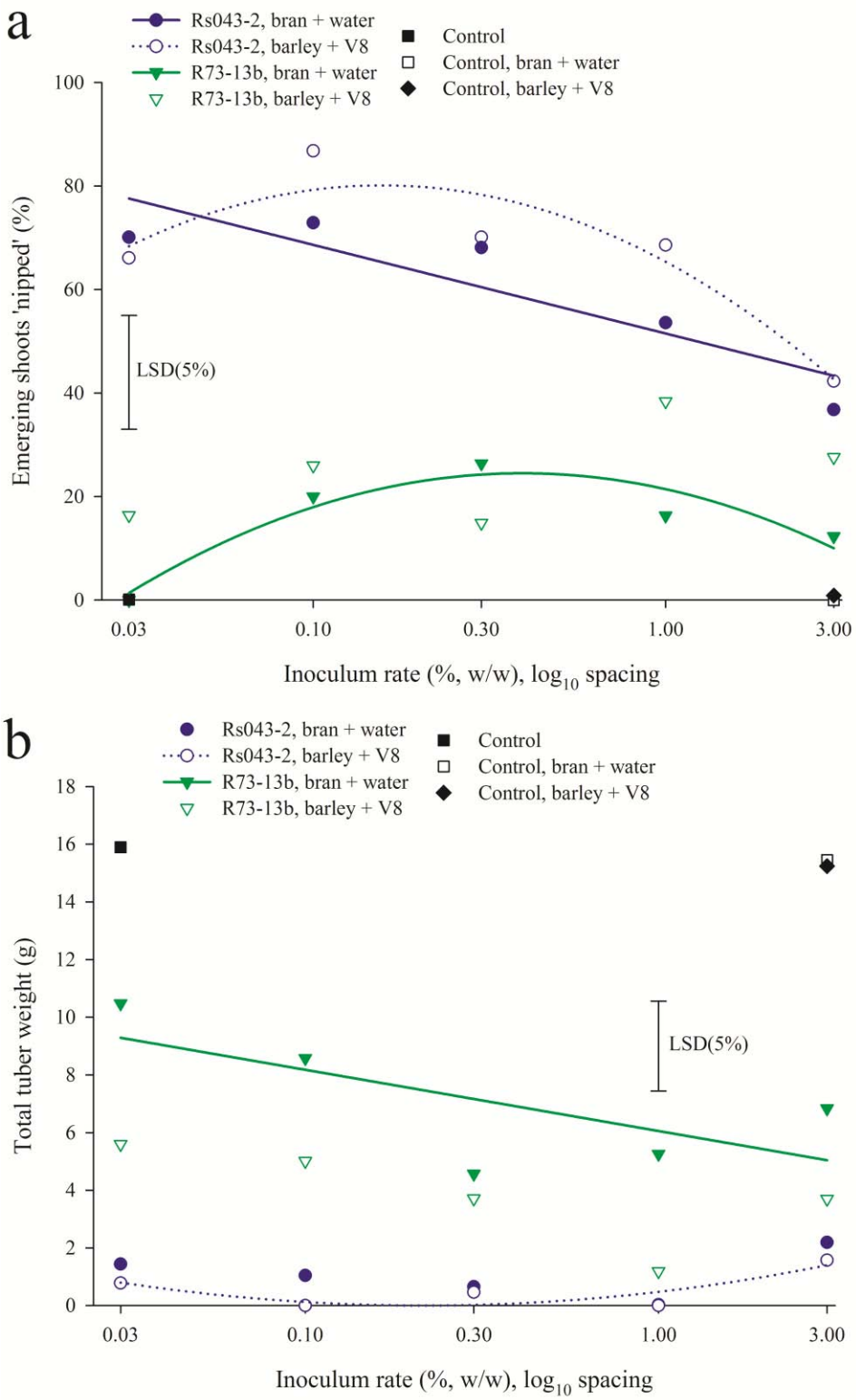

Figure 3.6 Mean (a) percentage of 'nipped' shoots and (b) number of daughter tubers (square root transformed and averaged over two inoculum media), on potato plants grown from minitubers. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.4.5 Number of stolons

In both experiments, the data of number of stolons per plant were square root transformed to satisfy the ANOVA requirement of normal distribution.

In the tissue-cultured plant experiment, the (transformed) mean number of stolons per plant (Figure 3.7 a) was affected by isolate and inoculum rate but not inoculum medium. Isolate Rs043-2 produced a significant quadratic but not linear trend across the inoculum rates, while isolate R73-13b produced a significant linear trend.

In the minituber experiment, the (transformed) mean number of stolons per plant (Figure 3.7 b) was affected by isolate, inoculum type and inoculum rate. Isolate Rs043-2 produced a significant linear trend across the inoculum rates from bran + water inoculum, but a significant quadratic trend (but not a significant linear trend) from barley + V8® inoculum. From bran + water inoculum, isolate R73-13b produced a significant quadratic, but not linear, trend across the inoculum rates. While from barley + V8® inoculum, only the linear trend across inoculum rates was significant.

On plants from tissue culture, the mean number of stolons from isolate Rs043-2 treatments was, with the exception of the lowest inoculum rate, considerably greater than from isolate R73-13b (Figure 3.7). However, for the same measure on plants from minitubers, Rs043-2 gave fewer stolons than R73-13b, with this difference reducing at the higher inoculum rates.

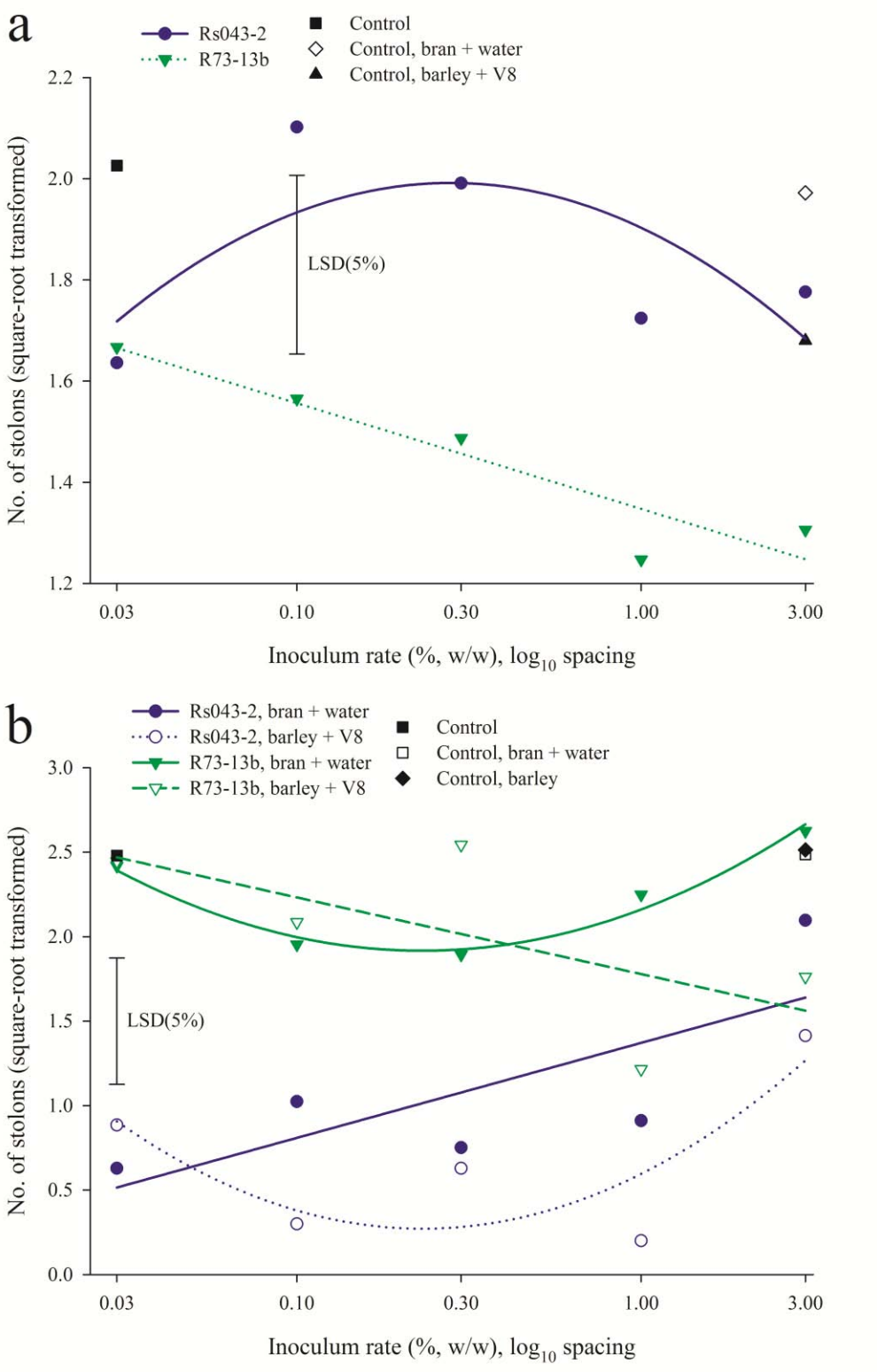

Figure 3.7 Mean number of stolons on potato plants grown from (a) tissue-cultured plantlets (averaged over two inoculum media) and (b) minitubers. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.4.6 Weight of daughter tubers

In the tissue-cultured plant experiment the tuber weight data were $\log_{10}$ transformed to satisfy the ANOVA requirement of normal distribution. This was not required for the minituber experiment data.

In the tissue-cultured plant experiment, there was no significant effect of pathogen isolate, inoculum type or inoculum rate on the (transformed) mean weight of daughter tubers per plant (Figure 3.8 a). The linear component of the interaction between isolate and inoculum rate was significant, however, which justified separate analyses of pathogen isolates with inoculum rates. Isolate Rs043-2 gave a significant linear trend across inoculum rates, while linear and quadratic trends were both significant for isolate R73-13b.

In the minituber experiment the mean weight of daughter tubers per plant (Figure 3.8 b) was affected by isolate, inoculum type and rate. There was no significant trend between isolate Rs043-2 from bran + water inoculum and inoculum rate, while the quadratic trend (but not linear) was significant from barley + V8® inoculum. For isolate R73-13b from bran + water inoculum, the linear trend across inoculum rates was significant, but there was no significant trend from barley + V8® inoculum.

For the mean weight of daughter tubers from plants from tissue culture, the trend for increasing isolate Rs043-2 inoculum rate was an increase in tuber weight, but with no significant difference between isolates Rs043-2 and R73-13b treatment means (except at the 0.03% inoculum rate). Again, as noted for the mean numbers of stolons (Figure 3.7), on plants from minitubers isolate Rs043-2 gave smaller daughter tuber weights than those from R73-13b treatments. This pattern was also evident in mean numbers of tubers in the tissue-cultured plantlet experiment, with plants from Rs043-2 treatments producing significantly more tubers than those from R73-13b treatments, but the converse was true for the minituber experiment (Figure 3.6).

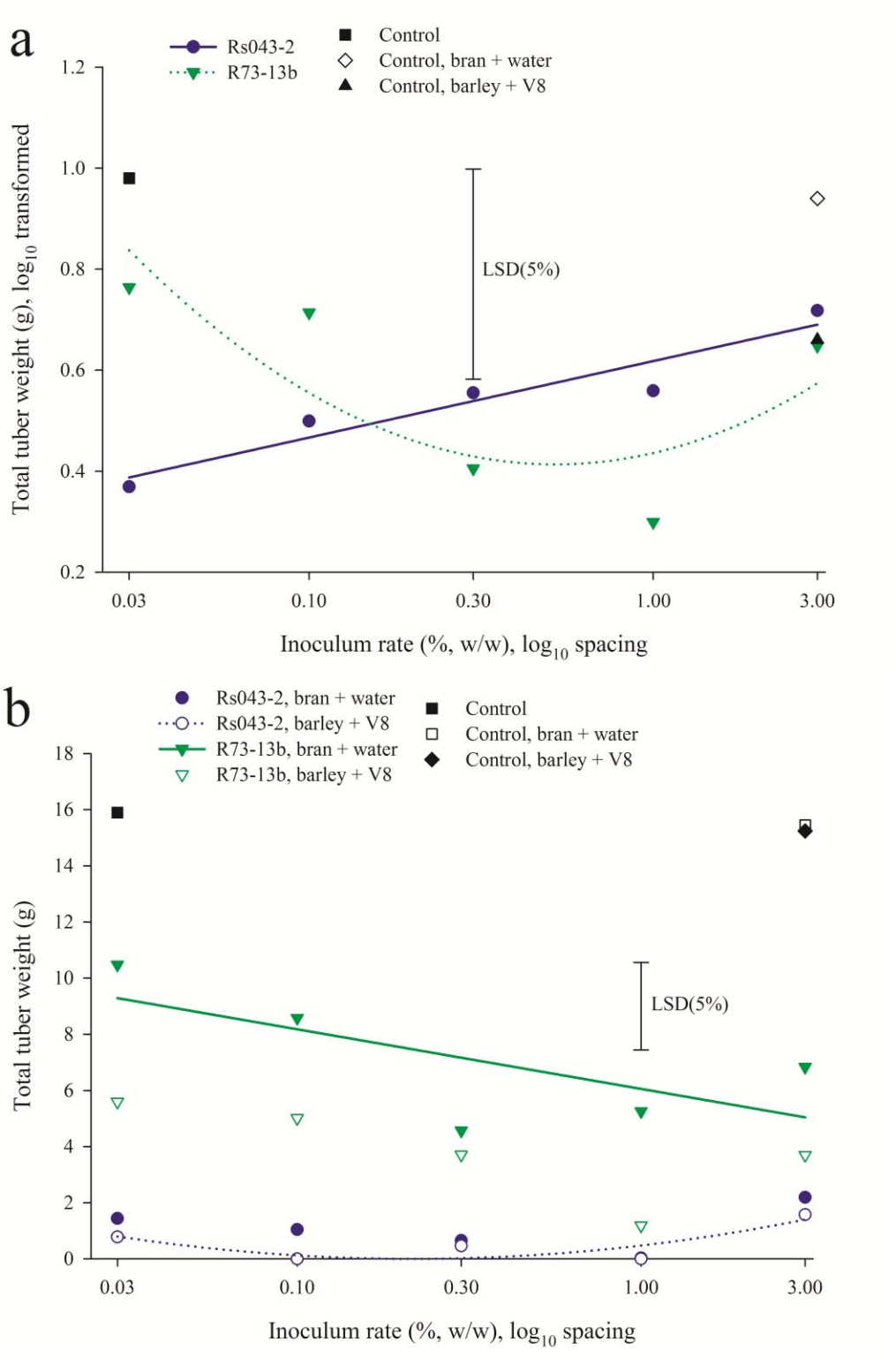

Figure 3.8 Mean total weight (g) of daughter tubers on potato plants grown from (a) tissue-cultured plantlets, weights $\log_{10}$ transformed (averaged over two inoculum media) and (b) minitubers. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.4.7 Plant dry weights

In the tissue-cultured plant experiment, the mean shoot dry weight per plant (Figure 3.9 a) was affected by pathogen isolate, inoculum type and rate. Isolate Rs043-2 from bran + water inoculum produced no significant trend across inoculum rates, but the linear trend from barley + V8® inoculum was significant. Isolate R73-13b from bran + water inoculum produced no significant trend, but the linear trend was significant from barley + V8® inoculum of this isolate.

In the minituber experiment, mean shoot dry weight (Figure 3.9 b) was affected by pathogen isolate, inoculum type and rate. Isolate Rs043-2 from bran + water inoculum produced a significant quadratic trend across the inoculum rates, but no significant linear trend, whereas both linear and quadratic trends were significant for Rs043-2 from barley + V8® inoculum. For isolate R73-13b from bran + water inoculum the quadratic trend across inoculum rates was significant, but the linear trend was not, while the converse was true for R73-13b from barley + V8® inoculum.

In the tissue-cultured plant experiment, the mean root dry weight per plant (Figure 3.10 a) was affected by pathogen isolate and inoculum type but not rate. However, the linear component of the interaction between inoculum type and rate was significant, justifying separate analyses of each pathogen/inoculum type combination with inoculum rates. Only the linear trend between isolate Rs043-2 from barley + V8® inoculum and inoculum rate was significant.

In the minituber experiment, the mean root dry weight per plant (Figure 3.10 b) was affected by pathogen isolate and inoculum rate but not by inoculum type. For both isolates Rs043-2 and R73-13b, the quadratic trend across inoculum rates was statistically significant, but the linear trend was not.

Shoot and root dry weight of plants from tissue culture were the only parameters for which the inoculum type made a significant difference, with consistency between the patterns of expression between the two isolates. For shoot dry weight, both isolates from bran + water inoculum gave no significant trends with increasing inoculum rate, while from barley + V8® inoculum the trend for both isolates was a decrease in dry weight with increasing initial inoculum concentration (Figure 3.9 a). For isolate Rs043-2, these trends in shoot dry weight held true for root dry weight, but neither trend was significant for isolate R73-13b although the barley + V8® trend was still negative (Figure 3.10 a). For the plants from tissue culture, Rs043-2 gave mean root dry weights which were often greater than those from R73-13b

treatments, especially from bran + water inoculum, but this pattern was reversed in the minituber experiment.

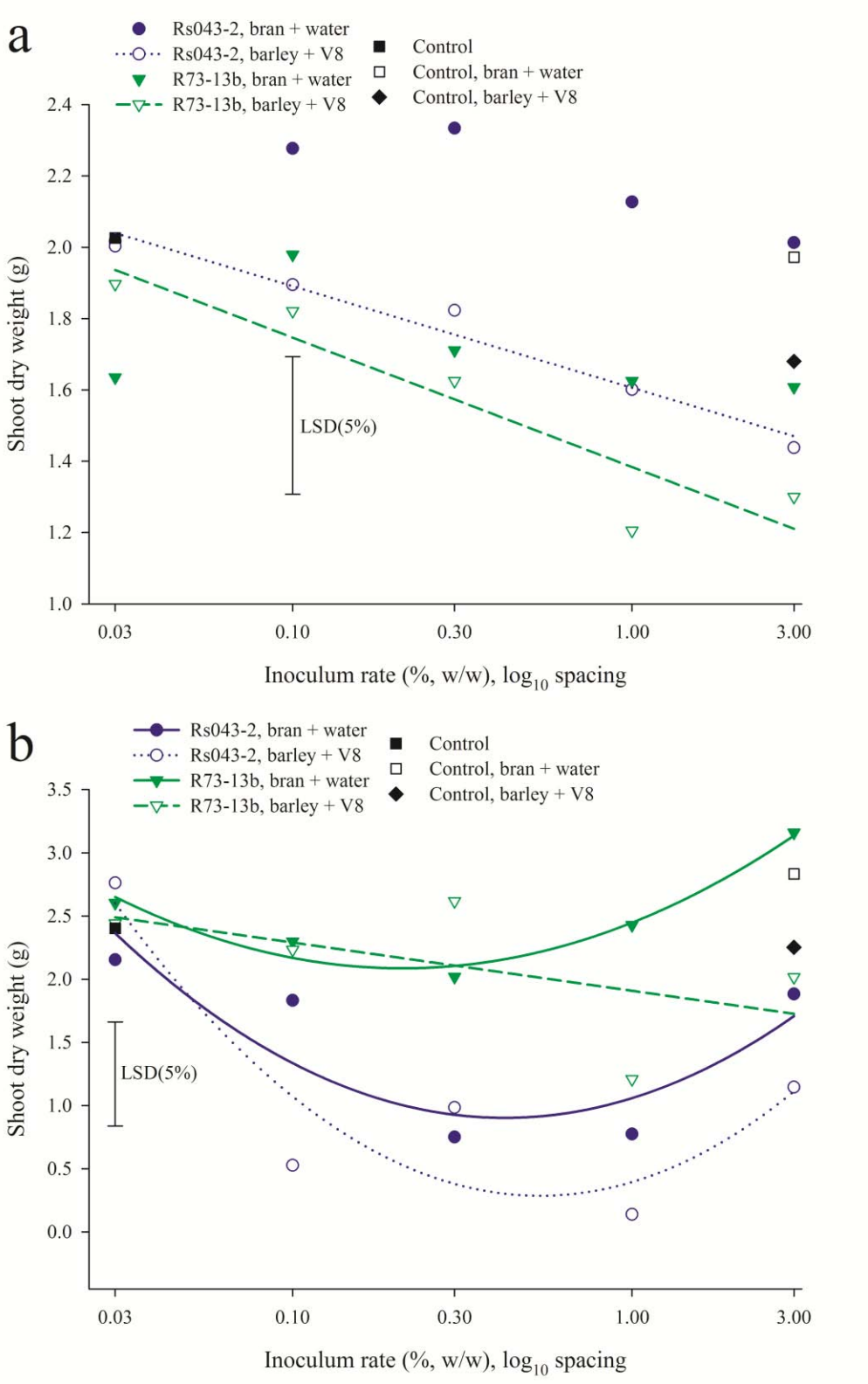

Figure 3.9 Mean dry weight (g) of potato plant shoots grown from (a) tissue-cultured plantlets and (b) minitubers. Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

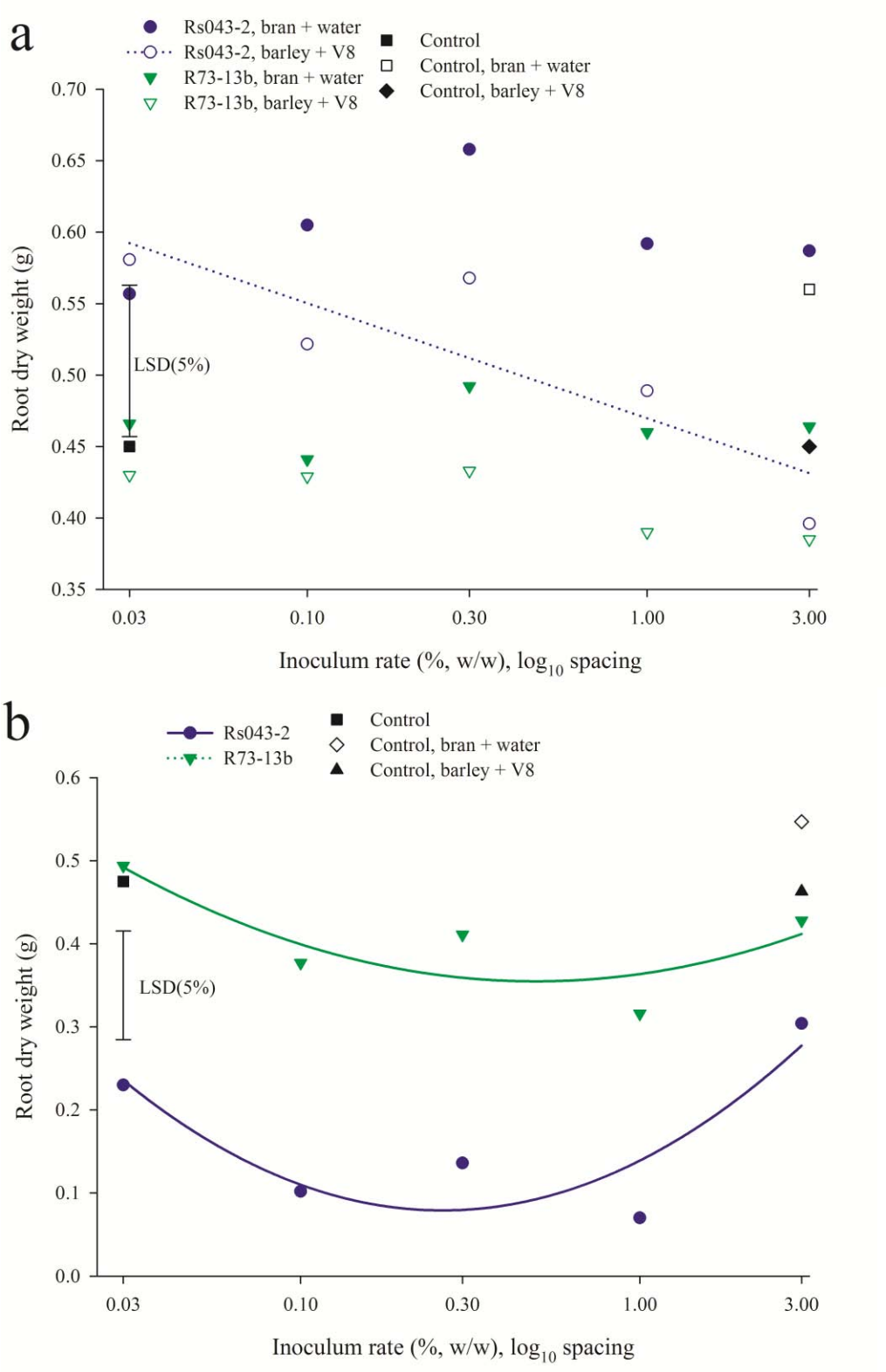

Figure 3.10 Mean dry weight (g) of potato plant roots grown from (a) tissue-cultured plantlets and (b) minitubers (averaged over two inoculum media). Plants inoculated with two different *Rhizoctonia solani* isolates cultured on two different media at five rates of inoculation. Bars are LSDs ($P = 0.05$) for comparisons betwen "non-control" points.

3.5 Discussion

3.5.1 Optimal bioassay parameters

The direct measures of the canker phase of Rhizoctonia disease of potato, including the proportion of diseased stolons (Figure 3.4) and the number of symptomless stolons (Figure 3.5), different methods of analysing the same data, and the proportions of 'nipped' shoots (Figure 3.6), demonstrated consistent patterns of disease expression for each isolate between the two host plant propagule types: For isolate R73-13b disease levels were largely constant across all inoculums rates, while there were reduced levels of disease for isolate Rs043-2 at high inoculums rates. This suggests that the disease was expressed similarly on both host propagule types, making either suitable for use in a bioassay. For the proportion of diseased stolons in the minituber experiment the Rs043-2, barley + V8®, 0.10% inoculum rate datum runs contrary to this pattern. This could be an artefact due to the low number of total stolons present from that treatment (Figure 3.7). As an alternative method for measuring the canker disease on stolons, the number of symptomless stolons offers a measure less likely to generate misleading data when few stolons are produced.

For the plants from tissue culture, the total number of stolons was affected less by isolate Rs043-2 than R73-13b at most inoculum rates, making analysis of proportion of diseased stolons less likely to give misleading results from low total numbers of stolons for Rs043-2. This indicates that Rs043-2 would be a more suitable isolate for bioassay than isolate R73-13b for plants from tissue culture. For the plants from minitubers, isolate Rs043-2 demonstrated greater capacity for shoot nipping than R73-13b, providing an additional measure of disease severity which is potentially economically important. Shoot nipping caused yield reductions through reducing number and weight of daughter tubers, and recovery of these yield parameters could, for isolate Rs043-2, provide supporting evidence of disease suppression in a bioassay. A drawback of using Rs043-2 in a bioassay with plants from minitubers is the greater reduction in the numbers of stolons compared with isolate R73-13b, which could, as previously mentioned, result in misleading results when considering proportion of diseased stolons. However, the measure 'number of symptomless stolons' could provide a more reliable measure of disease pressure in experiments where few stolons are produced, and this parameter should be measured in assessment of pathogen effects.

Accepting that isolate Rs043-2 is the preferred isolate for future bioassays, an appropriate inoculum culture medium and initial inoculum concentration must be selected. For the

majority of treatments, the culture medium for Rs043-2 did not affect disease expression, so either medium would be appropriate for future use. However, grain-based culture media have been more commonly used in published bioassays (Table 3.1), so for consistency with previous research, grain-based medium is favoured.

The consistent trends for lower disease incidence at greater inoculum rates for isolate Rs043-2 is of interest, as this indicates that disease suppression was occurring at high inoculum rates. These greater rates should be avoided in future bioassays, as providing initial conditions which already favour disease suppression could potentially mask biocontrol activity of a treatment. Furthermore, if the effects of biocontrol and high inoculum act synergistically, treatments could appear to be more effective than they would otherwise be. As the lowest inoculum rate of isolate Rs043-2 from barley + V8® inoculum was sometimes the most severe in terms of disease incidence, and was a time-consuming inoculation method when ensuring even inoculum distribution between replicates, the mid-range inoculum concentrations (0.10 and 0.30%, w/w) are the most practically appropriate, and either would be suitable for future use.

3.5.2 Rhizoctonia disease expression

Direct comparisons between the levels of disease presented here and from greenhouse assays reported in published literature is problematic. The majority of previous studies assessed the canker symptoms using severity scales, while disease incidence was determined in the present study. Further, if tuber yield parameters are measured, these manifest when plants are much more than 7 weeks old (Table 3.1). What is clear is that Rhizoctonia disease potato bioassays reported in the literature have used a large range of inoculation rates to achieve moderate disease severity, from as few as three sclerotia per plant (Boogert and Jager, 1984) up to 10% (v/v) infested barley grain (Campion *et al.*, 2003). Most studies use *R. solani* isolates of AG 3, and for the AG 3 isolate used in this research (isolate R73-13b) the measures of canker symptoms remained constant over the 100-fold range of inoculation rates (Figures 3.4, 3.5 and 3.6). This would suggest that for many isolates of this anastomosis group, expression of disease symptoms is independent of the total hyphal mass present in the system. Relationships between hyphal mass and disease levels could exist at inoculum rates below those tested here, but further experiments with reduced inoculum rates would be required to confirm this.

For the AG 2-1 isolate (Rs043-2), the greatest inoculation rates often presented less disease than the lower rates. This result fits with meta-analysis by Bonanomi *et al.* (2010), which found that suppression of *R. solani* diseases by organic amendments rarely correlated with pathogen population decline, and often the inverse relationship held true. Suggestions for the

mechanisms underpinning this relationship include induced resistance of host plants and/or fungistasis, but these suggestions have rarely been investigated further. Either of these hypotheses could explain the trend for isolate Rs043-2 of decreasing disease incidence with increasing inoculum concentration. Fungistasis could potentially be induced through the action of other organisms responding to the increased nutrient availability, assuming that the pathogen has not used all the resources of the inoculum medium prior to the bioassay inoculation. Alternatively, the increased presence of specific carbon sources could be directly regulating the pathogenicity of isolate Rs043-2. Weinhold and Bowman (1974) demonstrated that, for *R. solani* pathogenic to cotton seedlings, applying glucose inhibited disease development, potentially by suppressing pectinase production, while 3-*O*-methyl glucose suppressed infection cushion formation. The disease decline could also be due to an isolate-specific population density effect regulating pathogenicity, perhaps quorum sensing or intra-population competition. However, quorum sensing has so far only been reported for fungi with yeast-like phases in their lifecycles (Hogan, 2006), and this is therefore a less likely explanation for disease suppression than other explanations outlined above.

A final note on the AG of Rs043-2 is pertinent: AG 3 is the most frequently isolated AG from potato exhibiting Rhizoctonia disease symptoms in most countries, including New Zealand (Anderson, 1982; Farrokhi-Nejad *et al.*, 2007; Justesen *et al.*, 2003; Lehtonen *et al.*, 2008b). This is likely to be because members of AG 3 produce the most tuber-borne sclerotia (Campion *et al.*, 2003; Woodhall *et al.*, 2008). However, a study from Finland (Lehtonen *et al.*, 2009) found that while AG 3 isolates form far more black scurf on tubers than AG 2-1 or AG 5 isolates and have greater potential for dissemination of inoculum, AG 2-1s and AG 5s have broader host ranges than AG 3s. Broad host ranges would imply that the AG 2-1s and AG 5s may have increased capacity to persist in fields during the absence of a potato crops, potentially making them economically more important in situations where control of tuber-borne inoculum is effective.

3.6 Conclusions

The results from this study have demonstrated consistency in disease susceptibility between plants grown from tissue-cultured plantlets and minitubers, which support the use of either type as host propagules for assays investigating disease suppression. The *R. solani* isolate Rs043-2, at an inoculum rate of 0.1 or 0.3% (w/w) was selected as the most practically appropriate inoculum option for future disease suppression studies, based on severity of disease and impact on plant physical parameters. The two pathogen inoculum media gave similar levels of disease, but the barley medium gives consistency with previous studies. Plant

pots of 0.9 L capacity, a peat:pumice based plant growth medium and a growth period of 7 weeks were also confirmed as suitable for a greenhouse disease suppression assay. The use of a combination of direct measures of disease symptoms (proportion of stolons with cankers and proportion of emerging shoots 'nipped') and measures of plant physical parameters (especially tuber weight) was useful in assessing the total impact of the pathogens to the plants, and should be maintained in the future biological suppression bioassays. These results provide a sound basis for studies on biological suppression of Rhizoctonia diseases of potato.

Chapter 4
Screening potential biocontrol agents in a greenhouse disease assay

4.1 Abstract

This study tested 89 microbial isolates for suppression of Rhizoctonia diseases of potato in a greenhouse assay, as either spore (*Trichoderma*/*Gliocladium* isolates) or cell (Pseudomonads or endospore-forming bacteria) suspensions. While several isolates gave significant reductions in measures of canker symptoms, or improvement of other plant parameters, none were consistent enough between assays, or demonstrated strong enough disease suppression, to warrant further investigation of their biocontrol potential. Compared with similar greenhouse assays in the literature, disease pressure was high in the pathogen controls, which may have contributed to poor performance of potential biocontrol isolates. Alternatively, the results support the hypothesis that the single isolate inundative approach to biocontrol is insufficient for consistent suppression of Rhizoctonia diseases of potato.

4.2 Introduction

To determine if the selected isolates (Chapter 2) possessed biocontrol activity for Rhizoctonia canker diseases of potato, they were screened using a greenhouse bioassay (Chapter 3). To validate the results, selected isolates were re-tested in an assay more reflective of field conditions. The ultimate goal of this study was to select one to three isolates demonstrating the strongest levels of *in vivo* disease suppression for further studies on their physical limitations, optimium biocontrol potential and mode(s) of biocontrol action.

Many recently published studies have aimed to identify microbial isolates with potential to be developed as inundative biocontrol treatments for Rhizoctonia diseases of plants. These have focused on extensive *in vitro* candidate selections, commonly on the basis of antibiosis, mycoparasitism or production of enzymes linked to this mode of action, antagonism in culture assays or decreased *R. solani* sclerotial viability. In these studies, only a few isolates progress to *in vivo* tests of disease suppression, usually in greenhouse pathogenicity assays. For example Grosch *et al.* (2006) screened 390 fungal isolates *in vitro*, and only six *in vivo*. Similarly, Faltin *et al.* (2004) screened 434 bacterial isolates *in vitro*, and highlighted six for further study. However, this process of hierarchical selection to narrow the collection screened to a few 'best' isolates requires strong relationships between *in vitro* selection

criteria and *in vivo* disease suppression. Otherwise a large proportion of isolates with good *in vivo* disease suppressing capacity that perform less well in *in vitro* assays will be overlooked. For example, in a study of rhizosphere Pseudomonads, Bautista *et al*. (2007) found no correlation between *in vitro* suppression of *R. solani* and *in vivo* suppression of Rhizoctonia diseases of *Solanum phureja*. In the present study, the decision to screen a relatively large number of isolates (89) was made, firstly to avoid limiting the modes of action to only those that previous studies have uncovered, and secondly, the limitations of *in vitro* screening was acknowledged and strong *in vitro* pathogen suppression was not completely relied upon for narrowing the choice of isolates to progress into further studies. Isolate selection based on the information generated in Chapter 2 (Table 2.3) provided isolates possessing one or a combination of traits (growth promotion, production of anti-fungal compounds, strong physical competition on agar, potential endophytic habit in potato plants, previous biocontrol of fungal phytopathogens), which would possibly inform as to which of these characteristics is the most important indicator of potential biocontrol activity against Rhizoctonia diseases of potato. To this end, a biocontrol score was created to allow analysis across the seven screens and three validation assays with regards to test isolate selection criteria and the level of disease suppression achieved.

Five of the seven initial greenhouse screens (screens 3 to 7) described in Chapter 4 were conducted on plants from tissue-culture, which, because they already possess an above-ground shoot at the time of assay initiation, are not susceptible to the emerging shoot 'nipping-off' stage of Rhizoctonia disease of potato. Therefore, validation of the results for many of the isolates demonstrating some suppression of disease was performed using minitubers as the plant propagation material, over three assays (validation assays 1 to 3). Plants from minitubers exhibit the shoot 'nipping-off' symptom. Validations were also conducted both in the seed raising mix and a soil-based mix to compare the screen assay conditions with a system closer to a 'real world' soil environment. The third validation assay included a ten-fold greater rate of inoculation of three of the potential biocontrol isolates being tested, as well as a variation of the application procedure (direct to minitubers rather than pre-mixed through the soil). This provided a preliminary test of whether variation of application procedure might improve disease suppression, possibly indicating whether investigation into optimisation of biocontrol would be worthwhile in future experiments. The candidate isolates were also tested for *in vitro* suppression of sclerotial production by *R. solani* on agar. Furthermore, three isolates were tested for suppression of black scurf in a shadehouse experiment where plants were grown to maturity, and severity of sclerotium development on tubers was assessed.

Regression analyses over the seven disease suppression screens were conducted to determine if an isolate's likelihood of achieving statistically significant disease suppression was related to the severity of disease in the assay. The results of the experiments were also compared with those of recent published studies which employed similar greenhouse assays, and conclusions were drawn as to whether further investigations would be worthwhile into the biocontrol potential of any of the test isolates applied in an inundative fashion.

4.3 Methods

4.3.1 Culture of microbial inoculum

The bacterial and fungal isolates screened (as detailed in Chapter 2, Table 2.3) and pathogen isolate were cultured as described below.

4.3.1.1 Bacterial inoculum

For each bacterial isolate tested for suppression of disease, the relationship between Optical Density (OD) of broth culture and concentration of colony forming units (CFUs) was determined prior to greenhouse assay initiation. For each isolate, 18 mL capacity Universal bottles each containing 5 mL Luria Bertani broth (LB, Appendix A.1.6) were inoculated from bacterial colonies grown on NA (Appendix A.1.2 inoculated from -80°C stock culture), then capped and incubated (25°C, darkness, 180 rpm orbital shaking) for 24 h. From these cultures, 2 mL aliquots were each dispensed into 98 mL of LB in 250 mL capacity conical flasks. Flasks were plugged with sterile cotton wool and capped with tinfoil prior to incubation at 30°C in darkness at 180 rpm for 18 h. Broth culture (20 mL) was dispensed into a 50 mL screw-cap tube (Axygen® Scientific), and then subjected to 3220 rcf for 10 min at 18°C in a centrifuge (5810 R, Eppendorf). The supernatant was removed and the bacterial pellet was re-suspended in 20 mL of physiological peptone solution (PPS, Appendix A.1.8) by vortex mixing. This re-suspension underwent 2-fold and 10-fold serial dilutions in PPS, with the OD at 600 nm recorded (Geneysis 10uv Scanning, Thermo Fisher Scientific) for each dilution step. The CFU concentration of the re-suspension was determined by plating aliquots of the serial dilutions onto NA, with subsequent colony counting once colonies formed, after incubation (25°C, darkness). The calculated CFU concentration for each dilution step was plotted against the recorded OD value, and linear regression was used to produce the formula describing the relationship between CFU concentration and OD. This was:

$x = (y+a)/b,$

where x = CFU/ mL, y = OD and a and b are constants supplied by the regression.

Culture broths (100 mL) were prepared to be ready for the day of assay initiation. A 2 mL aliquot of each 100 mL of culture broth was centrifuged, the supernatant removed, and the bacterial pellet re-suspended in 2 mL of PPS and diluted 10-fold in PPS prior to OD reading. The pre-determined equation and OD value were then used to calculate CFU concentration of the broth culture, and the required volume dispensed, centrifuged, supernatant removed and pellet re-suspended in 50 mL of the selected application solution. For screen assays 1, 3 and 4 (Table 4.1) the bacterial application solution was 1:1 (v/v) LB:PPS. For screen 5, 6 and 7, PPS was used. A 10-fold serial dilution of the bacterial application solution was also made in PPS and aliquots plated onto NA so that colony counting after incubation (25°C, darkness) could be used to determine actual CFU concentrations.

4.3.1.2 Fungal inoculum

Fungus isolates were first inoculated from refrigerated slopes onto PDA (Appendix A.1.1) plates and incubated (25°C, darkness) for 5 days. For screens 2 to 5, ten PDA plates per isolate were each inoculated with a 1 cm^2 agar square of mycelia from a PDA culture, sealed with cling wrap (GLAD®) and incubated at room temperature in a blue-light box (12/12 h light/dark cycle with blue NARVA® LT 18W/018 lighting) for 2 weeks. For screen 6 onward the culture method was inoculation of bran:peat media flasks (Appendix A.2.3) with five mycelia PDA squares as detailed previously in this section, and incubated in a blue-light box as previously detailed. On the morning of assay initiation, spores were harvested with sterile tap water; agitated from PDA culture surfaces or mixed through bran:peat medium with a sterile glass rod in *ca* 50 mL, then filtered through Miracloth® (Calbiochem, Merck KGaA). An aliquot of spore suspension was diluted 100-fold in water and spore concentration determined by performing haemocytometer counts on 10 µL. The required volume of spore suspension was then dispensed and made up to 50 mL with sterile water and maintained on ice until required. For suppression of sclerotial formation agar assays, 7 mm diameter agar plugs from the growing *R. solani* colony margin on PDA were used to inoculate water agar (WA, Appendix A.1.9), which was incubated for 5 days (25°C, darkness) and 7 mm diameter agar plugs from WA growing colony margins were used to inoculate the assay plates.

4.3.1.3 Pathogen inoculum

The *R. solani* isolate used for the screening and validation assays was Rs043-2 (see Chapter 3). For the agar plate sclerotial suppression assay isolates Rs043-2 and Rs018-2 (an AG 3 provided by Subha Das, Plant and Food Research Ltd., Lincoln) were both used, and for the assay for the *in vivo* suppression of black scurf, isolate Rs018-2 was used. PDA plates were inoculated from refrigerated infested barley grains and incubated (25°C, darkness) for 5 days. For *in vivo* assays, conical flasks of barley grain + V8® juice (Appendix A.2.1) were each

inoculated with five 7 mm diameter PDA plugs from growing colony margins. Flasks were plugged with cotton wool, capped with tinfoil and incubated at 25°C in darkness for 14 days. For agar plate assays, 7 mm diameter mycelia plugs of PDA cultures were used to inoculate WA plates, and after 5 days incubation (25°C, darkness), 7 mm diameter WA plugs from the growing colony margins were used to inoculate the assay plates.

4.3.1.4 Inoculum concentrations

In screens 1 to 5, the target concentration of pathogen inoculum was 0.30% w/w. The mean weight of 0.9 L of peat mix was determined as 430 g, so for treatments of ten replicates (4.3 kg), 12.9 g of infested barley grains was dispensed. The target inoculation rate for potential biocontrol agents (BCAs) for these assays was 1×10^6 CFUs/spores per g peat mix, equating to 4.3×10^9 total CFUs or spores for a treatment of ten replicates. As screen 6 contained a mixture of peat mix and soil mix treatments (Table 4.1), the inoculation rate for screens 6 and 7, all three validations and the assay of suppression of black scurf, was changed to a w/v ratio. This was because of the difference in density between the two growing media, and so that each treatment would receive the same weight of inoculum or number of CFUs/spores: For these assays a pathogen inoculum concentration of 0.10% w/v was selected, as the total weight of inoculum per treatment was close to that of the first five screens (9.0 g), but was still one of the selected concentrations used as described in Chapter 3. The target inoculation rate of potential BCAs for these later screens was 1×10^6 CFUs/spores per mL plant growth medium (9.0×10^9 CFUs/spores per treatment), with a few exceptions (see Results, below).

4.3.2 Fungicide treatment

The fungicide pencycuron (Monceren® 250 FS, Bayer CropScience) was used as a treatment for comparison in some of the assays. The required volume of a 250 g pencycuron/L concentrate with recommended in-furrow application rate of 3 – 5 kg a.i./ha, was calculated on a per plant basis as follows: One square hectare of commercially planted potatoes consists of 133 rows of plants with 0.3 m spacing between plants, equalling 44,333 seed potatoes planted per hectare. The 5 kg a.i./ha rate of pencycuron would require 20 L of Monceren® product per hectare, which is the equivalent of 0.45 mL per plant. Therefore, for a treatment of ten replicate plants (pots), 4.5 mL of concentrate was dispensed and made up to 50 mL with tap water on the morning of assay initiation.

4.3.3 Screening and validation assay conditions and procedures

The seven disease suppression screens and three validation assays (Table 4.1) were set up as follows: Each treatment consisted of ten 0.9 L capacity pot replicates, so 9 L of plant growth

medium was dispensed onto clean plastic sheeting per treatment, either seed raising mix (peat mix, Appendix A.3.1) or John Innes shrub mix (soil mix, Appendix A.3.2). For treatments requiring pathogen inoculation (all except no-pathogen controls), the pre-weighed infested barley + V8® media was mixed thoroughly through the plant growth medium by hand (with latex gloves). Next, for potential BCA or chemical treatments the prepared application suspensions were gently shaken then evenly distributed over the plant growth medium and thoroughly mixed through by hand (gloves were changed between treatments to avoid cross-contamination). The inoculated plant growth medium was then divided between ten 0.9 L capacity (120 mm diameter) plastic plant pots with individual saucers. In the final validation there were three treatments where potential BCA application was not performed by this method, but by pipetting 1/10 of the suspension (5 mL) direct to each minituber when planted, before the minituber was covered with potting mix. Pots were then arranged in a randomised block design (ten blocks, one replicate of each treatment per block) on mesh tables in a temperature-controlled greenhouse unit set to 16.5°C with supplementary lighting (16:8 h light:dark, 7 h total supplement from ten SON-T AGRO 400 lamps - Philips, Belgium). One potato propagule was planted per pot: for screens 1 and 2 and the three validation assays, minitubers undersized for field planting (cv. 'Desiree', Alex McDonald Merchants, Lincoln) were pre-sprouted (2 weeks at 18°C, 12:12 h light:dark, lighting: two 30W/860 Lumilux® Daylight lamps - Osram, Germany), then planted at 30 mm depth. For screens 3 to 7, tissue-cultured plantlets (cv. 'Russet Burbank', Aspara Pacific Ltd, Canterbury) were rinsed free of agar in tap water before planting. Pots were watered as required when mix appeared dry. For soil mix treatments, once plants had reached 200 mm in height they received 30 mL of a 0.01% solution of High NK™ liquid fertiliser (8:6:13.5 N:P:K, Agrichem, Australia) twice a week to prevent nutrient deficiency.

4.3.4 Screening and validation assays harvest procedures

After 7 weeks growth, all plants were washed free of plant growth medium and total number of stolons and number of stolons with visible lesions were recorded for each plant. For assays using minitubers, the number of emerged shoots and number and proportion of non-emerged shoots with rotted or dead apices ('nipped') were also determined for each plant. The number and fresh weight of tubers, and root and shoot dry weights, were also recorded for each plant. All data were subjected to analysis of variance for randomised block designs, employing Fisher's unrestricted LSD at $P = 0.05$ for multiple comparisons.

4.3.5 Sclerotial suppression assay

All potential BCAs included in the *in vivo* disease suppression screen were also tested for *in vitro* suppression of *R. solani* sclerotial formation. For *Trichoderma* (and one *Gliocladium*) test isolates, 7 mm diameter WA plugs from growing colony margins (section 4.3.1.2) were co-inoculated onto PDA in Petri plates along with *R. solani* from WA (section 4.3.1.3) in the same manner as outlined for dual plate assays in Chapter 2. Bacterial isolates were loop inoculated from NA cultures (4.3.1.1) onto four points on PDA:NA 1:1 (v/v, Appendix A.1.10) plates as described for dual plate assays in Chapter 2, with one plug of *R. solani* in the centre of each plate. Each test isolate was co-inoculated onto two plates with *R. solani* isolate Rs043-2 and two plates with Rs018-2. Plates were sealed with plastic film (GLAD®) and incubated (25°C, darkness) for 4 weeks, then the coverage of the agar plates with *R. solani* sclerotia was assessed on a 0 to 3 scale: 0 = no sclerotia, 1 = light coverage, 2 = medium coverage, 3 = heavy coverage (similar to experimental controls).

4.3.6 Black scurf suppression and harvest procedures

Each treatment in this experiment consisted of ten replicate planter bags (PB 8; 4.5 L capacity, Egmont commercial), each containing 4 L of plant growth medium and one potato plant. For each treatment, 40 L of soil:pumice mix (Appendix A.3.3) was dispensed onto clean plastic sheeting, then for treatments requiring pathogen inoculation (all except non-pathogen control), 40 g of infested barley + V8® media (isolate Rs018-2, cultured as detailed 4.3.1.3) was mixed thoroughly into the plant growth medium by trowel. Treatments receiving fungal (4×10^{10} spores in 300 mL sterile tap water) or bacterial inoculum (4×10^{10} CFUs in 300 mL PPS), or pencycuron (90 mg made to 300 mL with tap water), had application solutions dispensed evenly over the plant growth medium, which was then mixed thoroughly by trowel. Treatment plant growth medium was then divided equally between ten PB 8 planter bags and one small seed potato (cv. 'Russet Burbank', mean weight 16.7 g) free of sclerotia and surface sterilised (2 min in 2% sodium hypochlorite solution then rinsed twice in tap water), was planted per bag at 50 mm depth. Bags were arranged in a randomised block design, ten blocks each with one treatment replicate per block, in a shade house and watered as required. Plants were grown for 18 weeks, after which time they were cut at soil level. After a further 3 weeks, daughter tubers were removed from each bag, washed free of soil and the number and total weight recorded, as well as marketable tuber yield (excluding malformed tubers and tubers <45 mm or >85 mm in length (NIAB, 2009)). The severity of black scurf on each tuber was assessed using the standard severity diagrams described by James (1973). All data were

subjected to analysis of variance for randomised block designs, employing Fisher's unrestricted LSD at $P = 0.05$ for multiple comparisons.

Table 4.1 Screen and validation assay details, including plant propagule type, plant growth medium and project identifier codes of isolates tested

Assay	Plant Propagule	Plant growth medium	Isolates tested (RSP/RSPT code unless otherwise stated)
Screen 1	Minituber 'Desiree'	Peat mix	RSP0002, 0017, 0144, 0170, 0268, 0362, 2004, 2020, 2042, 2086, 2139 & 2183
Screen 2	Minituber 'Desiree'	Peat mix	RSPT028, 037, 085, 105, 106, 113, 116, 119, 122, 124, 125 & LU806
Screen 3	Tissue-culture plantlet 'Russet Burbank'	Peat mix	RSP0268^{+}, 0362^{+}, 2002, 2009, 2014, 2015, 2110, RSPT018, 037^{+}, 084, 5030, 5080 & LU140
Screen 4	Tissue-culture plantlet 'Russet Burbank'	Peat mix	RSP2002, 2009, 2013, 2016, 2023, 2024, 2026, 2027, 2053, 2057, 2067, 2071, 2089, 2116, RSPT001, 005, 007, 029, 097 & 110.
Screen 5	Tissue-culture plantlet 'Russet Burbank'	Peat mix	RSP2072, 2090, 2094, 2110^{+}, 2120, 2158, 2013, RSPT003, 031, 036, 060, 079, 093, 5182, LU144, LU1187, SS573, SS1635, SS1708 & SS1902
Screen 6	Tissue-culture plantlet 'Russet Burbank'	Peat mix and soil mix	RSP2083, 2090^{+}, 2125, 2193, RSPT106^{+}, 124^{+}, LU540, LU713, LU740, SS1708^{+} & pencycuron
Screen 7	Tissue-culture plantlet 'Russet Burbank'	Soil mix	RSPT037^{+}, 084^{+}, 085^{+}, 106^{+}, 119^{+}, 122^{+}, 5075, 5163, 5182, LU 132, LU144^{+}, LU151, LU297, LU298, LU540^{+}, LU547, LU549, LU569, LU593, LU753, LU806^{+}, LU1370 & pencycuron
Validation 1	Minituber 'Desiree'	Peat mix and soil mix	RSP2002, 2193, RSPT113, 119, 125, LU806 & pencycuron
Validation 2	Minituber 'Desiree'	Peat mix and soil mix	RSP2009, 2139, RSPT 106, 122, 125, SS1708 & pencycuron
Validation 3	Minituber 'Desiree'	Peat mix and soil mix	RSP2139, RSPT106, LU144 & LU549

$^{+}$Isolate repeated from previous screen

4.3.7 Analysis of trends across screens

In order to determine if a relationship existed between the degree of canker in a disease suppression assay and the proportion of isolates in a screen which significantly reduced disease, linear regression analyses were performed on the data from the seven screens. The mean percent diseased stolons, and mean percent reduction in number of symptomless stolons in pathogen controls were paired with the percent of isolates that significantly reduced those measures of disease.

A general biocontrol points system was created so that the results across the seven screens and three validation assays could be analysed together with regards to the original criteria for which the test organisms were selected. For each assay, comparisons were made of test isolates means relative to the pathogen control mean, but only if there was a significant difference between the pathogen and no pathogen control means. If a test isolate improved ($P \leq 0.05$) one of the measures of canker disease (percent diseased stolons, number of symptomless stolons or percent emerging shoots nipped) it was given +1 score, and the same for improvement of a physical parameter (shoot or root dry weight, number or total weight of tubers, total number of stolons), giving a maximum possible score of 2. If a test isolate increased one of the measures of canker disease, it was given -1 score, and the same for any physical parameter, giving a possible minimum score of -2. If an isolate made no significant difference to any measure of canker disease it was given score 0, and the same for any physical parameter. The -2 to +2 scale was then subjected to unbalanced ANOVA, with possession of the five selection criteria (growth promotion, antibiotic production, physical competition, endophytism and evidence of previous biocontrol) as factors.

4.4 Results

4.4.1 Canker suppression screens and validation assays

Tables 4.2 to 4.8 present the results for experimental controls and any treatment which statistically ($P \leq 0.05$) improved any parameter negatively impacted in the pathogen controls, for the seven disease suppression screens. These tables also include isolates which were repeated between screens (for assessment of consistency of results) irrespective of whether they positively improved disease parameters. Tables 4.9, 4.10 and 4.11 present results from all the treatments included in the three validation assays. If data sets did not meet the parametric requirement of normal distribution, data were transformed (square root transformation for count data, $\log_{10}$ transformation for weights) before ANOVA. The group means presented are from the pre-transformed data. For each parameter, group means which share a designated letter are not statistically different at $P \leq 0.05$. Data sets without designated lettering could not be analysed in this manner.

4.4.1.1 Canker suppression screens

Of the twelve potential biocontol isolates tested in disease suppression screen 1, only three positively impacted on one or more measure of canker symptoms (Table 4.2). Two isolates decreased percent emerging shoots nipped (RSP0268 and 2004) and one decreased percent diseased stolons (RSP2139). No isolate increased total number of symptomless stolons and isolate RSP2004 gave no symptomless stolons on any plant. Only RSP0268 improved any of the plant parameters, increasing both total number of tubers and stolons. Five isolates increased the percent diseased stolons, including RSP2004, and every isolate except for one gave total tuber weight significantly less than the pathogen control. Full results for screen 1 are presented in Appendix B, Table 7.2.

In the second disease suppression screen, five of the twelve tested isolates reduced the mean percent diseased stolons (Table 4.3), but of these only one (RSPT122) also increased the total number of symptomless stolons. Four isolates also increased one or more of the plant parameters, although none increased total tuber weight. Three isolates increased the percent diseased stolons. Full results for screen 2 are presented in Appendix B, Table 7.3.

Table 4.2 Screen 1, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell suspensions as treatements, with target 1 × 10^6 CFU/g plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (CFU/g)	Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.0 a	00.0 a	9.4 a	9.4 a	1.99 d	0.21 c	4.7 a	4.61 b
Pathogen control		48.2 e	82.7 cd	3.9 d	0.75 bcd	1.14 ab	0.11 a	1.4 c	3.09 ab
Appl^n solution (LB:PPS)		36.9 cde	47.1 b	4.0 cd	1.5 bc	0.85 a	0.08 a	1.7 bc	0.86 cde
RSP0268 *B. thuringiensis*	8.53×10^5	13.6 abc	88.0 cdef	7.3 abc	1.8 bc	1.83 bcd	0.15 abc	2.8 ab	0.82 cd
RSP2004 *Flavobacterium* sp.	1.85×10^6	19.9 abcd	99.3 f	6.2 abcd	0.0 d	1.37 abcd	0.14 abc	1.6 bc	0.56 def
RSP2139 *Paenibacillus peoriae*	8.45×10^5	48.6 de	33.5 b	4.3 cd	1.8 bc	0.79 a	0.08 a	0.9 c	0.24 g

Table 4.3 Screen 2, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected spore suspensions as treatements, with target 1 × 10^6 spores/g plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (spores/g)	Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.0 a	00.48 a	7.9 a	7.8 a	1.86 a	0.22 a	4.4 a	4.18 a
Pathogen control		65.1 bc	79.75 cd	0.9 de	0.2 cd	0.23 de	0.04 d	0.5 c	0.22 bc
RSPT113 *Trichoderma* sp.	1.00×10^6	67.5 bc	42.51 b	1.8 bcde	0.9 bc	0.44 cde	0.04 cd	0.2 bc	0.03 bc
RSPT119 *Trichoderma* sp.	1.00×10^6	63.9 bc	50.01 b	1.9 bcd	0.5 bcd	0.70 bcd	0.10 bcd	0.7 bc	0.14 bc
RSPT028 *Trichoderma* sp.	1.00×10^6	55.9 bc	72.61 c	2.7 b	0.5 bcd	0.73 bcd	0.11 bc	0.3 bc	0.16 bc
RSPT122 *Trichoderma* sp.	1.00×10^6	56.4 bc	43.38 b	2.2 bcd	1.0 b	0.76 bc	0.07 bcd	0.2 bc	0.16 bc
LU806 *T. atroviride*	1.00×10^6	80.2 c	02.92 a	0.1 e	0.1 d	0.04 e	0.03 d	0.0 c	0.00 c
RSPT106 *Trichoderma* sp.	9.77×10^5	45.2 b	90.03 de	2.6 b	0.3 bcd	1.19 b	0.14 b	0.7 b	0.70 b
RSPT116 *Trichoderma* sp.	1.00×10^6	46.4 b	73.15 c	1.0 bc	0.7 bcd	0.94 bc	0.10 bc	0.5 bc	0.18 bc
RSPT125 *Trichoderma* sp.	7.21×10^5	60.1 bc	04.21 a	0.8 bcde	0.2 bcd	0.43 cde	0.05 cd	0.1 bc	0.01 bc

In disease suppression screen 3 (Table 4.4), of the 14 isolates tested, only one isolate (RSP2009) reduced canker symptoms. It decreased mean percent diseased stolons and increased total number of symptomless stolons. Two isolates (RSP0268 and RSPT084) also increased one plant parameter each, number of stolons and number of tubers, respectively. Isolates RSP2002 and LU132 were tested in other screens, but neither positively or negatively affected any measure of canker or plant parameter relative to pathogen controls in screen 3. Full results for screen 3 are presented in Appendix B, Table 7.4.

In disease suppression screen 4 there were insufficient tubers at harvest for analysis (Table 4.5). Of the 22 isolates tested, two (RSP2002 and 2013) reduced the percent diseased stolons, of which one (RSP2013) also increased the total number of symptomless stolons. No isolate improved any of the plant parameters, while one decreased total number of stolons and two increased the percent diseased stolons. Isolate RSP2009 was a repeat treatment from a previous screen but in this experiment did not improve any canker symptom or plant parameter. Full results for screen 4 in are presented in Appendix B, Table 7.5.

Disease suppression screen 5 (Table 4.6) tested 20 isolates, of which two (RSP2072 and 2090) decreased the mean percent diseased stolons, but no isolate increased the total number of symptomless stolons. Isolates RSP2072 and RSP2090 also improved one plant parameter each (number of stolons and total tuber weight, respectively), and one further isolate (SS1708) increased the number of tubers. Isolates RSP2110, 2013 and LU144 were also tested in other screens, but none improved any canker symptom or plant parameter in this experiment. Full results for screen 5 are presented in Appendix B, Table 7.6.

Table 4.4 Screen 3, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1 × 10^6 CFU or spores/g plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)$^+$	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.4 a	4.6 a	4.4 a	4.18	0.95 abc	4.0 a	9.01 a
Pathogen control		91.0 de	2.7 cde	0.3 d	5.06	0.94 abc	0.3 cd	0.29 b
Appl^n solution (LB:PPS)		72.3 bc	3.7 abc	1.2 b	3.60	0.62 ef	0.2 cd	0.04 b
RSP0268 *B. thuringiensis*	1.01×10^6	94.7 de	4.0 ab	0.2 d	5.53	0.96 abc	0.3 cd	0.09 b
RSP2002 *B. mycoides*	6.59×10^5	88.3 cde	2.7 cde	0.4 cd	4.70	0.88 bcd	0.7 bc	0.17 b
RSP2009 *B. mycoides*	1.63×10^6	60.8 b	2.4 cde	1.2 bc	3.68	0.72 def	0.0 d	0.00 b
RSP2110 *Pseudomonas* sp.	3.88×10^5	78.7 cd	2.8 abc	0.6 bcd	4.16	0.73 def	0.4 bcd	0.03 b
RSPT084 *Trichoderma* sp.	1.00×10^6	98.2 e	2.9 abc	0.1 d	5.79	0.11 ab	0.9 b	0.46 b
LU132 *T. atroviride*	1.00×10^6	85.9 cde	1.9 e	0.4 bcd	3.84	0.570 f	0.1 cd	0.17 b

$^+$No differences ($P < 0.05$) between any of the shoot dry weight treatment means

Table 4.5 Screen 4, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1 × 10^6 CFU or spores/g plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	Total no. of Stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)
No pathogen control		1.0 a	2.5 a	2.4 a	1.78 abcde	0.39 abcde
Pathogen control		62.9 cdefg	1.6 ab	0.5 cdefg	1.86 bcde	0.35 de
Appl^n solution (LB:PPS)		80.7 fghi	2.3 ab	0.5 cdefg	1.77 abcde	0.33 e
RSP2002 *B. mycoides*	5.43×10^4	39.6 b	1.6 abc	0.8 bcde	1.89 abcde	0.37 bcde
RSP2009 *B. mycoides*	1.55×10^6	55.3 bcdef	2.3 ab	0.9 bcdef	2.06 e	0.45 a
RSP2013 *B. mycoides*	8.14×10^5	39.7 b	2.3 ab	1.4 b	1.62 abc	0.35 cde

Table 4.6 Screen 5, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 CFU or spores/g plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)$^+$	No. of tubers	Total tuber weight (g)
No pathogen control		1.9 a	5.0 fg	4.9 a	4.16 a	0.68	4.5 a	13.63 a
Pathogen control		95.8 de	3.2 abc	0.2 bcd	5.74 cdef	0.73	0.6 cd	0.36 d
Appl^n solution (PPS)		97.7 de	3.6 abcde	0.2 bcd	5.50 bcdef	0.69	0.6 bcd	0.57 d
SS1708 *Bacillus* sp.	9.57×10^5	90.2 bcd	3.9 bcdefg	0.4 bc	5.82 cdef	0.74	1.3 b	1.71 d
RSP2072 *B. mycoides*	8.45×10^5	89.2 bc	4.9 defg	0.3 b	5.03 b	0.62	0.3 bcd	1.79 d
RSP2090 Endospore bacterium	7.71×10^5	87.2 b	3.8 abcdefg	0.5 bc	5.18 bc	0.58	1.3 bc	3.62 c
RSP2110 *Pseudomonas* sp.	1.07×10^6	100 e	4.2 bcdefg	0.0 d	5.62 bcdef	0.72	0.6 bcd	1.62 d
RSP2013 *B. mycoides*	8.53×10^5	93.7 bcde	3.4 abc	0.3 bcd	6.87 g	0.83	0.2 d	0.05 d
LU144 *T. atroviride*	1.00×10^6	97.2 cde	2.7 ab	0.1 cd	6.17 fg	0.74	0.7 bcd	0.89 d

$^+$No differences ($P < 0.05$) between any of the root dry weight treatment means

In disease suppression screen 6, ten isolates and pencycuron were tested in the seed raising (peat) mix, and four of these isolates plus pencycuron and the experimental controls were also tested in soil mix (Table 4.7). For the pathogen controls, disease was less severe in the soil than the peat mix, both for percent diseased and number of symptomless stolons, with several plant parameters less affected in the soil mix. Pencycuron in both plant growth media reduced disease to levels not different ($P < 0.05$) to the no pathogen controls, and the same was true of most plant parameters. Isolates SS1708, RSP2090 and RSPT106 were repeated from previous screens, and only RSPT106 in soil mix reduced canker (reduced percent diseased stolons and increased total number of symptomless stolons), but not in peat mix, and none of the three isolates improved any plant parameter in either plant growth medium. Isolate RSP2083 increased the total number of tubers, but no other plant parameter was improved. Full results for screen 6 are presented in Appendix B, Table 7.7.

Of the 22 isolates tested in disease suppression screen 7 (Table 4.8), five isolates reduced canker parameters. Isolates LU132 and RSPT122 increased total number of symptomless stolons, RSPT106 decreased percent diseased stolons, and LU549 and LU144 achieved both. In screen 7 there was no difference ($P < 0.05$) between pathogen and no pathogen controls for the mean total number of stolons or total tuber weight. Isolates LU806, RSPT119 and RSPT084 were repeat treatments from previous screens, but in this experiment no treatment significantly reduced canker parameters. Full results for screen 7 are presented in Appendix B, Table 7.8.

Table 4.7 Screen 6, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix or soil mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1 × 10^6 CFU or spores/mL plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Plant growth medium	Applied conc^n (spores or CFU /mL)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control	peat mix		0.6 a	10.0 a	9.9 a	3.03 ab	0.74 c	6.6 a	8.54 a
	soil mix		4.5 a	7.3 bcd	7.1 b	3.20 abc	0.57 ef	6.7 a	7.52 ab
Pathogen control	peat mix		97.0 fg	4.8 hij	0.2 h	3.99 gh	0.78 ab	0.4 ef	0.17 g
	soil mix		80.6 cd	6.2 cdefgh	1.7 d	3.32 bcd	0.56 efg	4.3 ab	4.72 cd
Pencycuron	peat mix		0.0 a	9.0 ab	9.0 ab	2.92 a	0.70 c	7.1 a	8.12 a
	soil mix		1.1 a	7.5 bcd	7.4 b	3.11 ab	0.47 g	5.7 ab	5.44 c
Appl^n solution (PPS)	peat mix		100 fg	5.2 fghij	0.0 h	3.93 efgh	0.70 cd	0.3 def	0.00 g
SS1708 *Bacillus* sp.	peat mix	1.13×10^6	100 fg	4.3 ij	0.0 h	4.02 fghi	0.69 cd	0.9 def	0.14 g
	soil mix	1.06×10^6	76.3 c	6.6 cdefg	2.3 de	3.33 bcd	0.47 g	4.6 b	2.50 ef
RSP2090 Endospore bacterium	peat mix	1.19×10^6	100 fg	5.4 efghij	0.0 h	4.29 hi	0.74 abc	0.6 def	0.08 g
	soil mix	9.63×10^5	88.1 de	6.9 cdef	1.0 def	3.57 cde	0.53 fg	2.7 c	3.21 de
RSPT106 *Trichoderma* sp.	peat mix	1.00×10^6	95.0 efg	4.6 hij	0.1 h	4.54 i	0.82 a	0.1 f	0.03 g
	soil mix	1.00×10^6	59.9 b	7.1 cde	3.3 c	3.10 ab	0.50 fg	5.4 ab	4.47 cd
RSP2083 *P. brassicacearum*	peat mix	1.00×10^6	96.0 efg	5.3 fghij	0.2 gh	4.26 hi	0.76 abc	0.8 d	0.81 fg

Table 4.8 Screen 7, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in soil mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected spore suspensions as treatements, with target 1×10^6 spores/mL plant growth medium (only positive results plus any isolate repeated between screens are presented)

Treatment	Applied conc^n (spores/mL)	Diseased stolons (%)	Total no. of Stolons	No. of symptom-less stolons	Shoot dry weight (g)$^+$	Root dry weight (g)$^+$	No. of tubers	Total tuber weight (g)
No pathogen control		0.2 a	4.89 bc	4.75 a	3.95	0.38	3.28 ab	6.05 bcde
Pathogen control		66.2 defg	4.05 bcd	0.75 d	4.12	0.42	2.26 cd	4.08 def
Pencycuron		5.3 a	7.20 a	6.62 a	3.57	0.40	4.20 a	10.08 a
LU132 *T. atroviride*	1.00×10^6	45.3 bcd	5.00 bc	2.05 bc	3.94	0.36	2.60 bcd	7.09 abc
LU549 *T. virens*	1.00×10^6	37.2 b	4.60 bcd	2.03 bc	3.66	0.36	2.20 bcd	7.21 abc
RSPT106 *Trichoderma* sp.	1.00×10^6	39.2 bc	3.44 cd	1.75 bcd	4.11	0.38	2.78 abcd	4.28 bcdef
LU144 *T. atroviride*	1.00×10^6	35.7 b	4.80 bcd	2.41 b	3.39	0.35	2.00 cd	7.20 abc
RSPT122 *Trichoderma* sp.	1.00×10^6	50.2 bcdef	4.40 bcd	1.92 bc	3.82	0.36	3.20 abc	3.91 cdef
LU806 *T. atroviride*	1.00×10^6	60.4 bcdefg	4.40 bcd	1.33 bcd	4.75	0.49	2.50 bcd	4.24 bcdef
RSPT119 *Trichoderma* sp.	1.00×10^6	55.4 bcdefg	4.67 bcd	1.47 bcd	4.51	0.43	2.50 bcd	2.13 f
RSPT084 *Trichoderma* sp.	8.25×10^5	66.3 defg	4.50 bcd	0.79 cd	4.33	0.42	2.60 bcd	4.44 bcdef

$^+$No significant differences ($P < 0.05$) between shoot dry weight means or root dry weight means

4.4.1.2 Validation assay results

In the first validation assay (Table 4.9), only one isolate (RSP2193) reduced the percent of emerging shoots nipped, and only in the soil mix. Isolates RSP2193, LU806, RSPT113 and RSPT119 increased the percent emerging shoots nipped in the peat mix (but not the soil mix). Five isolates decreased the percent diseased stolons in the peat mix, but none in the soil mix. The only isolate to increase the number of symptomless stolons was RSPT125, and only for the soil mix. Isolate RSP2002 decreased the total number of tubers in the soil mix, and all six isolates decreased the total number of stolons in the peat mix. The effect of the disease on the pathogen controls was less in the soil mix than in the peat mix.

In the second validation assay (Table 4.10), no isolate reduced either percent emerging shoots nipped or diseased stolons, or increased the total number of symptomless stolons. The only isolate to affect a plant parameter was RSPT106, which increased the total number of tubers. Isolate RSP2139 increased percent diseased stolons and decreased total number of symptomless stolons in the soil mix. In the peat mix, RSPT122 decreased number of symptomless stolons and SS1708 increased the percent of emerging stolons nipped.

In the third validation assay (Table 4.11), which also included three isolate treatments at an increased inoculum rate (1×10^7 CFU/spores) and three treatments applied direct to minitubers, percent emerging shoots nipped was low in pathogen controls and no treatment reduced ($P < 0.05$) this disease parameter. Six treatments increased the mean percent emerging shoots nipped. These were LU549, LU144 and RSPT106 at 1×10^6 mixed through the soil mix, and LU549, RSPT106 and RSP2139 applied direct to minitubers. Isolate RSPT106 applied direct to minitubers was the only treatment to decrease mean percent diseased stolons, but no treatment increased total number of symptomless stolons, and LU5498 applied direct to minitubers decreased this parameter. No treatment increased the total tuber weight, but four treatments (all three LU549 treatments and RSPT106 applied direct to minitubers) decreased this parameter. For the soil:pumice + pathogen control the only parameters for which there was a significant difference to the soil mix was shoot dry weight (soil mix gave greater dry weight) and root dry weight (soil mix gave less root dry weight).

Table 4.9 Validation 1, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in soil mix or peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 CFU or spores/mL plant growth medium

Treatment	Appl^n conc (spores or CFU / mL)		Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control			0.0 a	0.7 a	4.4 bcd	4.3 a	3.37 cde	0.64 fg	3.0 a	14.53 a
Pathogen control			21.0 cd	98.0 h	3.7 cde	0.1 g	3.05 de	0.57 efg	0.2 f	0.53 g
Pencycuron		Peat mix	0.0 ab	2.5 a	4.2 bcde	4.1 a	3.33 bcde	0.72 g	2.1 ab	8.94 b
Appl^n solution (PPS)			29.2 cdef	95.5 fgh	3.0 def	0.2 defg	2.55 ef	0.39 cd	0.0 f	0.00 fg
RSP2002 *B. mycoides*	4.07×10^5		36.9 def	76.4 b	2.6 efg	0.7 defg	2.05 fg	0.43 cde	0.4 f	1.11 defg
RSP2193 *Flavobacterium*	1.22×10^6		50.0 f	90.0 cdefg	1.3 fg	0.2 defg	0.69 h	0.11 ab	0.4 ef	0.87 defg
LU806 *T.atroviride*	1.00×10^6		42.3 ef	100 h	1.5 fg	0.0 efg	1.21 gh	0.26 bc	0.2 f	0.9 fg
RSPT113 *Trichoderma*	1.00×10^6		49.2 f	82.6 bc	1.0 g	0.1 efg	1.32 gh	0.19 ab	0.1 f	0.03 fg
RSPT119 *Trichoderma*	1.00×10^6		47.5 ef	86.2 cde	0.8 g	0.1 efg	0.421 h	0.03 a	0.4 f	0.41 efg
RSPT125 *Trichoderma*	1.00×10^6		35.8 def	84.0 bcd	1.6 fg	0.3 efg	1.40 gh	0.24 bc	0.2 f	0.59 defg
No pathogen control			0.0 a	2.0 a	4.4 bcd	4.4 a	3.95 abc	0.53 def	3.1 a	16.43 a
Pathogen control			34.1 def	92.8 efgh	5.9 a	0.4 cde	4.34 a	0.60 fg	2.3 bc	5.10 c
Pencycuron		Soil mix	0.0 ab	0.0 a	4.6 abcd	4.6 a	3.768 abcd	0.51 def	3.2 a	17.52 a
Appl^n solution (PPS)			22.5 bcde	93.6 efgh	5.7 ab	0.4 defg	4.48 a	0.68 fg	2.2 bcd	4.36 cdef
RSP2002 *B. mycoides*	3.52×10^5		35.0 def	96.9 fgh	5.3 ab	0.2 defg	4.21 abc	0.58 defg	1.1 de	3.21 cdefg
RSP2193 *Flavobacterium*	8.89×10^5		5.0 abc	87.7 cdef	5.2 ab	0.9 bc	4.22 abc	0.60 efg	1.7 bcd	6.07 bc
LU806 *T.atroviride*	1.00×10^6		29.7 cdef	97.9 gh	5.8 ab	0.8 defg	4.33 ab	0.55 defg	1.4 cd	4.05 cdefg
RSPT113 *Trichoderma*	1.00×10^6		31.7 def	92.2 defgh	5.5 ab	0.3 defg	4.265 ab	0.57 defg	1.3 cd	4.87 bcde
RSPT119 *Trichoderma*	1.00×10^6		44.2 ef	90.9 cdefgh	6.3 a	0.6 bcd	4.68 a	0.72 g	2.2 ab	6.98 bc
RSPT125 *Trichoderma*	1.00×10^6		37.2 def	92.3 defgh	5.0 abc	0.9 b	4.432 a	0.60 efg	2.5 ab	4.98 bcd

Table 4.10 Validation 2, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in soil mix or peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 CFU or spores/mL plant growth medium (one exception)

Treatment	Appl^n conc (spores or CFU / mL)		Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers*	Total tuber weight (g)
No pathogen control			1.1 g	1.0 a	6.4 defg	6.3 h	3.27	0.69 a	2.3	4.56
Pathogen control			26.8 d	86.4 efgh	5.7 cdefg	0.87 cdef	2.94	0.52 bcdef	0.2 d	0.05
Pencycuron			0.0 efg	3.3 a	7.5 fg	7.3 h	3.08	0.72 a	2.4	2.48
Appl^n solution (PPS)			36.3 abcd	94.9 h	4.6 abcde	0.3 ab	2.67	0.38 efgh	0.2 d	0.01
RSP2009 *B. mycoides*	2.04×10^6	Peat mix	31.6 bcd	83.0 defgh	5.0 bcdef	0.9 bcdef	3.18	0.52 bcdef	0.1 d	0.38
RSP2139 *Paenibacillus*	1.72×10^6		40.7 abcd	86.2 defgh	5.0 bcdef	0.8 abc	3.51	0.45 cdefgh	0.0 d	0.00
RSPT106 *Trichoderma*	1.00×10^6		38.9 abcd	83.3 defgh	4.2 abcd	0.7 defg	2.33	0.34 ghi	0.4 bcd	0.08
RSPT122 *Trichoderma*	1.00×10^6		21.7 def	92.9 gh	3.0 ab	0.2 a	1.57	0.32 hi	0.0 d	0.00
SS1708 *Bacillus*	2.22×10^6		60.3 a	85.5 defgh	2.2 a	0.3 abcd	0.83	0.19 i	0.2 d	0.04
RSPT125 *Trichoderma*	1.00×10^6		25.3 cd	80.3 bcdefg	4.9 bcdef	0.8 bcdefg	3.20	0.46 cdefgh	0.2 d	0.03
RSPT125 *Trichoderma*	3.13×10^6		41.6 abcd	93.0 gh	3.6 abc	0.5 abcd	2.86	0.41 defgh	0.6 bcd	0.10
No pathogen control			3.3 fg	2.4 a	6.9 fg	6.7 h	3.49	0.57 bc	3.2	9.64
Pathogen control			37.1 bcd	71.6 bc	6.7 efg	1.9 g	3.21	0.52 bcde	1.1 abc	0.65
Pencycuron			0.0 efg	1.7 a	7.0 efg	6.9 h	3.39	0.64 ab	3.3	6.34
Appl^n solution (PPS)			51.0 ab	81.3 cdefg	5.2 bcdefg	1.2 bcdef	3.95	0.46 cdefgh	1.3 ab	1.18
RSP2009 *B. mycoides*	4.44×10^6	Soil Mix	34.3 bcd	68.1 b	5.7 bcdefg	1.8 fg	2.77	0.45 cdefgh	1.1 abc	0.78
RSP2139 *Paenibacillus*	1.48×10^6		39.3 abcd	90.6 fgh	7.8 g	0.9 abcde	3.73	0.56 abcd	1.6 a	0.83
RSPT106 *Trichoderma*	1.00×10^6		45.0 abcd	79.5 bcdef	4.8 bcdef	1.1 cdefg	2.54	0.32 hi	1.1 abc	1.23
RSPT122 *Trichoderma*	1.00×10^6		47.8 abc	69.7 bc	6.1 cdefg	2.7 fg	3.06	0.47 bcdefgh	0.9 abc	0.51
SS1708 *Bacillus*	1.67×10^6		32.7 bcd	73.5 bcd	6.2 cdefg	1.8 efg	3.51	0.51 bcdefg	0.4 cd	0.08
RSPT125 *Trichoderma*	1.00×10^6		38.2 abcd	76.0 bcde	5.1 bcdefg	1.5 defg	2.29	0.37 fghi	1.3 bcd	0.93
RSPT125 *Trichoderma*	3.13×10^6		24.0 cde	75.5 bcd	6.9 defg	1.7 defg	3.43	0.51 bcdeefg	0.4 bcd	0.77

*No-pathogen controls and pencycuron treatment data not included in analysis so parametric requirement of normal distribution satisfied.

Table 4.11 Validation 3, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in soil mix or peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 or 10^7 spores/mL plant growth medium

Treatment	Appl^n conc (spores or CFU / mL)		Emerged shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		Peat mix	5.0 def	9.2 a	8.4 bcdef	7.6 a	2.87 bcdef	0.56 cdef	3.4 abcd	23.29 a
Pathogen control		Peat mix	0.0 f	85.7 d	11.8 ab	1.7 bcd	4.44 h	0.70 ef	4.8 cdef	8.89 bcd
LU549 *T. virens*	1.00×10^6	Peat mix	15.8 cdef	88.4 d	9.4 abcde	0.9 bcd	3.93 fgh	0.68 ef	4.0 abcde	5.85 cdef
LU144 *T. atroviride*	1.00×10^6	Peat mix	21.7 bcde	89.6 d	6.9 defg	1.1 cd	3.84 efgh	0.59 cdef	4.1 abcdef	8.18 bcde
No pathogen control		Soil mix	0.0 ef	0.8 a	8.5 cde	8.4 a	3.35 defg	0.57 def	5.3 def	21.63 a
Pathogen control		Soil mix	10.3 def	77.4 cd	12.2 a	2.6 b	4.16 h	0.63 ef	6.0 f	9.82 bc
LU549 *T. virens*	1.00×10^6	Soil mix	40.5 ab	80.8 cd	6.9 defg	1.5 bcd	1.91 ab	0.28 a	2.5 abc	3.31 ef
LU144 *T. atroviride*	1.00×10^6	Soil mix	31.7 abc	74.0 cd	7.5 cdef	1.9 bc	2.67 abcd	0.41 abcd	4.0 abcde	6.70 cdef
RSPT 106 *Trichoderma*	1.00×10^6	Soil mix	33.8 abc	80.7 cd	5.7 efg	1.2 bcd	2.44 abcd	0.38 abc	3.2 abc	6.03 cdef
RSP 2139 *Paenibacillus*	1.00×10^6	Soil mix	5.0 def	79.4 cd	10.4 abcd	2.2 b	3.87 efgh	0.68 ef	3.8 abcde	7.30 cdef
LU549 *T. virens*	1.00×10^7	Soil mix	25.0 bcd	75 cd	8.3 bcdef	1.8 bcd	3.08 cdefg	0.51 bcde	3.1 abc	4.68 def
RSPT 106 *Trichoderma*	1.00×10^7	Soil mix	11.7 cdef	74.7 cd	11.7 ab	2.5 b	3.83 efgh	0.62 def	4.8 cdef	8.17 bcde
RSP 2139 *Paenibacillus*	1.00×10^7	Soil mix	5.0 def	76.2 cd	11.2 abc	2.5 b	4.22 gh	0.73 f	4.3 bcdef	6.55 cdef
LU549, to tuber	1.00×10^6	Soil mix	49.6 a	81.1 cd	4.6 fg	0.4 d	1.94 abc	0.31 ab	2.1 ab	2.76 f
RSPT 106, to tuber	1.00×10^6	Soil mix	54.1 a	51.1 b	3.5 g	1.0 bc	1.64 a	0.22 a	1.8 a	5.08 def
RSP 2139, to tuber	1.00×10^6	Soil mix	39.6 ab	67.1 bc	6.3 efg	1.7 bc	2.28 abc	0.32 ab	2.4 abc	7.26 cdef
Soil:pumice +pathogen			12.5 cdef	78.5 cd	11.4 abc	2.7 b	2.75 abcde	0.41 abcd	6.1 ef	13.11 b

4.4.2 Sclerotial suppression agar assay

All *Trichoderma* (and one *Gliocladium*) isolates tested completely prevented *in vitro* sclerotial formation by both *R. solani* isolates, except for LU753 which achieved mean scores of 1.5 against isolate Rs043-2 and 0 against isolate Rs018-2. Mean suppression scores of the bacterial isolates tested are presented in Appendix B.2.2, Table 7.9.

4.4.3 Black scurf suppression assay

The results in Table 4.12 demonstrate that the isolate Rs018-2 (AG 3) produced no significant effect on number of tubers or yield. None of the biological control isolates reduced percent sclerotial coverage of tubers. The only treatment to reduce ($P < 0.05$) black scurf severity was the pencycuron fungicide treatment.

Table 4.12 Black scurf suppression *in vivo*, mean yield parameters and black scurf severity for tubers produced by potato plants grown from seed potatoes in soil:pumice mix inoculated with *Rhizoctonia solani* isolate Rs018-2 and selected cell/spore suspensions as treatements, with target 1×10^6 spores/mL plant growth medium

Treatment	No. undersized tubers	No. marketable tubers	Total tuber weight (g)	Average marketable tuber weight (g)	Black scurf coverage (%)
No pathogen control	2.1 ab	4.5 a	235.5 a	55.87 b	0.025 a
Pathogen control	1.8 a	4.4 a	205.2 a	48.2 ab	7.57 b
SS1708 *Bacillus*	2.3 ab	5.4 a	240.8 a	44.5 ab	7.740 b
RSPT125 *Trichoderma* sp.	1.3 a	4.8 a	195.5 a	41.37 a	6.270 b
RSPT106 *Trichoderma* sp.	2.5 ab	5.3 a	238.1 a	48.9 ab	6.548 b
Pencycuron	3.1 b	4.5 a	218.3 a	50.23 b	0.030 a

4.4.4 Multi-screen analysis

The number and percent of isolates which decreased the number of symptomless stolons relative to the pathogen control negatively correlated with the percent reduction in number of symptomless stolons in an assay (Table 4.13). For this parameter, there was a trend for more isolates able to reduce disease symptoms in assays with lower disease levels. This relationship was not found for percent diseased stolons.

The analysis of the general biocontrol points data (Table 4.14) showed that of the six factors tested, only antibiotic production was a significant, and the interactions between antibiotic production and strong competition as well as between growth promotion and potential endophytism, gave significance levels $P < 0.1$. Other interactions could not be analysed as there were no data for all factor combinations.

Table 4.13 Regression analyses of levels of canker disease in experimental controls against number and percent of isolates tested that decreased disease

		'Positive' isolates			'Positive' isolates	
Screen	Diseased stolons (%)	Number	% of total	Reduction in number of symptomless stolons (%)	Number	% of total
1	82.7	1	8.3	97.7	1	8.3
2	79.8	5	41.7	99.5	1	8.3
3	91.0	1	7.1	98.6	1	7.1
4	62.9	2	9.1	88.4	1	4.5
5	95.8	2	10.0	99.4	0	0.0
6[A]	97.0	0	0.0	99.7	0	0.0
6[B]	80.6	1	25.0	84.6	1	25.0
7	66.2	3	13.0	84.2	4	17.4
	R^2	0.20	0.07	R^2	0.45	0.59
	P	0.31	0.54	*P*	0.10	0.03

[A]Data from peat based plant growth medium, [B]Data from soil mix

Table 4.14 Unbalanced factorial ANOVA of biocontrol points data *P* values

Factor	*P* value
Taxon*	0.256
Growth Promotion (GP)	0.991
Previous biocontrol evidence (BC)	0.941
Antibiotic producer (AB)	0.066
Strong competitor	0.356
Potential endophyte	0.246

**Trichoderma*/*Gliocladium*, fluorescent Pseudomonad, or endospore forming bacterium

Interaction	*P* value
Taxon×GP	0.780
Taxon×AB	0.318
GP×AB	0.623
Taxon×Comp.	0.870
GP×Comp.	0.542
AB×Comp.	0.022
Taxon×Endo.	0.261
GP×Endo.	0.079
AB×Endo.	0.338
Comp×Endo.	0.692
Taxon×BC	0.921

Results from the unbalanced ANOVA (Table 4.15) demonstrate that isolates that produced antifungal compounds on agar scored lower on the biocontrol points scale than isolates that did not ($P < 0.1$).

Table 4.15 Factor and interactions ($P < 0.1$) from unbalanced ANOVA of biocontrol points data

Antibiotic production	Mean biocontrol points	Number
Antibiotic producer	-0.258	n=31
Non antibiotic producer	0.026	n=115
LSD(5%)	0.303	
Antibiotic production x competitive ability	**Mean biocontrol points**	**Number**
Antibiotic producing strong competitors	-1.500	n=2
Antibiotic producing non-competitors	-0.172	n=29
Non antibiotic producing strong competitors	0.078	n=64
Non antibiotic producing non-competitors	-0.039	n=51
Average LSD(5%)	0.692	
Growth promotion x Potential endophytism	**Mean biocontrol points**	**Number**
Growth promoting potential endophyte	-0.750	n=4
Growth promoting no- potential endophyte	0.083	n=24
Non growth promoting potential endophyte	-0.091	n=22
Non growth promoting non-potential endophyte	-0.021	n=96
Average LSD(5%)	0.591	

4.5 Discussion

4.5.1 Suppression screens and validation assays

In the Rhizoctonia disease suppression screens there were isolates in each screen which positively impacted on either a direct measure of canker symptoms (percent emerging shoots nipped, percent diseased stolons, number of symptomless stolons) or one of the plant

parameters affected in the pathogen control (number of stolons or tubers, total tuber weight, dry weight of shoots or roots). However, rarely did any isolate achieve positive results both for canker and plant parameters. Only isolate RSP0268 (*Bacillus thuringiensis*) in one assay and RSPT106 (*Trichoderma* sp.), if results over several assays are considered, gave reduced disease and increased plant parameters. Isolates which were repeatedly tested between screens often demonstrated conflicting results, exhibiting some capacity for disease suppression in one assay but none in another. Of the 22 isolates which demonstrated positive impacts on canker disease symptoms or affected plant parameters, 13 were tested in more than one screen, of which only three demonstrated positive effects (RSP0268, RSPT122 (*Trichoderma* sp.) and RSPT106) in more than one assay.

In the three validation assays, 13 isolates had their screen assay results tested in the minituber system, both in peat mix and soil mix. Twelve of these had positive impacts in at least one screen assay, and one (RSP2193, *Flavobacterium* sp.) had shown no positive impact. Across the validation assays, isolates tested rarely improved the assessed parameters, and often had a negative effect on one or more. For example, isolate RSPT122, one of the few isolates to give positive results in more than one screen assay, decreased the number of symptomless stolons in the second validation assay. Isolate RSPT106 was the only partially consistent positive isolate from the screens to have a positive impact in the first or second validation assay. Therefore isolate RSPT106, along with two other isolates, was included in the third assay at a 10-fold higher application concentration (1×10^7 spores/mL plant growth medium), as well as at the previous concentration, but as an application direct to the minitubers at planting. This was to determine if either of these approaches could improve the level of disease control and therefore form the basis of investigations into optimisation of biocontrol studies for selected isolates. This third assay demonstrated, again, that the selected isolates performed poorly with regards to disease control, with all three applications direct to minitubers actually increasing the percent emerging shoots nipped along with three of the other treatments. The validation assays did not provide evidence that any isolate would be a worthwhile candidate for future work on biocontrol of Rhizoctonia diseases of potato.

The final validation assay also included a plant growth medium with no peat to determine if this component of the plant growth medium was increasing the disease pressure, as peat has been found to be conducive to Rhizoctonia diseases in previous studies (Bonanomi *et al.*, 2007). No significant difference was found, however, between the isolate treatment and the pathogen control in soil mix, for the direct measures of canker disease, for number of stolons/tubers or for total tuber weight, indicating that the peat was not promoting disease.

4.5.2 Black scurf suppression

In spite of the ability of the biocontrol isolates tested in the shade house assay to suppress *R. solani* sclerotial production *in vitro*, they failed to reduce black scurf severity. Expecting an isolate incorporated into the soil at time of planting to suppress the action of the pathogen several months later may be unrealistic, and applications later in the potato growth period (perhaps near the vine kill stage of crop growth) could have a greater chance of success than application at planting. However, experiments described here offer no evidence that the selected isolates possess biocontrol potential.

4.5.3 Multi-screen analyses

The regression analyses between levels of canker disease in pathogen controls and the number of isolates achieving control suggest that reduction in disease by an isolate was more likely when disease pressures in the assay were less, at least for symptomless stolons. The disease levels in the screens were generally high, and so more isolates may have demonstrated some disease control capacity had the disease pressure been less. Ultimately, however, if an isolate is unable to offer control under high disease pressures, its suitability as a BCA would be difficult to justify. The efficacy of the fungicide pencycuron in all the experiments that included it as a treatment, demonstrates that disease control was achievable in the assay system, but that the isolates tested were not up to this standard.

The analysis of assigned biocontrol score demonstrated that most selection criteria made no difference to the potential of an isolate to reduce Rhizoctonia canker disease *in vivo*. What was unexpected was that isolates which produced anti-fungal compounds *in vitro* were more likely than others to make disease parameters more severe or reduce plant parameters. While the results in Table 4.15 suggest that antibiotic producing strong competitors and growth promoting potential endophytes were statistically more likely to increase disease symptoms, the number of data points for those groups was very low (n = 2 and 4, respectively), so this conclusion does not have a solid foundation.

4.5.4 Results of published studies

Published studies (Table 4.16), which used similar assay systems to the present study, have tested many fewer isolates in *in vivo* greenhouse disease suppression assays, and demonstrated that far greater proportions of isolates reduced disease symptoms than were observed in the present study.

Table 4.16 Comparison between Rhizoctonia disease suppression and plant parameter impacts from assays in the present study and a selection of assays from published sources

Study	Disease parameter	Disease level in control	Number of isolates tested	Number of 'positive' isolates	'Positive' isolates (%)	Disease reduction achieved[B]
Present study	Diseased stolons (%)	63-97%	89 (118[A])	15	12.7%	Up to 76%
	Symptom-less stolons reduction	84-100%		9	7.6%	Up to 35%
	Tuber weight reduction	37-100%		1	0.8%	24%
	Tuber number reduction	31-100%		4	3.4%	Up to 18%
Brewer and Larkin (2005)	Stem canker incidence	83-100%	28 (84[A])	22[A]	34.5%	Up to 75%
	Stem canker severity	50-70%		31[A]	36.9%	Up to 71%
	Black scurf severity	50-74%		35[A]	41.7%	Up to 100%
Grosch *et al.* (2005; 2006)	Stem canker severity	46-70%	9 (24[A])	12[A]	50%	Up to 60%
Tariq *et al.* (2010)	Black scurf incidence	Control = 100%	9	7	77.8%	Up to 74%
Lahlali and Hijri (2010)	Stem canker severity	89%	6	6	100%	Up to 69%
	Reduction in yield	80%		6	100%	Up to 86%

[A]Number including isolates repeated within screens.
[B]Presented data are percentage reduction compared with positive experimental control.

As Table 4.16 shows, of the isolates tested in previous similar greenhouse assays investigating suppression of Rhizoctonia disease of potato, a much greater proportion demonstrate disease suppression than was found in the present study. Direct comparison between published results and those from experiments conducted here is difficult, as different measurements of disease were made elsewhere, often based on 'severity scales'. It would appear, however, that the disease pressure in the screens in the present study often reached levels greater than those experienced in the other studies. This could be related to differences in the strain of *R. solani*, with AG 3s the most common in previous studies, while this bioassay used an AG 2-1 isolate which was very proficient at causing cankers on, and 'nipping' of, plant organs. Other differences that might have weighted these published assays more in favour of the biocontrol isolate include: Firstly, two methods (Lahlali and Hijri, 2010; Tariq *et al.*, 2010) used sterilised plant growth medium, which might favour the BCA by removing competition from non-pathogen suppressive isolates; Secondly, two methods (Grosch *et al.*, 2006; Lahlali and Hijri, 2010) inoculated the BCA into the plant growth

medium on a pre-colonised substrate, both at rates which could be considered high (1:10 v/v and 1:20 w/w respectively), which would give unrealistically high suppression levels if this application method could not be employed in the field. The assay by Brewer and Larkin (2005) was closest to the one employed in the present study, but none of the treatments tested reduced stem canker incidence and severity in all tests (this included isolates used in commercial biocontrol products as well as the fungicide azoxystrobin), with similar results for black scurf incidence and severity.

Taken together, the results of these experiments do not offer much support for potential biocontrol of Rhizoctonia diseases of potato, using a single microorganism inundative approach. It is not surprising that, in spite of the global importance of potato crops and the ubiquity of pathgenic *R. solani* strains and important Rhizoctonia diseases in potato producing regions, there are no commercial biocontrol products marketed for the control of these diseases. It may be, as other authors have suggested, that successful suppression of Rhizoctonia diseases relies on a suite of suppressive organisms, rather than selected individuals (Bonanomi *et al.*, 2010; Mendes *et al.*, 2011). Organic amendments which support antagonistic communities may also have to be applied along with antagonistic organisms in order to support strong, consistent biological control (Hoitink and Boehm, 1999; Noble and Coventry, 2005).

In many of the assays in the present study which used plants from tissue culture, the shoot dry weight of the pathogen control was often equal to, or greater than that of the no pathogen control, in spite of severe disease on below-ground plant parts. Increases in dry weight are likely to be due to lesion development on stolons preventing the transport of photosynthetic products to tubers, resulting in their redistribution elsewhere, as described by Hartill (1989). For this reason when examining potato crops, shoot biomass should not be relied upon as a proxy measure of yield, as in one recent high impact report on organic agriculture employed (Crowder *et al.*, 2010).

4.6 Conclusion

Ultimately, the main finding of these experiments was that none of the 89 isolates tested, demonstrated sufficiently strong or consistent reduction in Rhizoctonia canker diseases of potato to make further investigations into their biocontrol potential worthwhile. This was in spite of the fact that isolates were were selected by targeted isolations and possession of traits potentially important for biocontrol, and were applied to potato plant growing media in an inundative manner in *in vivo* greenhouse screens and subsequent greenhouse validation

assays. It is possible that disease pressures from the chosen *R. solani* isolate were too great for moderate suppression of disease to be detected in the assays. Furthermore, individual antagonistic isolates may be insufficient to control disease, and control could possibly be achieved with a suite of suppressive organisms or by the application of an isolate(s) along with a substrate to support biocontrol activity. Nevertheless, the results from this study strongly indicate that effective, practical inundative biocontrol of Rhizoctonia diseases of potatoes is likely to be an elusive goal.

Chapter 5
Investigation of organic matter amendments for suppression of Rhizoctonia diseases of potato

5.1 Abstract

Organic matter (OM) amendments were selected based on published reports of suppression of Rhizoctonia diseases. These OMs were tested over two greenhouse assays, at several rates, to determine if any possessed the capacity to suppress the Rhizoctonia cankers of potato. Pine bark compost was selected to be tested in a third greenhouse assay at four rates, to generate a range of disease suppression levels. The aim of the third assay was to examine biological parameters which correlated with disease suppression, to indicate the mechanism underpinning disease suppression. The third experiment gave insufficient disease suppression to warrant a full investigation of underlying biological parameters. However, microbial community substrate utilisation data for the growth medium revealed that general increases in total community metabolic activity or diversity were insufficient to suppress Rhizoctonia cankers of potato.

5.2 Introduction

Chapter 4 focussed on 'inundative biological control' (Eilenberg *et al.*, 2001), where specific organisms are applied to the environment with the expectation that they will directly suppress the pathogen (or disease) but not persist in the environment. As the results did not provide support for that strategy, the final two experimental chapters of this study focus on the 'conservation biological control' strategy. This strategy was defined as "Modification of environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests" by Eilenberg *et al.* (2001). The experiments in this chapter deal with the potential for general disease suppression, which is reliant upon establishment or enhancement of soil communities whose presence and activities reduce a pathogen's capacity to inflict damage to its host. As appropriate soil conditions are required to support general suppression (Hoitink and Boehm, 1999), this type of suppression is not transferable in the way that occurs with specific suppression (e.g. by inundative applications of specific organisms). One method by which general suppression can be achieved is through the use of organic matter (OM) amendments to soils (Bonanomi *et al.*, 2007; Huber and Sumner, 1996), which may provide substrates which increase the size or activity of

suppressive microbial populations, or introduce those populations to the soils with an appropriate nutrient base.

The aim of the experiments reported here was to test some OMs, selected from a review of literature on the subject, rather than screen a wide variety of OM amendments. These were to be assessed as potential Rhizoctonia disease suppressors, and their mechanisms of disease suppression were to be investigated. Economic viability of potential amendments was also taken into consideration in selecting the few OMs tested, as investigating amendments without any feasibility of practical use would not be consistent with the overall direction of this research. Experiments described here tested several concentrations of selected OMs, to determine if a range of levels of disease suppression could be generated. Investigation of the biological origin of disease suppressive conditions is easier when correlations can be made between disease severity and biological factors measured, for example, by combining disease suppressive with disease conducive soils in varying proportions, or by increasing the concentration of a suppressive amendment (Borneman and Becker, 2007), as discussed in Chapter 1, section 1.3.4.4.3.

5.2.1 Overview of published research

There have been many studies examining the potential of a wide variety of OM amendments to suppress soil-borne fungal plant pathogens, including *Rhizoctonia* spp. In a thorough review of this field, Bonanomi *et al*. (2007) divided OMs into four categories; green manures (crop residues), animal manure, composts and peats. *Rhizoctonia solani* was overall the most studied species, and green manures were suppressive in 41% of cases, but conducive to disease in 43% of cases; manures were suppressive 41% of cases and conducive in 23%, compost was suppressive in 32% of cases and conducive in 20%; peats were only suppressive in 4% of cases and conducive in 60%. Populations of *R. solani* increased in the majority of crop residue studies, but disease suppression was found only rarely to correlate with decreases in pathogen populations, and often the inverse relationship was true. In terms of phytotoxicity, green and animal manures were often phytotoxic at concentrations much lower than composts or peats. This is probably linked to their decomposition causing anaerobic conditions, although anaerobic conditions also negatively affects phytopathogenic fungi, which is potentially how they achieve some of their disease suppression. The authors recommended avoiding crop residues or organic wastes for *R. solani* control, as they provide erratic control, can be phytotoxic and *R. solani* can often use them as substrates. This leaves compost as the OM group most likely to provide consistent control of Rhizoctonia diseases.

In a follow up to their 2007 review, Bonanomi *et al.* (2010) correlated OM analytical parameters and disease suppressiveness for the most studied soilborne phytopathogens in the literature. For *R. solani*, decreases in disease levels were often correlated with increased pathogen populations, which is possibly related to induction of disease resistance in host plants or soil fungistasis, although these explanations have rarely been explored further. The 40 OM chemical and physical variables analysed were, in general, poor predictors of suppressiveness, and less informative than enzymatic and microbiological predictors. For example, the C:N ratio was not informative for *R. solani* suppressiveness, which is not surprising as *R. solani* can utilise materials with a broad range of C:N ratios (Bonanomi *et al.*, 2007). Contrasting results were reported for trends linking fluorescein diacetate (FDA) hydrolysis (a method for determining total microbial activity) and *R. solani* suppressiveness: for crop residues the correlation was negative, while for composts it was positive, so composts may suppress Rhizoctonia diseases by increasing microbial activity. For all pathogens and OM types, 95% of the studies reviewed demonstrated that sterilisation reduced disease suppression, and this indicates that the biotic component of OMs is essential for control. Overall, the measures which most consistently positively correlated with disease suppression were populations of fluorescent Pseudomonads (73% of cases), spore-forming bacteria (60% of cases) and *Trichoderma* spp. (56% of cases), with none of the three ever negatively correlating with suppressiveness. This gives good reason to theorise that OM amendments that reduce Rhizoctonia diseases achieve success by stimulating antagonists within the soil microbial communities to induce biological control of *R. solani*. Therefore, OM amendments fit well into this research project.

5.2.2 Selection of organic matter amendments active against *Rhizoctonia* spp.

Based on the following information gathered from a review of the literature, three groups of OM; composted wood products, chitin/chitosan and biochar, were selected for investigation of their potential to suppress Rhizoctonia diseases of potato.

5.2.2.1 Composted bark products

Based on the recommendations of Bonanomi *et al.* (2007; 2010) summarised above, composted OMs were targeted. When Scheuerell *et al.* (2005) tested 36 different composts for control of soilborne phytopathogens in a containerised (peat/perlite) system, six were found to suppress Rhizoctonia damping-off of cabbage. Of these, the most consistent were hemlock bark, dairy fir-bark compost, mushroom compost and nursery regrind compost. However, suppression was not related to any single physical, chemical or biological factor, making it difficult to predict which composts will suppress Rhizoctonia diseases. Of the variety of OMs

reported to suppress *R. solani* reviewed by Litterick *et al.* (2004), the wood products (wood chips and tree barks) stand out as often inducing disease suppression. In a review by Noble and Coventry (2005), one of the largest reductions of Rhizoctonia disease (81%) was with a hardwood bark. Composted barks, therefore, appear to be strong candidates for suppressing Rhizoctonia diseases. Supporting this is the study by Krause *et al.* (2001), which demonstrated that composted pine bark/peat potting medium was more frequently suppressive to Rhizoctonia diseases of radish and poinsettia, compared with sphagnum peat media of different decomposition stages. An explanation of why composted wood products may offer more consistent disease suppression than fresh material was summarised in a review by Hoitink and Boehm (1999). They suggested that low cellulose levels in composted bark encourage antagonism towards *R. solani*, as lignocellulosic substances are colonised by *Trichoderma* spp., and while fresh hardwood bark stimulates populations both of *R. solani* and *Trichoderma* spp. parasitic to them, damping-off severity is still increased. However, composted bark controls the pathogen even though the populations of parasitic *Trichoderma* spp. are far fewer than in fresh bark. If cellulose is combined with composted bark it returns to being conducive to Rhizoctonia diseases, even though the *Trichoderma* spp. populations rise considerably. Therefore, low cellulose levels promote fungal interactions, where the *R. solani* is suppressed. These authors also suggested that it is likely that production of chitin degrading enzymes decreases when the substrate cellulose, preferred by *Trichoderma* spp., is available. Van Beneden *et al.* (2010) demonstrated that the addition of lignin (1% w/w) reduced *R. solani* sclerotial viability in one soil type but not another, which was linked to differences in the response of the soil microbial communities to the amendment. In New Zealand, Monterey Pine (*Pinus radiata* D.Don) is grown extensively for the building and paper industries, so by-products such as bark are widely available, and likely to be economic sources of OM amendments.

5.2.2.2 Chitin and chitosan

Chitin is another OM with potential as a commercial soil amendment. Chitin is the second most abundant bio-polymer in the world, and a massive quantity of chitin-rich waste is regularly discarded by the seafood industry. Because it is a major component of fungal cell walls, it also has potential for increasing the activity of chitin-degrading communities. A study by Sneh and Henis (1971) found that while *R. solani* is able to colonise chitin particles, it was displaced from them within 30 days in non-sterile soil, suggesting that it is not a strong competitor for chitin. There is evidence from a variety of sources that chitin soil amendments reduced Rhizoctonia diseases. Huber and Sumner (1996) summarised that increasing the incubation time after chitin amendments to soil decreases the saprophytic growth of

Rhizoctonia. This suppression was also greater for the chitin amendment compared with a variety of plant residues, which the authors suggested was due to the accumulation of anti-fungal substances as a result of chitin degradation. There have been several more recent studies in which chitin amendments have reduced Rhizoctonia disease severity. Glasshouse assays (Rajkumar *et al.*, 2008) have shown that the addition of chitin increased the populations and disease suppressive capacities of two fluorescent Pseudomonads antagonistic to an *R. solani* isolate causing damping-off of pepper. The addition of chitin to the biocontol treatments of two *Bacillus* spp. isolates and one *Trichoderma harzianum* isolate improved the suppression of *R. solani* pepper root-rot (Sid Ahmed *et al.*, 2003). Sultana *et al.* (2000) demonstrated that amending soil with crustacean chitin reduced *R. solani* infection on chickpea and sunflowers. Ellis *et al.* (1998) reported that a fine dust formulation of crab waste (270 to 1350 g chitin per m^2 equivalent) improved sugarbeet seedling emergence in *R. solani* infested soil. On potato plants, Davies *et al.* (2002) found that the addition of chitin at 'realistic commercial rates' reduced potato black scurf severity but not length of stem canker lesions. Lewis *et al.* (1996) successfully used chitin as a nutrient base in alginate prills of two biocontrol strains of *Trichoderma* spp. and one *Gliocladium virens* strain, and improved biocontrol of *R. solani* (AG 4) damping-off on zinnia. In addition, chitin was one of the best nutrient bases for *Trichoderma* spp. to reduce the survival of *R. solani* in infested beet seed. In addition to evidence of effect of chitin as an amendment, Sadeghi *et al.* (2006) demonstrated that the biocontrol potential of some *R. solani* antagonists (*Streptomyces* spp.) was linked, in part, to their chitinase producing capacity. Incubation of chitin in soil has been shown to disrupt both pathogenic and saprophytic activity of *R. solani* and was linked to increases in the population of actinomycetes (Henis *et al.*, 1967). Also, transgenic expression of fungal chitinolytic genes can confer resistance of plants to fungal pathogens, including *R. solani* (Kumar *et al.*, 2009). Introduction of a novel chitinase gene into a fluorescent pseudomonad enhanced its levels of control against rice sheath blight and cotton damping-off caused by *R. solani* (Xu *et al.*, 2004).

Chitosan, the de-acetylated form of chitin, is produced as a bio-fertiliser in Asia (El Hadrami *et al.*, 2010), and is more water soluble than chitin. Chitosan, like chitin, demonstrates antimicrobial (including suppression of several phytopathogenic fungi) and plant defence promoting capabilities. Mazaro *et al.* (2009), investigating chitosan as an elicitor of plant defence genes, found it reduced Rhizoctonia damping-off of beet seedlings. Chitosan can also chelate minerals and metals, reducing their availability to pathogenic fungi, but has also been reported to bind mycotoxins (El Hadrami *et al.*, 2010). When Palma-Guerrero *et al.* (2008) investigated the mycotoxic effects of chitosan, they found that chitosan amended fungal

growth agar reduced *R. solani* growth by about 30%, but the fungus would not grow on Water Agar amended with chitosan. However, the growth of two mycoparasitic *Trichoderma* spp. was strongly inhibited by chitosan amendments in agar media. Applications of chitosan to potato tubers have also been reported to decrease black scurf incidence (Kurzawinska and Mazur, 2008). If chitosan is able to reduce Rhizoctonia diseases of potato, it could potentially be through direct mycotoxic activity, stimulation of acquired resistance in host plants or possibly through mediating other physical processes in the soil, perhaps including changes in the microbial community. If use of chitosan becomes more popular globally, investigating the mechanisms of action of this compound would be of interest.

5.2.2.3 Biochar

Soil amendments with biochar (charcoal produced from biomass pyrolysis) have been the focus of recent research, as this material can act as a carbon sink as well as having positive effects on soil quality, fertility and leachates (McHenry, 2011). One biochar has been shown to reduce the leaching of at least one pesticide from soils, which can protect the surrounding environment, but it also slowed pesticide biodegradation and could reduce pesticide efficacy (Jones *et al.*, 2011). Biochar has also been shown to induce plant defences and protect against foliar pathogens when used as a soil amendment (Elad *et al.*, 2010). Graber *et al.* (2010) found that wood-derived biochar amendments enhanced pepper plant development, possibly due to promotion of plant health promoting rhizosphere communities. There have been several reports that biochars/charcoals, including wood charcoal, can increase beneficial plant-microbe interactions such as mycorrhizal partnerships, although these materials often had to be 'charged' with fertilizers, and could have a substantial impact on soil microflora (Ogawa and Okimori, 2010). Because biochars are porous and can retain soluble nutrients, 'charging' them with fertilisers means that factors such as the C:N ratio can be easily adjusted for studies. For example, Huber and Sumner (1996) summarised that the addition of N fertilisers to amendments with high C:N ratios and suppressive to *Rhizoctonia* spp. counteracted the suppressive effect. They also noted that nitrate sources of N tended to reduce disease severity, while ammonium sources increased severity. This could offer an interesting area of study should biochars demonstrate any suppression of Rhizoctonia diseases of potato. While there is little published work on the effects of biochar on suppression of soilborne phytopathogens, the influence they can have on soil structure, plant health and soil communities means they have potential to yield positive results in this field. If their use in agriculture grows, then understanding their influence on plant-pathogen interactions will be important.

5.2.3 Experimental design

Three experiments were carried out. Experiments 1 and 2 tested selected OMs at several concentrations to determine if relationships existed between concentration and disease expression. Experiments 1 and 2 were similar in design to the disease suppression bioassay designed in Chapter 3 and used in Chapter 4. Treatments consisted of pathogen and selected OM, applied to 0.9 L plastic pots filled with growing medium, prior to planting of minitubers. In Experiment 3, Pine bark compost was selected and tested for disease suppression at four concentrations, both non-sterile and sterilised. The concentrations were selected to produce treatments covering a range of Rhizoctonia disease severities, so that underlying microbial community factors could be investigated and correlated with disease expression as a way of identifying the mechanism of disease suppression (Borneman and Becker, 2007). The set-up of Experiment 3 also attempted to separate the growing medium under the influence of the plant roots (rhizosphere) from the medium not under direct plant influence (bulk) by growing the plants in pots with 20 µm nylon mesh covering their bases, sitting in larger planter bags of medium, to exclude plant-roots from the medium in the planter bag. This was to determine if the factors for disease suppression (if present) were due to changes in rhizosphere or bulk growth medium, or in both.

5.3 Methods

5.3.1 Pathogen inoculum

This was prepared as detailed in Chapter 4. Barley grain + V8® juice medium (Appendix A.2.1) was inoculated with agar plugs from the edge of a growing colony of isolate Rs043-2, and incubated for 2 weeks at 25°C in darkness. The inoculation rate used in all three experiments was 0.10% (w/v).

5.3.2 Organic matter details

The following seven OMs were used in these experiments:

1. Pine bark (*Pinus radiata*) compost: horticultural bark, grade 2 (South-Hort, NZ), sieved to 3.5 mm maximum particle size.
2. Garden Grow compost: composted pine bark plus cow and pig manures (South-Hort, NZ).
3. Organic Compost: composted pine bark plus cow manure (Daltons™, NZ).
4. Biochar: from a batch purchased in 2009 from David J. Underwood and Don Slater, made from pyrolysis (280-285°C at 5 Pa for 16 – 24 hours) of mainly kanuka wood (*Kunzea ericoides* A. Rich.), but also wood of pine (*Pinus radiata* D. Don.), macrocarpa (*Cupressus*

macrocarpa Hartw. ex Gord.) and black wattle (*Acacia mearnsii* De Wild.). The biochar was sieved to 3.5 mm maximum particle size.

5. 'Charged' Biochar was created as follows: Once sieved, 460 g of biochar was placed in 1 L of a 0.5% (v/v) solution of Nitrosol® original blood and bone fertiliser (N:P:K 8:3:6, Yates®, Auckland, NZ) for 3 h prior to experiment initiation.

6. Chitin: Poly-[1→4]-β-N-acetyl-D-glucosamine, practical grade from crab shells (Sigma®, St Louis, Mo).

7. Chitosan, which was prepared as follows: 30 g batches of chitin were added to 60 mL of a 50% (w/v) NaOH solution in RO water, and then made to 450 mL with acetone. This was maintained at room temperature for 4 days and stirred five times a day. The chitosan was separated from the deacetylating solution by vacuum filtration and rinsed five times with 100 mL RO water, then placed in a drying cabinet overnight.

8. Liquid Chitosan: Armour-Zen®, 144 g/L (Botry-zen Ltd, Dunedin, NZ), supplied by Dr Tony Reglinski, Plant and Food Research, Hamilton.

5.3.3 Fungicide treatment

Pencycuron (Monceren® 250 FS; Bayer CropScience) was used at full label rate, as calculated in Chapter 4, as 4.5 mL of concentrate for ten plants. In Experiment 1, the full label rate, made to 50 mL with tap water, as well as treatments of 50% (2.25 mL concentrate), 25% (1.13 mL), 12.5% (0.56 mL) and 6.25% (0.28 mL) label rate, each made to 50 mL with tap water, were used as treatments. In Experiment 2, only 4.5 mL concentrate made to 50 mL with tap water (full label rate) was used. In Experiment 3, 4.5 mL of concentrate (full label rate) was also used, made to 200 mL with tap water.

5.3.4 Potato plant propagules

In Experiment 1, minitubers (cv. 'Russet Burbank') were used. These were grown from tissue-cultured plantlets (Aspara Pacific Ltd., NZ) in seed-raising mix (Appendix A.3.1) in an insect-exclusion cage, and stored at 4°C for 8 months before planting. The minitubers were incubated at 18°C, 12:12 h light:dark, lighting: two 30W/860 Lumilux® Daylight lamps (Osram, Germany), for 2 weeks prior to planting to break dormancy. In Experiment 2, minitubers (cv. 'Desiree', Alex McDonald (Merchants) Ltd., NZ) were incubated as above prior to planting. In Experiment 3, small seed potatoes (cv. 'Russet Burbank', mean weight 16.7 g, Alex McDonald (Merchants) Ltd., NZ) were first washed free of soil, and tubers with noticeable damage or disease were discarded. Remaining tubers were surface sterilised (5 min

in 2% sodium hypochlorite solution, then rinsed in tap water) before incubation as above prior to planting to break dormancy.

5.3.5 Amendment selection assays (Experiments 1 and 2)

For **Experiment 1**, required weights of OM were pre-weighed into plastic buckets (58, 115 and 230 g for chitin, pine bark compost, biochar and biochar+fertiliser, plus 460 g for all except chitin) and buckets made to 10 L with soil:pumice mix (Appendix A.3.3) – giving concentrations of OM of 5.8, 11.5, 23 and 46 g/L. These concentrations were calculated to be equivalent to 0.25, 0.5, 1.0 and 2.0 t/ha respectively. The contents of each bucket was tipped onto clean plastic sheeting, and pre-weighted pathogen-infested barley (10.0 g) was mixed in by hand. Ten 0.9 L plastic pots with individual saucers were then filled with the inoculated medium and placed in a randomised block design (ten blocks, one replicate per block) in a temperature controlled greenhouse cell at Plant and Food Research Ltd. (Lincoln), with glasshouse conditions as detailed in Chapter 3. After 2 weeks, pre-sprouted minitubers (cv. 'Russet Burbank') were planted one per pot at *ca.* 30 mm depth. Pots were watered as required to maintain moisture, and once plants had reached approx. 20 cm in height they each received 30 mL of a 0.01% solution of High NK™ liquid fertiliser (8:6:13.5 N:P:K, Agrichem, Australia) twice each week to prevent nutrient deficiency. Plants were harvested 8 weeks after planting (see 5.3.7).

Experiment 2 was conducted using the same methods as Experiment 1, using the following OMs; three composts (pine bark, garden grow and Dalton's organic) each at 46 and 92 g/L (equivalent to 2.0 and 5.0 t/ha), chitin and chitosan both at 58 g (equivalent to 0.25 t/ha) as well as 1.6 mL and 16 mL of liquid chitosan concentrate (equivalent to 7 and 70 L/ha concentrate, both made to 50 mL with tap water). After 2 weeks, pre-sprouted minitubers (cv. 'Desiree') were planted one per pot at *ca.* 30 mm depth. Pots were watered, fertilised and harvested as in Experiment 1.

5.3.6 Pine bark compost experiment (Experiment 3)

Four weights of pine bark compost (0.69, 1.38, 2.76 and 5.52 kg) and sterile (autoclaved at 121°C, 15 min, 15 psi) pine bark compost were dispensed into containers and made up to 30 L with soil:pumice mix (Appendix A.3.3). Final concentrations of the OMs were 23, 46, 92 and 184 g/L, equivalent to 3, 6, 12 and 24 t/ha. Pre-weighed pathogen inoculum (30 g) was mixed thoroughly into each treatment by hand. For each treatment, ten 3 L planter bags were half filled with medium, then a 0.9 L plastic plant pot with base removed and replaced with 20 μm nylon mesh (Sefar Filter Specialists Ltd., Auckland) was placed in each bag. Each pot and

remaining bag volume were filled with medium, and each placed in a temperature controlled greenhouse unit in a randomised block design (ten blocks, one replicate per block) along with positive and negative controls and fungicide treatment. After 2 weeks, pre-sprouted small seed potatoes (cv. 'Russet Burbank', detailed above) were planted at *ca.* 50 mm depth, one per pot. Plants were watered, fertilised and harvested as in Experiment 1.

5.3.7 Harvest procedure

For all three experiments, plants roots were washed free of growing medium and the following parameters recorded for each plant: number of stolons (total and number with lesions), number of stolon tips (total and number 'nipped'), number of tubers (total and number malformed or damaged by lesions), total tuber weight and dry weight of roots and shoots. For Experiment 3, first the 0.9 L pots were removed from the planter bags, growing medium samples (*ca.* 100 g) were them collected from each planter bag, directly below the pot. The potato plant was then removed from the pot and a further sample taken from the growing medium within the pot.

5.3.8 Community metabolic profiling (Experiment 3)

The growing medium samples collected at harvest (Experiment 3) were processed as follows: For each treatment, 2 g sub-samples of medium were taken from each of the ten replicate pots (bulk and rhizosphere separately) and combined in two 250 mL capacity conical flasks, five sub-samples per flask. Each flask was made up to 100 mL with 0.1% physiological agar (Appendix A.1.11), sealed with Parafilm® M (Brand GmbH, Germany) and shaken at 500 oscillations/min on a Stuart® SF1 flask shaker (Keison International Ltd., UK) for 5 min, creating two replicate 1×10^{-1} g/ mL suspensions for bulk and rhizosphere growth medium for each treatment. The two replicates were then combined to make one suspension each for bulk and rhizosphere. These were both serially diluted by pipetting 2 mL into 18 mL of sterile physiological salt solution (0.85% NaCl) three times to achieve 1×10^{-4} g/ mL. This final dilution was used to inoculate a 96 well EcoPlate™ (Biolog Inc., Hayward, CA, Figure 5.1), at 100 µL per well, with two plates per treatment – one bulk growth medium, one rhizosphere growth medium. Plates were incubated at 25°C in darkness and the Absorbance at 590 nm for each well was recorded every 24 h for 9 days on a Microplate reader – SpectraMax 190 (Molecular Devices, LLC, US), using Software – SoftMax Pro v5.4.3.001 (Molecular Devices, LLC, US).

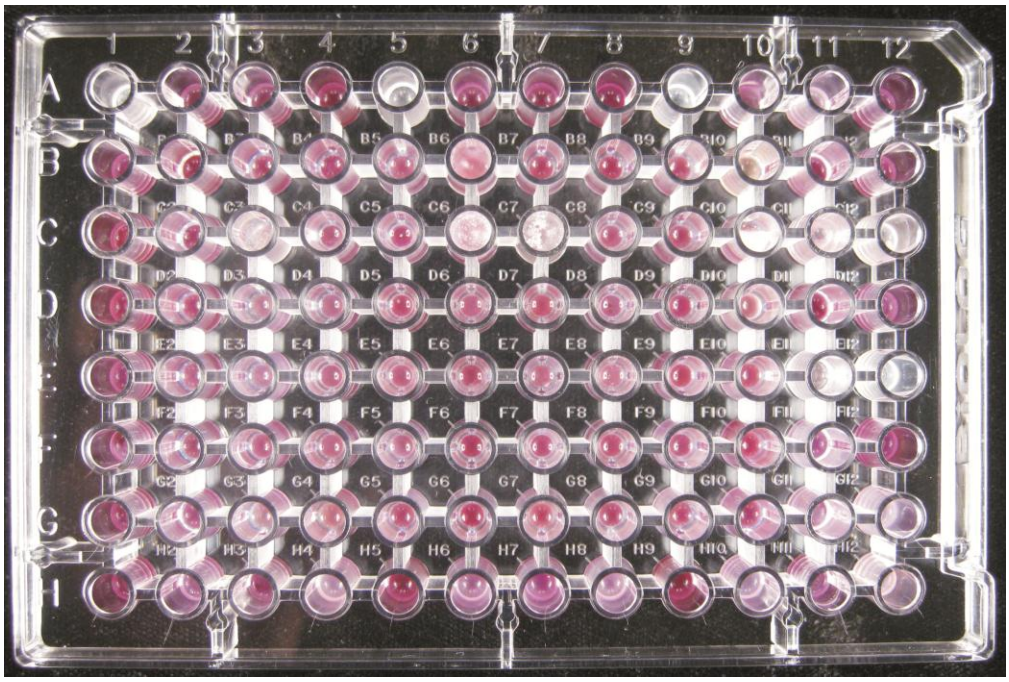

Figure 5.1 Example of a 96 well EcoPlate™ (Biolog Inc., Hayward, CA) for analysis of microbial community metabolic profiling.

5.3.9 Statistical analysis

All data were analysed by ANOVA and treatment means were compared using Fisher's unrestricted LSD at $P < 0.05$. If data did not satisfy the parametric assumption of normal distribution they were transformed (square root for count data, $\log_{10}$ for weights) prior to analysis. In Experiment 1, the data was also analysed as a 4 × 4 factorial experiment, four treatments (Pine bark compost, biochar, biochar+nitrosol and pencycuron) and four rates, for which linear and quadratic components were included in the analysis.

For Experiment 3, EcoPlate™ data for day 3 reading time first had average blank value (water well) subtracted from all plate values. Then mean well absorbance (average well colour development; AWCD) as well as number of wells exceeding a value of 0.25 (community metabolic diversity; CMD) were calculated for each of the three replicates per plate. AWCD and CMD data were then subjected to ANOVA using Fisher's unrestricted LSD ($P < 0.05$) to compare treatment means. Regression analyses were conducted for group means of each Rhizoctonia disease or plant parameter with rhizosphere and bulk AWCD and CMD group means.

5.4 Results

5.4.1 Experiment 1

In Experiment 1, the majority of OM treatments had no significant positive effects on the disease parameters measured (Table 5.1). The greatest rate of chitin increased the number and proportion of symptomless stolons, but this did not translate into a yield benefit, as no tubers resulted from this treatment. The greatest rate of pine bark compost increased the total number of symptomless stolons, and also increased the number and total weight of tubers as well as the proportion of symptomless tubers.

When the data were separately subjected to factorial analysis of variance (four amendments and four rates; arbitrarily represented by 12.5, 25, 50 and 100) with linear and quadratic polynomial components included, the overall effect of amendment type was significant (all $P < 0.001$) for all parameters measured, but rate was not. However, the linear component probabilities with rate were $P = 0.061$for percent diseased stolons, $P = 0.069$ for the number of healthy stolons (square root transformed), and $P = 0.062$ for the number of symptomless tubers (square root transformed). So for these three parameters, data were subjected to separate analyses for each amendment type with rate, and linear and quadratic components included in the analyses. Only the linear component of the pine bark compost treatment with rate was significant for percent diseased stolons ($P = 0.05$, Figure 5.2 a) and number of symptomless stolons ($P = 0.01$, Figure 5.2 b). The linear component was also significant for number of symptomless tubers ($P = 0.04$), as was the quadratic component of pencycuron with rate ($P = 0.05$, Figure 5.2 c).

5.4.2 Experiment 2

In Experiment 2, overall shoot nipping was low in the positive control, and two OM treatments (pine bark and Garden Grow composts at 5 t/ha) had significantly more nipped shoots than the positive inoculum control (Table 5.2). Both concentrations of pine bark compost, as well as the chitin treatment reduced percent diseased stolons, but of these only the chitin (plus Dalton's compost at 5 t/ha) increased the number of symptomless stolons, compared to the positive control. Only the pine bark compost treatments reduced the percent of nipped stolon tips, and only Dalton's compost increased the number of symptomless tubers. The treatment of liquid chitosan at 7 L/ha increased the number of tubers, and pine bark compost at 5 t/ha produced low numbers of tubers, but was not significantly different to the positive inoculum control. No OM treatment was significantly different to the positive control for total weight of tubers.

Table 5.1 Experiment 1 treatment means and ANOVA results for Rhizoctonia disease and physical parameters of potato plants grown from minitubers in growth medium amended with several rates of selected OMs

Treatment	Rate	Nipped shoots	No. stolons	Diseased stolons (%)	No. symptom-less stolons	Stolon tips nipped (%)	No. tubers	No. symptom-less tubers	Total tuber weight (g)	Shoot dry weight (g)	Root dry weight (g)
Neg. control		0.0 a[+]	8.1 abcd	0.2 a	8.1 a	0.2 a	3.7 f	3.7 b	10.4 g	3.2 abc	1.03 a
Pos. control		2.7 cde	6.28 efg	76.7 defg	1.4 cde	54.5 cd	1.4 bc	0.3 ef	1.3 cde	2.7 de	0.79 cdefg
Chitin (t/ha)	0.25	2.6 cde	6.6 cdefg	66.6 cd	2.4 bc	58.6 cd	0.3 ab	0.1 ef	0.1 ab	3.2 abc	0.83 cdefg
	0.50	1.8 cd	5.8 efg	67.5 cde	1.8 bcd	55.8 cd	0.0 a	0.0 f	0.0 a	3.5 abc	0.70 efg
	1.00	2.2 cd	8.8 abcd	31.4 b	5.8 a	20.1 b	0.0 a	0.0 f	0.0 a	3.8 a	0.94 ab
Pine bark compost (t/ha)	0.25	1.8 cd	6.6 cdefg	84.9 g	0.9 de	51.1 cd	2.4 de	0.3 def	1.4 ef	2.6 de	0.72 defg
	0.50	2.0 cd	6.2 efg	84.7 fg	0.8 e	53.0 cd	2.1 bcd	0.4 def	1.6 bcde	2.5 de	0.78 g
	1.00	1.8 cd	6.1 efg	64.2 c	2.1 bc	44.7 cd	1.9 abcd	0.7 def	1.5 cde	2.5 de	0.76 bcdefg
	2.00	3.1 de	7.4 bcdef	68.2 cde	2.4 b	39.9 c	3.3 ef	1.0 c	4.5 f	2.8 bcde	0.84 bcdefg
Biochar (t/ha)	0.25	1.8 cd	7.6 abcdef	82.1 defg	1.2 cde	50.4 cd	2.0 cde	0.3 def	1.8 def	2.9 bcde	0.92 abc
	0.50	2.1 bc	6.2 efg	83.7 efg	1.0 bcde	55.1 cd	1.6 bcd	0.2 def	1.6 cde	2.9 bcd	0.79 bcdefg
	1.00	2.3 cde	8.1 abcde	73.8 defg	2.5 bcde	59.1 cd	1.7 cd	0.7 cd	1.6 cde	2.8 bcde	0.82 cdefg
	2.00	2.6 cd	6.1 efg	68.5 cdef	1.9 bcd	50.1 cd	1.6 bcd	0.2 ef	1.5 cde	2.6 de	0.77 bcdefg
Biochar + Nitrosol (t/ha)	0.25	4.3 e	4.8 g	72.1 defg	1.1 bcde	63.8 d	1.0 abc	0.1 ef	1.1 bcde	2.2 e	0.70 fg
	0.50	3.0 cde	6.5 defg	69.6 cdefg	1.9 bcde	48.3 cd	1.6 bcd	0.4 de	0.5 abcd	2.9 bcde	0.86 bcdef
	1.00	2.3 cde	5.4 fg	76.7 cdefg	1.3 bcde	47.3 cd	1.5 bcd	0.2 def	0.8 bcde	2.8 cde	0.82 bcdefg
	2.00	2.7 cde	6.5 defg	78.4 cdefg	1.2 bcde	56.0 cd	1.4 bcd	0.1 ef	0.3 abc	3.0 bcd	0.82 bcdefg
Pencycuron (L/ha)	2.5	0.2 ab	9.9 a	12.6 a	8.7 a	11.5 ab	4.0 fg	3.4 b	10.9 g	3.5 ab	1.08 a
	5.0	0.1 ab	8.9 abc	7.9 a	8.4 a	4.0 ab	4.3 fg	4.3 ab	11.2 g	3.0 abcd	0.93 ab
	10	0.0 a	9.3 ab	5.6 a	8.9 a	0.0 a	5.1 g	4.9 a	10.5 g	3.2 abcd	0.90 abcd
	20	0.0 a	8.7 abcd	3.0 a	8.5 a	0.0 a	4.5 fg	4.5 ab	11.0 g	3.2 abcd	0.90 abcde

[+]Means accompanied by the same letter are not significantly ($P < 0.05$); using Fisher's unrestricted LSD.

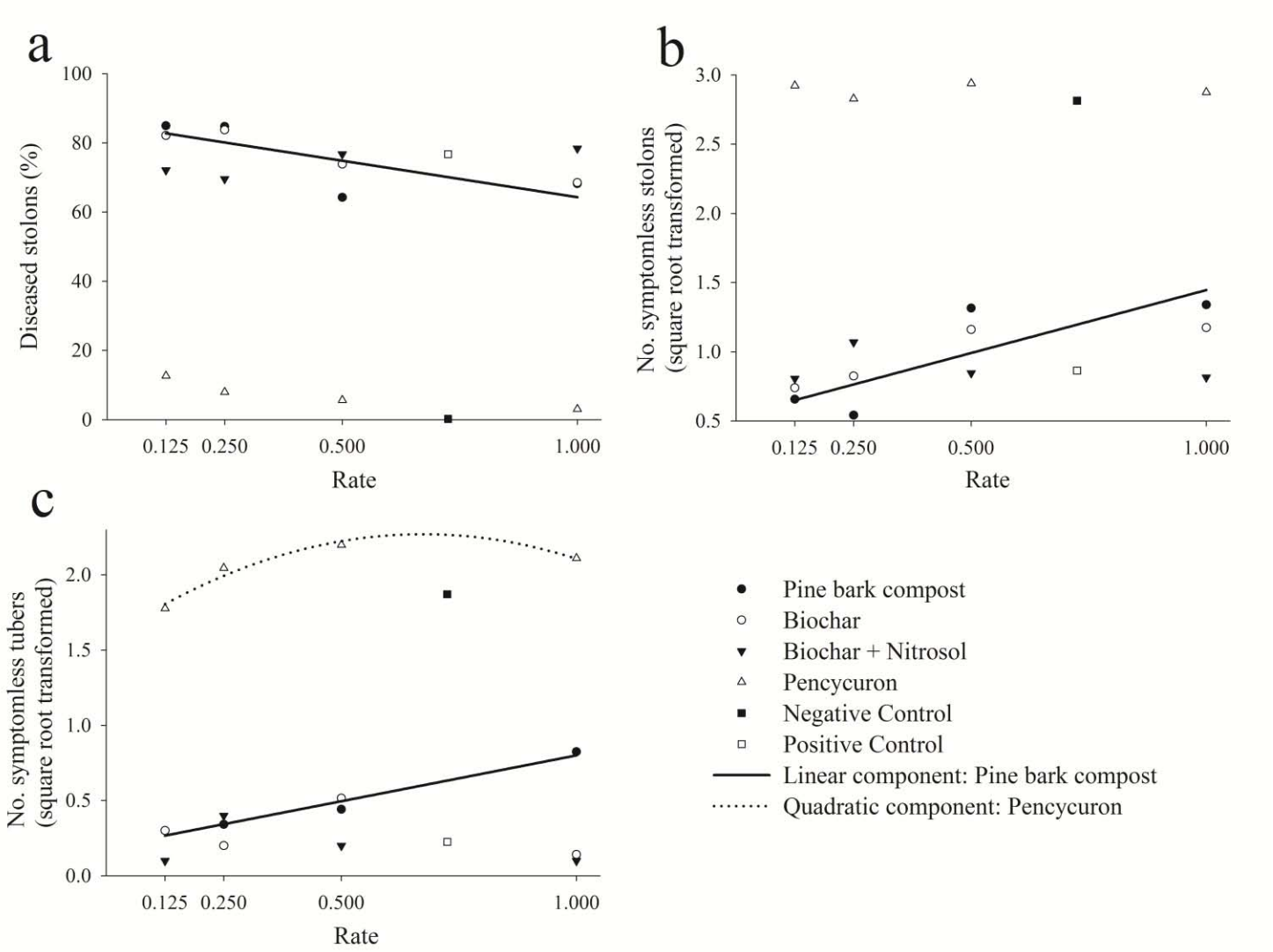

Figure 5.2 Treatment means of Rhizoctonia disease parameters from factorial analysis of Experiment 1 data, including significant poynomial components of rate, for four rates of selected organic amendments and the fungicide pencycuron

Table 5.2 Experiment 2 treatment means and ANOVA results for Rhizoctonia disease and physical parameters of potato plants grown from minitubers in growth medium amended with selected OMs

Treatment	Rate	Nipped shoots	No. stolons	Diseased stolons (%)	No. symptom-less stolons	Stolon tips nipped (%)	No. tubers	No. symptom-less tubers	Total tuber weight (g)	Shoot dry weight (g)	Root dry weight (g)
Neg. control		0.0 e[+]	23.9 a	1.3 a	24.1 a	0.4 a	11.6 a	11.5 a	33.8 a	1.7 ab	0.29 bcd
Pos. control		0.4 de	18.2 a	81.6 d	3.0 cd	52.8 c	7.2 cde	2.9 de	6.9 bc	1.6 bc	0.29 cd
Pine bark	2	0.9 abcd	15.8 a	57.8 bc	4.3 bcd	31.8 b	7.1cde	3.3 de	9.2 bc	1.1 cd	0.20 d
compost (t/ha)	5	1.3 ab	14.7 a	57.4 bc	3.8 bcd	33.4 b	3.1e	1.5 de	5.2 bc	0.8 d	0.18 d
Dalton's Org.	2	0.1 e	14.0 a	73.5 bcd	3.1 bcd	47.7 bc	5.0 de	1.5 e	3.1 c	1.7 abc	0.28 abcd
Comp. (t/ha)	5	0.7 bcde	19.4 a	65.2 bcd	7.1 b	43.8 bc	9.3 abcd	6.8 bc	9.5 bc	1.3 bcd	0.25 cd
Garden Grow	2	1.0 abcd	15.3 a	75.6 cd	2.1 d	50.2 bc	7.6 bcde	1.8 de	7.1 bc	1.9 ab	0.29 abcd
Comp. (t/ha)	5	1.7 a	16.1 a	80.1 cd	4.2 bcd	50.2 c	7.5 de	3.8 cde	5.2 c	1.6 abc	0.27 abcd
Chitin (t/ha)	0.25	1.2 abc	17.9 a	53.5 b	7.0 b	39.9 bc	5.4 cde	1.8 de	6.9 bc	2.2 a	0.41 a
Chitosan (t/ha)		0.4 cde	24.0 a	82.9 d	3.7 bcd	52.6 c	7.1 cde	1.8 de	4.2 c	2.2 a	0.40 ab
Liquid chitosan	7	0.0 e	20.8 a	85.2 d	3.4 bcd	46.5 bc	11.5 ab	4.1 bcd	9.0 bc	2.3 a	0.38 abc
(L/ha)	70	0.7 bcde	20.9 a	75.9 cd	5.8 bcd	46.3 bc	8.9 abcd	4.2 cde	12.5 b	2.0 ab	0.36 abc
Pencycuron(L/ha)	20	0.0 e	21.3 a	0.5 a	21.2 a	0.2 a	10.4 abc	9.6 ab	28.8 a	1.6 abcd	0.25 cd

[+]Means accompanied by the same letter are not significantly ($P < 0.05$); using Fisher's unrestricted LSD.

5.4.3 Experiment 3

In Experiment 3, shoot nipping was noticeably greater than in Experiments 1 and 2. At 3, 6 and 12 t/ha, neither OM significantly altered the number of shoots nipped, while both OMs at 24 t/ha gave more shoots nipped than the positive inoculum control (Table 5.4). Neither OM recovered the reduction in number of stolons in the positive inoculum control at any rate, and non-sterile compost at 24 t/ha gave significantly fewer stolons. Non-sterile compost at 3 and 6 t/ha, and sterilised compost at 3 and 24 t/ha, reduced percent diseased stolons and percent stolon tips nipped. No OM treatment gave numbers of symptomless stolons that were significantly different to the positive inoculum control. The shoot and root dry weights from both composts at 24 t/ha were significantly less than from the positive control. Very few tubers were present on the plants at harvest.

From the pattern of AWCD and CMD over the course of 9 days after inoculation (Figure 5.3), the day 3 time point was selected for ANOVA and regression analysis, as the CMD (Figure 5.3 c, d) generally began to plateau after that point.

The analysis of overall soil community metabolism at time of harvest in this experiment (Table 5.3) showed that in the rhizosphere, the greatest rate of compost, and the two greatest rates of sterile compost gave greater metabolic activity than either the inoculated or non-inoculated controls, or the pencycuron treatment. In the bulk soil the greatest rates of both composts gave greater metabolic activity than either control, but the other rates did not. Analysis of the community metabolic diversity demonstrated that in the rhizosphere samples, only the two greatest rates of sterilised pine bark compost gave diversities greater than both of the control means. In the bulk samples, only the greatest rate of sterile compost gave greater metabolic diversity than either control.

Table 5.3 Catabolic profile analyses of growing media from Experiment 3. Mean values for average well colour development (AWCD) and community metabolic diversity (CMD) for rhizosphere and bulk growing medium samples.

		AWCD		CMD	
Treatment	**Rate**	Rhizosphere	Bulk	Rhizosphere	Bulk
Neg. control		0.19 de	0.23 cd	9.7 d	11.0 b
Pos. control		0.18 de	0.24 cd	10.7 cd	12.7 b
Pine bark compost (t/ha)	3	0.23 bcd	0.26 bc	12.3 bc	13.0 ab
	6	0.22 bcd	0.18 d	12.3 bc	10.7 b
	12	0.20 cde	0.24 cd	11.3 bcd	12.3 b
	24	0.25 abc	0.35 a	11.7 bcd	14.0 ab
Sterile pine bark compost (t/ha)	3	0.22 bcd	0.28 abc	12.0 bc	13.3 ab
	6	0.20 cde	0.25 bcd	12.0 bc	12.3 b
	12	0.26 ab	0.24 cd	13.0 ab	12.7 b
	24	0.27 a	0.32 ab	14.7 a	16.3 a
Pencycuron(L/ha)	20	0.17 e	0.25 bcd	11.7 bcd	14.0 ab

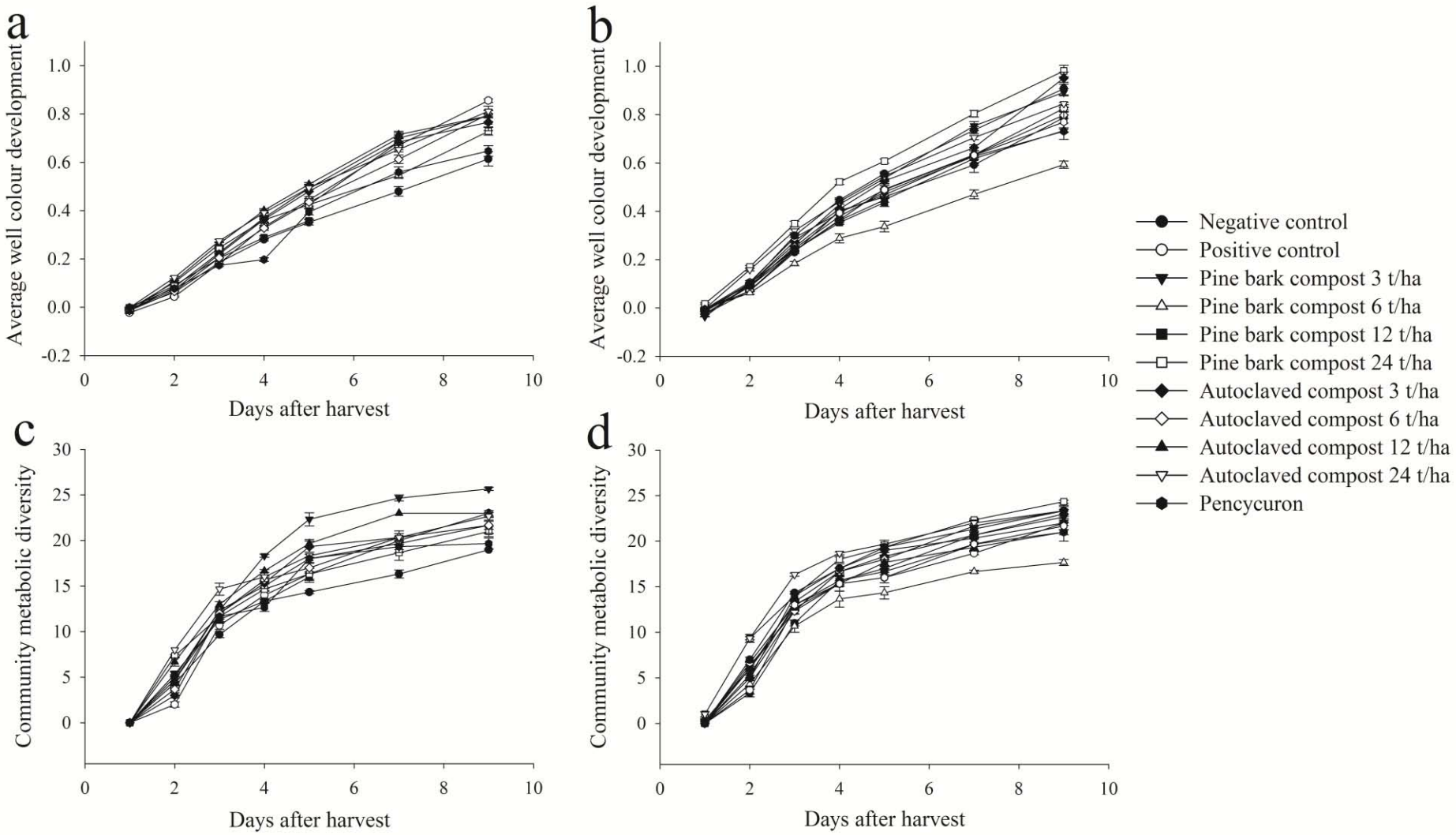

Figure 5.3 Experiment 3 substrate utilisation analysis of soil samples using Ecoplate™ (Biolog Inc., Hayward, CA). Daily absorbance (590 nm) readings used to calculate average well colour development of (a) rhizoshere and (b) bulk growing medium samples. Community metabilic diversity of (c) rhisosphere and (d) bulk growing medium samples over 9 days of incubation.

Table 5.4 Experiment 3 treatment means and ANOVA results for Rhizoctonia disease and physical parameters of potato plants grown from seed tubers in soil mix amended with non-sterile and sterile pine bark compost at four rates[+]

Treatment	Rate	Nipped shoots	No. stolons	Diseased stolons (%)	No. symptom-less stolons	Stolon tips nipped (%)	Shoot dry weight (g)	Root dry weight (g)
Neg. control		0.0 a	7.6 a	2.5 a	7.5 a	2.5 a	4.4 e	1.07 e
Pos. control		4.3 b	3.7 b	44.6 e	2.3 cde	38.0 d	2.9 cd	0.69 d
Pine bark compost (t/ha)	3	4.6 bc	2.4 bc	20.5 abc	1.7 cde	14.7 abc	2.9 d	0.61 bcd
	6	6.7 bc	2.3 bc	23.5 bcd	1.5 de	12.4 abc	2.5 bcd	0.67 cd
	12	3.5 b	3.6 b	48.7 de	1.5 cde	34.2 cd	3.2 d	0.67 cd
	24	7.3 c	1.8 c	25.9 bcde	1.3 e	17.7 abcd	0.4 a	0.29 a
Sterilised pine bark compost (t/ha)	3	5.1 bc	3.6 b	16.0 abc	2.7 bc	12.7 abc	2.5 cd	0.61 bcd
	6	4.7 bc	3.7 b	34.5 cde	2.4 cd	26.4 bcd	2.5 bcd	0.70 cd
	12	4.8 bc	3.7 b	35.3 cde	2.5 cd	28.8 cd	2.0 bc	0.49 abc
	24	7.1 c	2.7 bc	6.4 ab	2.6 cde	3.1 ab	1.7 b	0.40 ab
Pencycuron(L/ha)	20	0.1 a	6.2 a	14.4 abc	5.1 ab	16.7 abc	4.4 e	1.07 e

[+]Means accompanied by the same letter are not significantly ($P < 0.05$); using Fisher's unrestricted LSD.

Table 5.5 Regression analyses of mean Rhizoctonia disease and plant parameters vs. mean catabolic parameters[+]

Plant parameter	Soil division	Catabolic parameter	*P* value	R^2 value	Correlation
Nipped shoots	Rhizosphere	AWCD	0.03	0.52	positive
No. stolons	Rhizosphere	AWCD	0.09	0.36	negative
Shoot dry weight	Rhizosphere	AWCD	0.08	0.38	negative
	Bulk	AWCD	0.03	0.52	negative
Root dry weight	Rhizosphere	AWCD	0.03	0.51	negative
	Bulk	AWCD	0.01	0.66	negative
		CMD	0.04	0.48	negative
Rate	Bulk	AWCD	0.06	0.48	positive
		CMD	0.06	0.47	positive

[+]Only statistically significant regressions are presented

5.5 Discussion

The results of Experiments 1 and 2 demonstrated that, at the rates tested, none of the selected OM amendments were strong suppressors of Rhizoctonia diseases of potato. However, because analysis of the application rates for individual OMs in Experiment 1 demonstrated that there was a significant linear relationship between increasing the rate of pine bark compost and decreasing Rhizoctonia disease symptoms (percent diseased stolons, number of symptomless stolons and number of symptomless tubers), this amendment was selected for testing in Experiment 3. The concentrations of pine bark selected for Experiment 3 were the greatest two from Experiment 1 (23 and 46 g/L) and two further doublings of concentration (92 and 184 g/L). This was to generate treatments covering a range of potential disease suppression levels, and to then determine if any physical or microbiological factors could be identified as mechanisms of disease suppression. The sterilised pine bark compost was included to help determine if any suppressive effects were due to the introduction of organisms on the OM, or alternatively through stimulation of soil communities. However, the results did not show a range of suppression levels which warranted further analysis of factors underpinning disease suppression (no treatment reduced shoot nipping or increased the number of symptom-less stolons). While it may appear that the greatest rate of sterilised compost reduced symptoms on stolons, this treatment also gave greater nipping of emerging shoots and reduced shoot and root dry weights, so this medium cannot be considered a disease suppressor. As described in a review by Hoitink and Boehm (1999), lignocellulosic material with greater cellulose content can stimulate *R. solani*, even though antagonist populations are increased, leading to disease. In spite of the pine bark having been composted, the total amount of cellulose remaining after composting may have been too great to stimulate antagonists of *R. solani* without also stimulating the pathogen. This may explain the greater shoot nipping at the greatest rates of pine bark compost in Experiment 3. It could be that more mature bark composts with lower amounts of cellulose, or lignin extracts, would not induce this response.

The analysis of the community metabolic activity and metabolic diversity of samples taken from the 'rhizosphere' and 'bulk' growth medium of each treatment in Experiment 3 was conducted at the time of harvest, before analysis of Rhizoctonia disease and plant physical parameters had ruled out the need for further study. One of the main findings from the regression analyses between mean disease or plant parameters and catabolic parameters was the lack of relationship between disease parameters and levels of metabolic activity and diversity. Several other studies have found absence of relationships between total microbial

activity or general diversity and levels of disease suppression. These results reinforce the assertion that suppression of Rhizoctonia diseases is due to the activity of specific components of soil communities, rather than stimulation of the whole communities (Bonanomi *et al.*, 2010). In addition, a study by Temorshuizen *et al.* (2006), which compared the suppressiveness of 18 composts against seven soilborne phytopathogens, demonstrated that one OM amendment could have contrasting effects on disease levels for different *R. solani* pathosystems: what was suppressive in one could be conducive in another. This is further indication that the suppression of *Rhizoctonia* spp. is quite specific, and unlikely to be governed by general increases in microbial activity. In the present study, increases in OM rate correlated with increases in the AWCD and CMD of bulk growth medium samples, but not rhizosphere samples. This indicates that the host plants influenced the microbial communities at levels strong enough to mask or negate the effects of the OMs. Even if an OM leads to background general community population increases, this might not be relevant in the infection courts where plant exudates dominate.

When Scheurerell *et al.* (2005) investigated factors responsible for Rhizoctonia disease suppression in their suppressive composts they found that disease levels were not related to any single physical, chemical or biological factor – including: compost temperature, water content, particle size distribution, pH, electrical conductivity, levels of ammonium- or nitrate-N, total C, N, Ca, Mg, K, P, Fe and Mn, ammonia volitization, respiration and respiration potential, concentrations of culturable bacteria, actinomycetes, yeast, fungi or *Trichoderma* spp. These authors did, however, report that the reduction of of Rhizoctonia damping-off from dairy fir-bark compost was increased (50 to 93%) when compost was inoculated with *T. hamatum* (T 382) 5 days prior to assay initiation. This reduction was less (71%) when inoculation occurred at assay initiation. This supported the hypothesis that *R. solani* suppression is not related to the general microbial populations, but corresponds to populations of specific fungal (and bacterial) antagonist species. Previous work on T 382 by Kwok *et al.* (1987) found that in plant growth media amended with composted hardwood tree bark, inoculation with combinations of the *Trichoderma* and antagonistic bacteria offered consistently greater suppression than T 382 alone. Similarly, in the study by Krause *et al.* (2001), addition of composted pine bark greatly enhanced the disease suppression capacity of two commercial biocontrol products ($C_{299}R_2$, a *Chryseobacterium gleum*, and T 382), while composted bark alone gave inconsistent disease suppression.

The conclusion that Rhizoctonia disease suppression is mediated by populations of specific microorganisms is supported by other studies. For example, when investigating differences

between fungal communities from hardwood bark composts suppressive and conducive to Rhizoctonia damping-off, Kuter *et al.* (1983) found that disease suppression was not associated with a single fungal group, although populations of *Trichoderma* spp. were implicated in suppression. Recently, Pugliese *et al.* (2011) demonstrated that composts (from urban sludge) without inherent capacity to suppress Rhizoctonia diseases became suppressive when inoculated with biocontrol *Trichoderma* strains.

Several authors have suggested that combinations of organic amendments and biocontrol organisms may be viable options for more consistent disease suppression. However, to attempt this with the amendments and isolates investigated in the present study would likely give negative results, for the following reason: None of the isolations were conducted with compatibility with specified organic matter amendments in mind, and there is therefore no information as to which, if any, would thrive in soil augmented with those amendments. The following section on future prospects covers a different potential starting point for a biocontrol strategy combining organic matter amendments and suppressive microorganisms.

5.5.1 Future research

It has been suggested that in order to ensure strong, consistent biological suppression of diseases caused by soil-borne pathogens like *R. solani*, it is necessary to combine suppressive microorganisms with organic matter amendments which will support their activity. The following strategy is suggested for targeted selection of OMs and organisms to generate appropriate combinations with a strong probability of achieving effective reduction of Rhizoctonia diseases of potato, with consideration of the economics of large-scale implementation. Firstly, abundant, low-cost organic amendments (e.g. chitin from seafood industries, lignin from paper production, barks and sawdust from timber production) should be selected. Selective media (agars) based on their most abundant component (e.g. chitin, lignin or cellulose) should be used for biocontrol agent selection. Thirdly, the amendment should be incubated in soils (perhaps from a variety of backgrounds, such as conventional and organic agricultural fields, as well as non-cultivated soils) along with pathogen inoculum. Next, potato plants would be grown in the incubated media, and plants not exhibiting disease symptoms identified. Isolations of microorganisms would then be conducted (using the developed selective media) from soil as well as from the host plant infection courts. This would generate a collection of organisms which utilise the amendment as a nutrient base. After discarding all isolates not amenable to common culture methods, those with phytotoxic effects, and those likely to be plant or human pathogens, the remaining isolates would be screened in rapid *in vivo* disease suppression assays. These assays should incorporate the

amendment (inoculated with test organisms) at concentrations which would be financially realistic when scaled up to crop areas used in conventional agricultural. The assays should have short run-times for high-throughput (e.g. only focus on shoot nipping, the first symptom of Rhizoctonia disease of potato), and isolates from different taxonomic groups should be tested in appropriate factorial design experiments. Thus, individual isolates, or combinations of isolates which suppress disease, could be determined. This process would be rapid, and should generate small cohorts of microbial isolates which perform well as biocontrol agents, directly supported by economically realistic amounts of organic amendment. These would then be tested and developed with good prospects for practical efficacy and use.

Conclusions

The types of organic amendments tested here were selected from results in published accounts of suppression of Rhizoctonia diseases. However, none provided sufficient disease control to be classified as suppressive, so no in depth investigation of underlying disease control mechanisms was warranted. Furthermore, no relationship was detected between most disease parameters and general microbial activity or metabolic diversity in the rhizosphere or bulk growth medium partitions from potato plants. Biological control of Rhizoctonia diseases is often associated with populations of specific taxa (rather than general increases in soil communities), which would either need to be already present in the soil, or in the OM amendment, for the amendment to have a suppressive effect. A commonly prescribed solution to produce, increase or reduce variability of biocontrol is to pre-inoculate OM with known BCAs. This was not investigated in the present study, as neither the isolates screened in Chapter 4, nor the amendments investigated here, demonstrated disease suppression strong enough to be considered suitable for further study.

Chapter 6
The impact of crop rotation on bacterial and fungal communities, *Rhizoctonia solani* populations and Rhizoctonia diseases of potato

6.1 Abstract

Crop rotations are regularly practiced to limit the build-up of soil-borne phytopathogens, including *Rhizoctonia solani*. The impact of different crop rotations on soil microbial communities is less well studied. In the present study, four different crop rotations from a long-term trial site in New Zealand were investigated for their impact on soil bacterial and fungal communities, using culture dependant and independent techniques, as well as for their impact on *R. solani* populations. The effect of crop rotation on the expression of Rhizoctonia canker symptoms and populations of inoculated *R. solani* was also investigated in a greenhouse assay. Since host plant monoculture has been demonstrated to occasionally lead to the induction of soils suppressive to soil-borne phytopathogens, the present study aimed to determine if potato monoculture had caused the development of a soil suppressive to Rhizoctonia diseases of potato. The results demonstrated that there had not been a measurable build-up of *R. solani* in any of the rotations, including one which had potato monoculture for eight successive growing seasons. Soil community structure was influenced more by the identity of the most recent crop than the rotation crop sequence. Rotation type did not influence the expression of Rhizoctonia canker symptoms in the greenhouse assay using soil from the field trial. This study demonstrates that soil bacterial and fungal communities can be very resistant to influence by crop rotation practice, and, for the soil type examined, potato monoculture did not result in either a build-up of *R. solani* populations, or the induction of Rhizoctonia disease suppression.

6.2 Introduction

6.2.1 Crop rotations and Pukokohe trial

Crop rotations can be a practical method for preventing build-up of pathogen inoculum in agricultural soils, as well as improving soil structure, soil microbial community diversity and activity (see Chapter 1). Historically, crop rotations were normal practice for maintaining soil "health". With the advent of synthetic fertilisers and pesticides, however, continuous monoculture or short intervals between specific crops have become commonplace in

agriculture, often with consequent build-up of soilborne pathogen inoculum. One phenomenon which can arise as a result of continuous high presence of pathogen populations is disease decline. In these situations, either pathogen populations rise to a peak and then subsequently recede to a level where they no longer cause severe disease, or populations remain high but disease symptoms on plants decline over time. In these situations there has been no alteration of land management practice, and the origins of this disease decline have been proposed to be biological in nature, and therefore fit well into the overall gamut of the present research project.

One of the best studied examples of monoculture leading to disease decline is take-all decline in wheat, where many examples of spontaneous decline in disease severity (caused by the pathogen *Gaeumannomyces graminis* var. *tritici*) were found to correlate with increases in populations of Pseudomonads antagonistic to the pathogen. Antagonism has been demonstrated both in *in vitro* and *in vivo* assays (Borneman and Becker, 2007).

While not as common, there have been a few reports of Rhizoctonia disease decline in agriculture. In Japan, monoculture of sugar beet resulted in decline of root rot severity caused by *R. solani* AG 2-2IV, as reviewed by Hyakumachi (1996). Wheat monoculture in Australia resulted in decline in severity of root rot caused by *R. solani* AG 8 (Roget, 1995). The most comprehensive investigation of Rhizoctonia disease decline to date was conducted by Mendes *et al*., (2011), who studied a soil from the Netherlands which had developed suppression to Rhizoctonia disease of sugar beet, resulting from continuous cropping. They employed bacterial/archeal phylochip analysis to determine which taxa of soil microorganisms were associated with suppressive soil, and subsequently highlighted γ-Proteobacteria, especially Pseudomonadaceae, for further investigation. Isolation of members of this group from beet rhizospheres, followed by a series of lab assays, revealed that protection of plants was conferred through antifungal activity exhibited by members of the Pseudomonadaceae, although other taxa were also likely to be involved.

Results from experiments in the present study (Chapter 2) found that the Pseudomonads isolated from one of the four soil locations (Pukekohe) were more suppressive to *R. solani in vitro* than those from other locations. Since this soil had been sampled from a continuous potato monoculture (part of a long-term trial), it was hypothesised that there could have been a build-up of suppressive soil populations due to the cropping history. Experiments were therefore conducted to determine if soil from the Pukekohe trial, from plots of continual potato culture, were suppressive to Rhizoctonia disease of potato relative to other rotations at

the same location, and to investigate if the soil history had led to the formation of a *Rhizoctonia*-suppressive soil.

Even in the absence of evidence of development of *Rhizoctonia*-suppressive soils at the Pukekohe rotation trial site, generating data on the impacts of the different crop rotation treatments on severity of Rhizoctonia diseases of potato will be useful to growers in this region, which is one of New Zealand's main potato producing areas (Aitken and Hewett, 2011). Knowing if there are differences in Rhizoctonia disease levels associated with certain crop rotations, as have been reported by other authors (Griffin *et al.*, 2009; Larkin and Honeycutt, 2006; Larkin *et al.*, 2010), will help inform growers as to which rotations are more likely to reduce economic losses from this pathogen. One hypothesis tested here was that soils from crop rotations with greater numbers of years between potato crops would harbour smaller populations of *R. solani* AGs pathogenic to potato. Populations of *R. solani* AG 3 and 2-1 were determined in soil samples using a quantitative PCR (qPCR) method.

Understanding the dynamic relationship between a pathogen, its host plants and the soil microbial communities is key to elucidation of how biological control of diseases is, or could be, achieved, as well as limitations of biocontrol over a range of environmental conditions and cropping practices. The experiments described here examined the effects of four different potato rotation practices on the populations of *Rhizoctonia solani* AGs 3 and 2-1, as well as the richness, diversity, evenness and total metabolic activity and diversity of bacterial and fungal soil communities. How these factors influence the levels of disease expression of Rhizoctonia cankers on potato was then investigated in a glasshouse assay, as well as their effect on the populations of *R. solani* over time. It was hypothesised that soil from rotations with a diverse succession of crops would harbour soil microbial communities with greater richness, diversity and evenness than soil from monoculture, or rotations with a more limited succession of crop species, and that these 'healthier' communities could possess greater capacity to suppress Rhizoctonia diseases of potato.

In the past, investigations into soil microbial communities have relied on culture-dependent techniques, such as agar plating of diluted samples, which carries with it the large bias of only detecting those organisms able to be cultured. These include approximately only 17% for known fungi (Bridge and Spooner, 2001), and approximately 1% for known bacteria (Kirk *et al.*, 2004). Also, in culture, fast-growing organisms can overgrow those which are less suited to the conditions, further biasing the results. Culture-independent methods of analysing soil microbial communities overcome some of these problems. Examples of these methods are: PCR based DNA fingerprinting techniques; denaturing or temperature-gradient gel

electrophoresis (DGGE and TGGE), amplified ribosomal DNA restriction analysis (ARDRA) and rRNA intergenic spacer analysis (RISA) (Ranjard *et al.*, 2000). The experiments described here employed both culture-dependent enumeration of bacteria and fungi on agar, as well as an automated RISA (ARISA) technique developed for bacterial community analysis by Fisher and Triplett (1999), and modified for fungal community analysis by Ranjard *et al*. (2001). This method allows rapid, cost effective, high resolution analysis to determine soil community richness, diversity and evenness. RISA exploits differences between taxa in the length of non-coding regions of the rRNA loci sitting between conserved coding regions of the genes. Organisms with the same intergenic spacer length therefore group together into Operational Taxonomic Units (OTUs), which can then be analysed for their presence and relative abundance in a sample, but cannot be identified *per se*. The experiments also utilised a recently developed method for extracting DNA from large quantities (50 g) of soil, so that analyses would be more representative of the different total communities in each treatment, especially for fungal populations and communities whose distribution can be heterogeneous throughout soils. Culture-dependent catabolic profiling was also performed (Biolog EcoPlate™) to assess soil metabolic parameters. Statistical analysis of the relationships between soil community factors and subsequent levels of Rhizoctonia disease in a greenhouse assay using the soils sampled was conducted to generate information regarding factors which might suppress disease.

6.3 Methods

6.3.1 Pukekohe rotation trial

In 2004, a crop rotation field trial was established at the Pukekohe Research Station (grid reference: 2675590E, 6442200N), managed by Plant and Food Research Ltd. The trial continues, and is being used to study the ecology of pathogens, pests and beneficial organisms as well as disease expression to demonstrate the benefits of crop rotation, focusing particularly on onion and potato. The trial site (Figure 6.1 and 6.2) was 65 × 140 m (0.78 ha) and divided into 24 plots, each 25 m long and eight rows (potatoes) / four seed beds (onions) wide, with 4.5 m buffer spaces between plots. The trial consists of six rotation treatments (Table 6.1), with four replicate plots per treatment (originally in a randomised block design, later converted to a fully randomised design).

6.3.2 Soil sampling and preparation

Soil was collected from all replicate plots of rotation treatments 2, 3, 4 and 6 (Table 6.1, Figure 6.1 and 6.2), on the 14th March 2012, after the respective vegetable crops had been

harvested (Table 6.2). Samples were taken from the inner four rows (potato plots) or inner two beds (onion plots) and consisted of 20 samples of *ca.* 350 mL taken by hand trowel from the top 150 mm of soil, five per row (potato) or ten per bed (onion), evenly spaced along each row/bed, with plant litter avoided. Soil samples from each plot were combined in a pre-labelled plastic sack, and the sacks were transported to Lincoln University. Upon arrival, soil from each plot was mixed thoroughly by tumbling in the sack for 5 min, before a *ca.* 200 g sub-sample was transferred from each into a zipper bag (Premium red line™ reclosable zipper bags, Minigrip®, GA) then placed in -20°C storage. Soil sacks were stored at room temperature in darkness until required (5 days).

Table 6.1 Crop rotation plan showing six treatments in the Pukekohe vegetable rotation trial during successive growing seasons since 2004/05. Samples were taken for the present study from plots of rotation treatments 2, 3, 4 and 6 at the 2011/12 growing season (bold) rotation.

Rot. Trt.[A]	Year									
	04/05	05/06	06/07	07/08	08/09	09/10	10/11	**11/12**[B]	12/13	13/14
1	Onion	Onion	Onion	Onion	Onion	Onion	Onion	Onion	Onion	Onion
2	Onion	Onion	Potato	Potato	Onion	Onion	Potato	**Potato**	Onion	Onion
3	Onion	Potato	Oats	Brassica	Onion	Potato	Brassica	**Potato**	Onion	Potato
4	Potato	Potato	Potato	Potato	Potato	Potato	Potato	**Potato**	Potato	Potato
5	Potato	Potato	Onion	Onion	Potato	Potato	Onion	Onion	Potato	Potato
6	Potato	Onion	Oats	Brassica	Potato	Onion	Squash	**Onion**	Potato	Onion

[A]Rotation treatments

[B]In bold are crops grown the season prior to soil sampling. From 09/10 onward, potato cultivar changed from 'Ilam Hardy' to 'Agria'.

Table 6.2 Management practices applied to the Pukekohe vegetable crop rotation trial plots during the 2011/12 growing season.

<table>
<tr><th></th><th colspan="2">Apr</th><th>May</th><th>Jun</th><th>Jul</th><th>Aug</th><th>Sep</th><th>Oct</th><th>Nov</th><th>Dec</th><th>Jan</th><th>Feb</th><th>Mar</th></tr>
<tr><td>Onion:</td><td colspan="3" rowspan="5">Lime, plough, fertilise, rotary hoe, bed formation</td><td colspan="10">Onion</td></tr>
<tr><td>Potato:</td><td></td><td colspan="5">Potato</td><td colspan="4"></td></tr>
<tr><td>Oats:</td><td colspan="10">2 crops of oats; 1st allowed to seed; 2nd sprayed off before seeding</td></tr>
<tr><td>Brassica:</td><td colspan="5">Cabbage / Broccoli</td><td colspan="5"></td></tr>
<tr><td>Squash:</td><td colspan="6"></td><td colspan="4">Squash</td></tr>
</table>

Standard regional agrochemical (including herbicide, fungicide and insecticide applications) and soil management practices were followed.

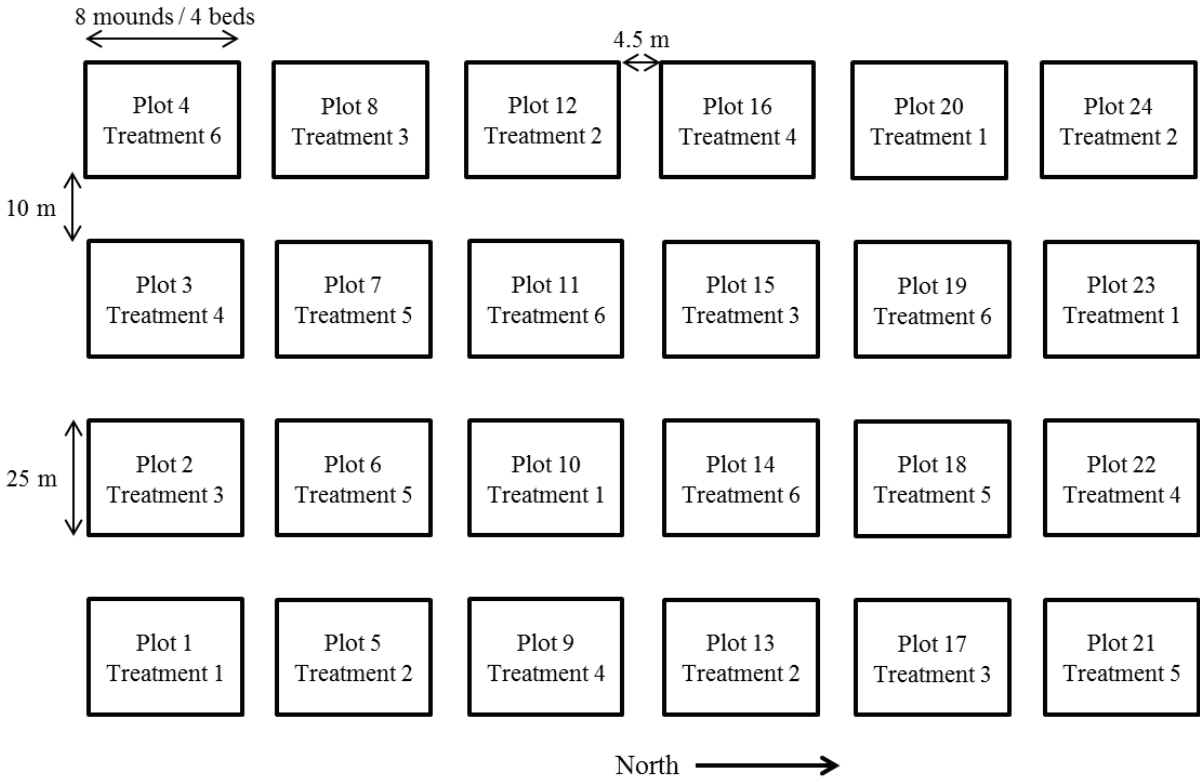

Figure 6.1 Layout of rotation treatment plots at the Pukekohe vegetable crop rotation trial (not to scale). Soil was sampled from all plots of rotation treatments 2, 3, 4 and 6 (total of 16 plots).

Figure 6.2 Pukekohe vegetable crop rotation trial on the day of soil sampling for the present study.

6.3.3 Soil analysis

6.3.3.1 Soil pH and moisture content

Soil pH for each plot sampled was determined by suspending 10 g of air-dried soil in 25 mL of Nanopure® water (10 min on an orbital shaker at 225 rpm, room temperature), and each sample was then left to stand overnight before the pH was measured (pH Benchtop Meter 2210, Hanna® Instruments). Triplicate samples of *ca* 5 g from each plot were weighed, dried in a 60°C oven (24 h) and then re-weighed. Mean soil moisture content was calculated for each plot from these weight data.

6.3.3.2 Enumeration of culturable microorganisms and catabolic profiling

The dilution and inoculation of agar plates and multi-substrate plates was conducted in plot order (random treatment). For each plot, 10 g of soil was dispensed into a 250 mL capacity conical flask and the volume adjusted to 100 mL with 0.1% physiological agar (Appendix A.1.11). Flasks were then sealed with Parafilm® M (Brand GmbH, Germany) and shaken at 500 oscillations/min on a Stuart® SF1 flask shaker (Keison International Ltd., UK) for 5 min, creating 1×10^{-1} g/ mL suspensions. Suspensions were then serially diluted (1 mL pipetted into 9 mL sterile physiological salt solution; 0.85% NaCl) four times to produce 1×10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions of the soil suspensions. Triplicate Petri plates containing fungal enumeration agar (Appendix A.1.12) were each inoculated with a 25 µL aliquot of the 1×10^{-2}, 10^{-3} and 10^{-4} dilutions, which were spread on the agar surface with a sterile glass rod. The 'microdot' method was used to enumerate bacteria (personal communication, J. Swaminathan, AgResearch Ltd., Lincoln) as follows. Three 10 µL aliquots of each of the 1×10^{-3}, 10^{-4} and 10^{-5} dilutions were each dotted onto separate nutrient agar (NA) containing Petri plates (Appendix A.1.2), which were then tilted to allowed aliquots to run the length of the plate, creating three 'lanes' per plate. Using a modified version of the Zak *et al.* (1994) microbial community substrate utilisation method, the 1×10^{-3} suspension from each plot was used to inoculate one replicate (⅓) of an EcoPlate™ (Biolog Inc., Hayward, CA) with 100 µL per well. All agar plates and EcoPlates were incubated at 25°C in darkness. The numbers of mycelial fungal colonies (fungal agar) or bacterial colonies (NA) were recorded once colonies had grown but were not overlapped. The numbers of (culturable) fungal and bacterial colonies per g (dry weight) of soil for each plot were then calculated. The Absorbance at 590 nm for each well of the inoculated EcoPlates was recorded every 24 h for 6 days, with a final reading on day 8: Microplate reader – Microskan®GO (Thermo Fisher Scientific Inc.), Software – SkanIt Software 3.2 (for Multiskan®GO, Thermo Fisher Scientific Inc.). At each time point, average well colour development (AWCD) was calculated for each EcoPlate replicate by

taking the mean absorbance (590 nm) reading over all 32 wells after the blank (water well) was subtracted (Larkin, 2003). The community metabolic diversity (CMD) was calculated as the number of wells with absorbance reading greater than 0.25 (Xiong *et al.*, 2010).

6.3.3.3 Culture independent community analysis and pathogen quantification

DNA extraction from soil samples was conducted as follows: Frozen soil samples (6.3.2) were thawed at 4°C for 48 h. Samples were then dried for 24 h in an oven at 40°C. For each field plot, 50 g of dried soil was weighed and combined with 50 g of a glass sand / glass bead (0.6 mm) 1:1 mix in a 350 mL BeadBeater® polycarbonate chamber. Extraction buffer (100 mL; Appendix A.4.1) was added to each chamber and this mixture was beaten for 2 min (BeadBeater® 1107900, Biospec Products Inc.). A 15 mL screw cap tube was filled with the homogenised mix and centrifuged at 3220 rcf for 20 min. The supernatant (1 mL) was combined with 100 µL of ammonium acetate (10M, Appendix A.4.2) in an Eppendorf tube and left at 4°C overnight. Tubes were centrifuged at 16,000 rcf for 5 min and the supernatant combined with 1 mL of binding buffer (PB, Qiagen) and mixed well. This mixture was centrifuged at 16,000 rcf through a spin column (EconoSpin™ All-in-1 Mini Spin Column for DNA/RNA extraction, Epoch Life Sciences Inc.) and the supernatant from the catch tube discarded. The spin tube columns were washed by twice adding wash buffer (750 µL then 500 µL, PE, Qiagen) and centrifuging at 16,000 rcf. DNA was recovered from the spin column by adding 50 µL of elution buffer (QF, Qiagen) to the spin tubes and centrifuging – this was repeated once. This process produced 100 µL of PCR ready DNA for each field plot sampled.

Quantitative PCR reactions were conducted as follows: Reaction mixtures (total volume 20 µL) contained 10 µL of mastermix (iTaq™ supermix with ROX, Bio-Rad Laboratories Inc., USA), 2 µL of each primer (3 µM concentration, primers for *R. solani* AG 3 and AG 2-1 cannot be detailed due to intellectual property agreement), 2 µL of probe (1.5 µM concentration, 5' is 6-FAM™ labelled with MBG-NFQ on 3' end) 2 µL of Nuclease-free water (UltraPure™, Invitrogen Ltd.) and 2 µL of DNA template. Amplification was performed under the following conditions: 95°C for 2 min, then 40 cycles of 95°C for 15 sec, 62°C for 20 sec, and 72°C for 20 sec, performed using a StepOnePlus™ Real-Time PCR Instrument (Applied Biosystems) with StepOne software (v2.2.2, Applied Biosystems). DNA extracts for qPCR from Pukekohe rotation treatment soils and the greenhouse disease assay were diluted in elution buffer, to reduce concentration of PCR inhibition factors, by1:10 and 1:20, respectively.

DNA amplification for ARISA was conducted as follows: Reaction mixtures (total volume 50 µL) for PCR contained 2× Master Mix (25 µL, Go TaqGreen, Promega Ltd. which

includes dNTPs), Nuclease-free water (17 µL, Promega Ltd.), bovine serum Albumen (2 µL of 10 mg/mL solution, Invitrogen Ltd.) forward- and reverse-primers (2 µL of 10 µM solutions for each) and DNA template (2 µL). For analysis of bacterial community structure (B-ARISA), the bacterial intergenic spacer (IGS) located between the small (16S) and large (28S) subunits of ribosomal RNA genes were amplified using the following primers as detailed by Ranjard *et al*., (2001): targeting the eubacterial rRNA small subunit (S-D-Bact-1522-b-S-20, 5'-TGCGGCTGGATCCCCTCCTT-3') and eubacterial large subunit (L-D-Bact-132-a-A-18, 5'-CCGGGTTTCCCCATTCGG-3'). The small subunit primer was labelled at the 5' end with HEX (6-carboxyhexafluorescein) fluorochrome (Invitrogen Ltd, NZ) for automated detection of amplified PCR product. To analyse the fungal community structure, the ITS1-5.S-ITS2 region was amplified using primers, again as detailed by Ranjard *et al*. (2001), which target the 3' end of the 18S (primer 2234C, 5'-GTTTCCGTAGGTGAACCTGC-3') and 5' end of the 28S (primer 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') genes in various organisms. The 18S primer was labelled at the 5' end with HEX fluorochrome (Integrated DNA Technologies Inc, US). Amplification was performed under the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 61.5°C for 30s and 72°C for 1 min 30s, with a final phase of 72°C for 5 min for extension of incomplete products. PCR products were visualised under UV on 1.5% (w/v) agarose gels made with 1×TBE (Tris/Borate/EDTA) buffer and SYBR® safe DNA gel stain (10 µL in 100 mL gel, Invitrogen Ltd.), and run for 40 min at 110V at room temperature. PCR products were purified for analysis using Zymo DNA Clean and Concentrator™ kits (Ngaio Diagnostics Ltd, NZ) to remove primers and small fragment DNA sequences. Purified PCR products were then processed on a 3130XL Capillary Genetic Analyser (ABI Ltd., Melbourne) using a 50 cm capillary with standard Genemapper protocol and increased runtime (15 kV, 3700 s), and the fluorescence data generated were converted to electropherograms (Figure 6.5) with Genemapper (v3.7, Applied Biosystems™), which also assigned a fragment length to peaks based on comparison with a standard ladder (LIZ1200, ABI Ltd.).

6.3.4 *Rhizoctonia solani* inoculum

Inoculum was prepared as detailed in Chapter 4. Briefly, barley + V8® juice medium (Appendix A.2.1) was inoculated with agar plugs from the edge of a growing colony of isolate Rs043-2 (AG 2-1), and incubated for 2 weeks at 25°C in darkness.

6.3.5 Greenhouse disease assay

Five litres of soil from each field plot sampled (Figure 6.1) was combined with 2 L of pumice (3-5 mm grade, Egmont Commercial, NZ) in a plastic sack and mixed by tumbling for 5 min. The soil/pumice mix for each sampled plot was then used to fill three 0.9 L plastic plant pots. A further three pots were initially ⅓ filled and three *R. solani* infested barley grains (section 6.3.4) were placed on top of the first layer of soil in each pot, roughly equally spaced from the pot edge and each other. The inoculated pots were then filled with the soil mix. All pots were planted with one pre-sprouted minituber (potato cv. 'Desiree', Alex MacDonald (Merchants) Ltd, NZ, stored at 4°C, incubated at 18°C, 12:12 h light:dark for 2 weeks to sprout, mean weight 2.3 g). Each minituber was planted at *ca.* 30 mm depth, then covered with mix. Pots were placed on greenhouse benches and arranged into four blocks. Each block contained all the pots from one plot of each of the four crop rotation treatments sampled from the field trial (treatments 2, 3., 4 and 6), e.g. block 1 had all pots of plot 5 (Treatment 2), plot 2 (Treatment 3), plot 3 (Treatment 4) and plot 4 (Treatment 6). Each block took up ½ of a greenhouse bench, and was divided into three sub-blocks of paired pots from each plot sample (each pair consisted of one pot with, one without added pathogen inoculum), four pairs in a table width, with pairs in a random order and order of pots within pairs also randomised. Plants were grown in a temperature controlled greenhouse unit at Plant and Food Research Ltd., Lincoln with supplemental lighting (conditions as detailed in Chapter 3). Plants were watered as required to maintain moisture, and from 6 weeks after planting to harvest, each pot was given 30 mL of a 0.01% solution of High NK™ liquid fertiliser (8:6:13.5 N:P:K, Agrichem, Australia) twice each week to prevent nutrient deficiency. The date of plant emergence for each pot was recorded. Plants were grown for 10 weeks, and at harvest the soil was shaken free from plant roots and soil from each of the two triplicate sets per plot sample (with and without added pathogen inoculum) were combined in separate plastic sacks and mixed. From these combined samples, *ca.* 300 g sub-samples were oven dried at 40°C then transferred to individual zipper bags, which were storage at -20°C. Plants were washed free of soil and number of stolons (total and number with lesions), number of stolons tips (total and number 'nipped'), number of tubers (total and number malformed or damaged by lesions), total tuber weight, and dry weight of roots (without tubers) and shoots were determined. Soil extraction and quantification of *R. solani* AG 2-1 was performed for all of the inoculated treatments (see section 6.3.3.3).

6.3.6 Statistical analyses

The pH, fungal and bacterial enumeration, AWCD and CMD (at each time point) as well as plant physical parameters, Rhizoctonia disease data and *R. solani* quantification data, were subjected to ANOVA for complete randomised designs, and treatment means compared using Fisher's unrestricted LSD ($P < 0.05$). Plant physical and Rhizoctonia disease data were also analysed as a 4 × 2 factorial ANOVA, with the four rotation treatments and +/- pathogen inoculation as factors. The EcoPlate™ substrate utilisation (SU) data at incubation day 2 were analysed by principal components analysis using the covariance matrix (Glimm *et al.*, 1997). Generated principal component scores were then subjected to ANOVA, as above, so mean treatment scores could be statistically compared. ARISA electropherogram peak data (fragment length and peak height) were first edited using the protocol described by Ramette (2009). This protocol uses a custom R binning script to remove background 'noise' (peaks under 0.09% of total sample fluorescence) so that only clear peaks remain, and aligns peaks across samples to account for run-to-run variation. Only peaks > 100 bp and < 1000 bp were included in the analyses, as this encompasses the predicted range of fragments produced by the PCRs (Ranjard *et al.*, 2001). Data were converted to spreadsheet format, then entered into PRIMER 6 (Plymouth Marine Lab, UK) with PERMANOVA add-on statistical software, licenced to Gavin Lear, Lincoln University. This was used to generate a pairwise Bray-Curtis similarity matrix (Bray and Curtis, 1957), and data were rendered to give visual representations of the matrices for the bacterial and fungal results. The software also generated OTU richness (number of peaks), diversity (Shannon index) and evenness (Pielou's index) parameters for each sample, which were also subjected to ANOVA as detailed above. Correlations between the different soil community factors measured, and between soil factors and Rhizoctonia disease or plant physical parameters, were analysed by regression analysis (Microsoft Excel™ 2010, Microsoft® Corporation, USA).

6.4 Results

It was discovered that plot 12 (one of the rotation treatment 2 replicates) had received an incorrect planting in the second year (2005/06) of the rotation trial, so data for this plot were removed from the analyses.

6.4.1 Effect of crop rotation treatment on soil communities

6.4.1.1 Culture-dependent analysis

The analysis of soil samples collected (Table 6.3) showed that there was no significant difference in pH or culturable fungal populations between the four rotation treatments. The

population of culturable bacteria in rotation treatment 6 was significantly smaller than for the other three rotations treatments, and the mean PC1 score, AWCD and CMD values for this rotation treatment were significantly less than those for treatment 3, but not for treatments 2 and 4. Graphically, the principal component analysis of substrate utilisation (Figure 6.3) demonstrated that individual plots were often closer in profile to plots from other rotation treatments than to plots from their own treatment. Plots from rotation treatments 6 and 3 clustered more closely compared with rotations 2 and 4.

Table 6.3 Mean pH, culturable fungal and bacterial populations, and substrate utilisation parameters for each rotation treatment from ANOVA

Rotation treatment	Reps	pH	Fungal cfu / g dry soil	Bacterial cfu / g dry soil	PC1[B] 52.3%	PC2[B] 13.4%	AWCD	CMD
2	3	6.3 a	1.08×10^5 a	1.27×10^6 b	0.22 ab	-0.45 a	0.38 ab	12.0 ab
3	4	6.5 a	7.89×10^4 a	1.21×10^6 b	1.15 b	0.37 a	0.53 b	15.8 b
4	4	6.4 a	1.02×10^5 a	1.30×10^6 b	0.34 ab	0.24 a	0.44 ab	14.0 ab
6	4	6.4 a	5.56×10^4 a	8.47×10^5 a	-1.65 a	0.27 a	0.15 a	7.3 a
LSD[A] Max-min		0.24	64923	343091	2.67	1.58	0.35	8.0
LSD[A] Max rep		0.22	60107	317641	2.47	1.46	0.32	7.4

[A]Fisher's unrestricted LSDs at $P = 0.05$.
[B]Principal component scores generated from analysis of EcoPlate™ data after 48 h incubation with percent variation explained by each component.

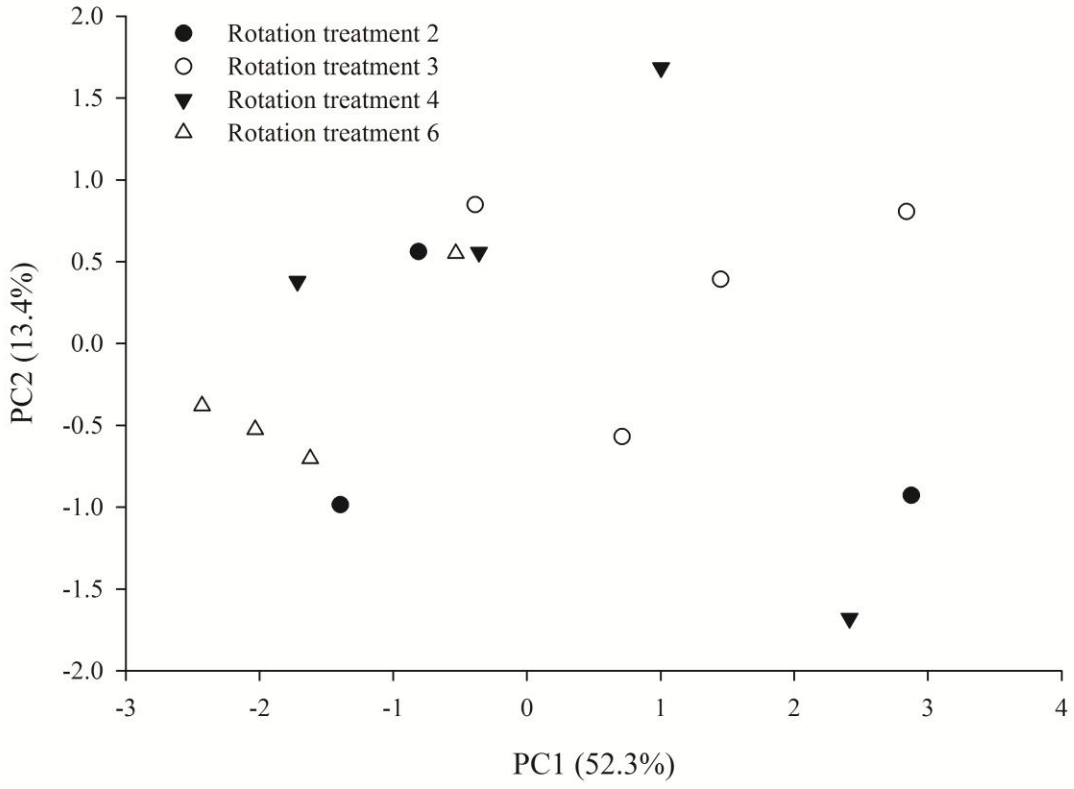

Figure 6.3 Scatter plot matrix of principal component scores for substrate utilisation (EcoPlate™) data after 48 h incubation, for microorganisms from soils from different crop rotation treatment plots.

6.4.1.2 Culture-independent analysis (qPCR and ARISA)

Of the rotation treatment composite soil samples tested for *R. solani* AG 3 and 2-1 DNA by qPCR, no plot had detectable AG 3 DNA (Appendix B.3.1). All four plots from rotation treatment 3 (mixed rotation ending with potato) had detectable AG 2-1 DNA, as did one plot from treatment 4 (potato monoculture, plot 22). However, determined values fell outside the range of the standard curve, so exact quantities of DNA could not be calculated (Appendix B.3.2).

The bacterial and fungal ARISA profiles (entered into PRIMER 6 software and Bray-Curtis similarity matrices generated, 6.3.6) are presented in Figure 6.4 as Non-Metric Multidimensional Scaling plots, displaying cluster analysis with resemblance levels of 40, 50 and 60% similarity.

There was at least 60% similarity between the bacterial community structures of all but two plots based on ARISA (Figure 6.4 a), with plots not grouping more closely with plots of the same rotation treatment than with plots of different rotations. There was at least 40% similarity between the fungal communities of all plots (based on ARISA, Figure 6.4 b), but rotation treatment 6 (mixed rotation ending with onion) plots grouped together, separate from the other plots at 50% similarity. The fungal communities of plots from rotation treatments 2 (alternating onion and potato in 2 year cycles), 3 (mixed rotation ending with potato) and 4 (potato monoculture) overall demonstrated no strong pattern of grouping together with other plots of the same rotations, and often had greater similarity with plots from other rotations.

ANOVA of bacterial and fungal community richness (peak/OTU number), diversity (Shannon index) and evenness (Pielou's index) demonstrated that there were no significant differences between rotation treatments for any of the three community measures for either bacteria or fungi (P values ranged from 0.62 to 0.94).

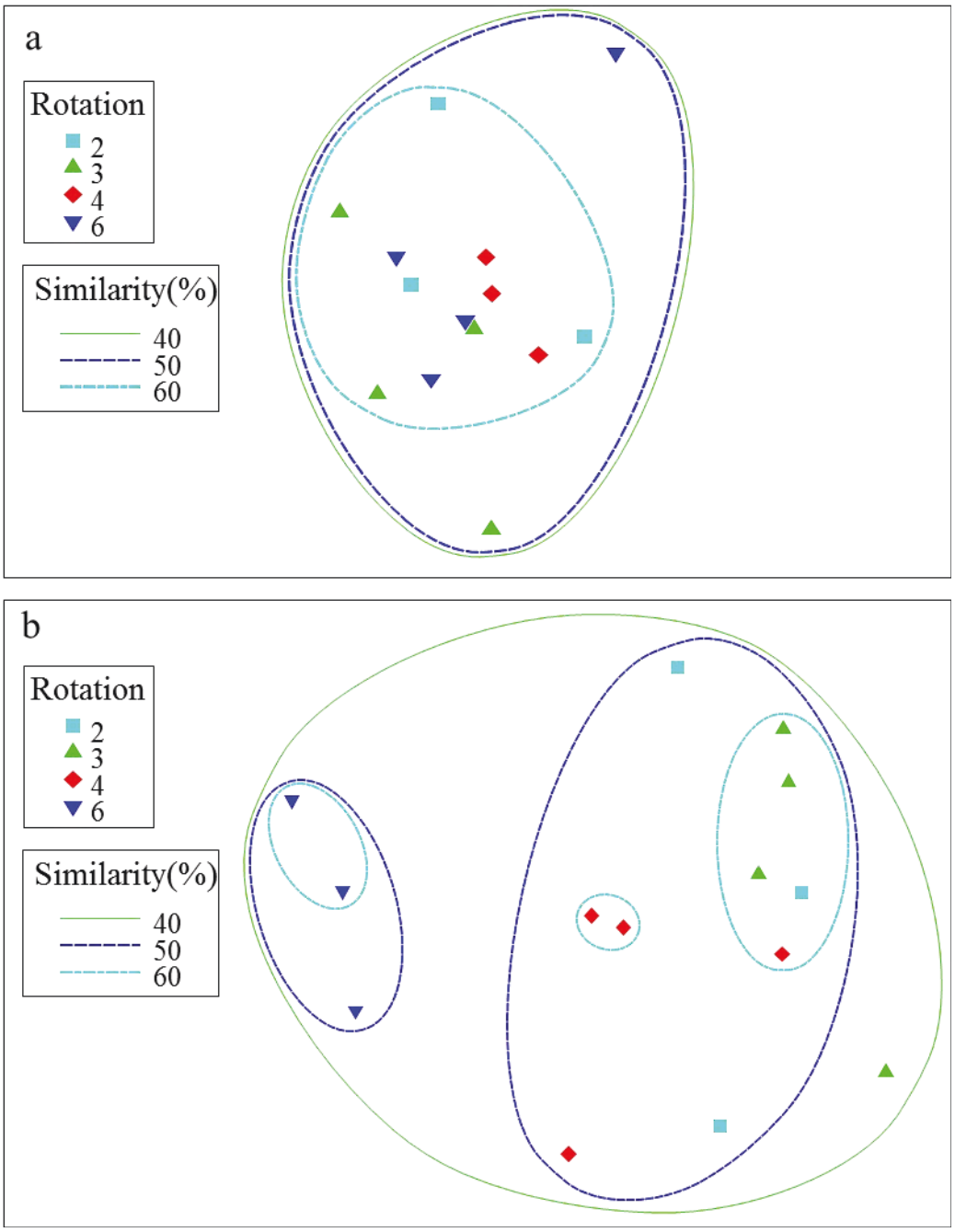

Figure 6.4 Multidimensional scaling plots of Bray-Curtis similarity matrices overlaying cluster analysis with resemblance levels of 40, 50 and 60%, for a) bacterial and b) fungal ARISA data

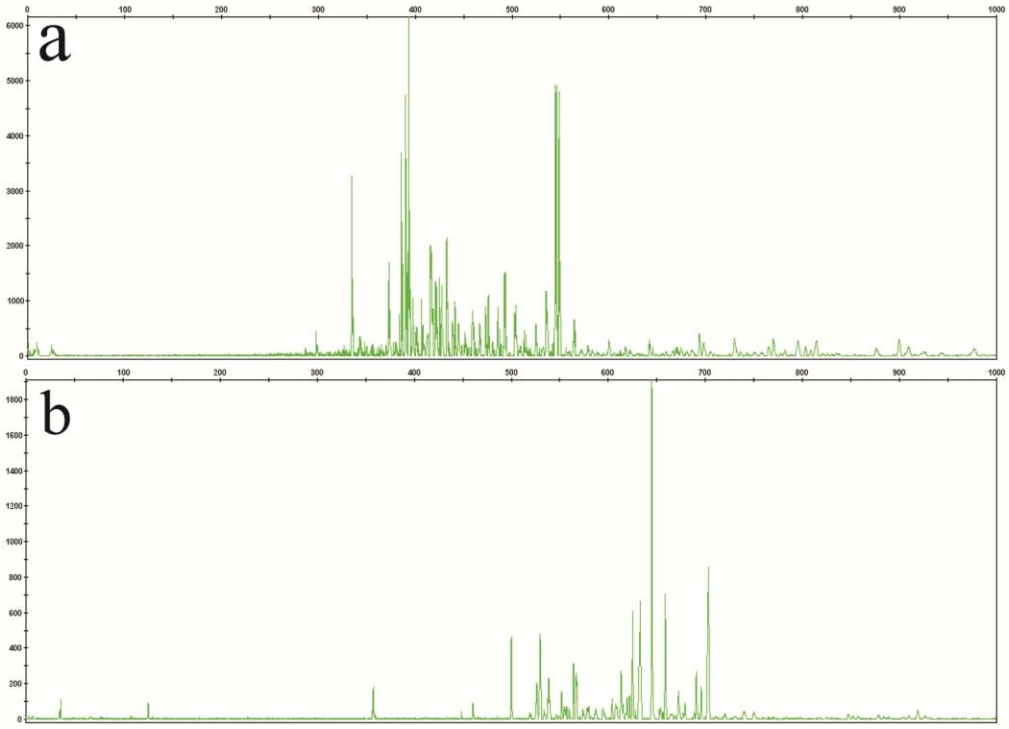

Figure 6.5 Examples of ARISA peak fluorescence data presented as electropherograms created by Genemapper software of (a) B-ARISA (plot 13) and (b) F-ARISA (plot 11) samples.

6.4.2 Effect of rotation treatments on Rhizoctonia cankers in greenhouse assay

The 4 × 2 factorial ANOVA (four rotation treatment soil samples, with or without *R. solani* isolate Rs043-2, AG 2-1) of the greenhouse Rhizoctonia canker assay (Table 6.4) showed that the only parameter for which there was an effect of rotation treatment ($P < 0.05$) was the number of symptom-less stolons. For this measure, rotation treatment 4 (potato monoculture) gave fewer symptomless stolons (mean = 3.9), than treatments 2 (5.7), 3 (5.1) or 6 (5.4). The addition of pathogen inoculum affected ($P < 0.05$) seven of the 12 parameters presented in Table 6.4, and the effect on number of shoots nipped was significant at $P > 0.1$. Addition of pathogen increased canker disease parameters (percent diseased stolons, number of symptom-less stolons, percent stolon tips nipped, number of symptom-less tubers). Inoculation also decreased the total tuber weight, and increased number of stolons and stolons tips. The addition of the pathogen did not affect the number of stolon tips which were not nipped, or the total number of tubers or average tuber weight.

Table 6.4 Mean parameters obtained from a glasshouse assay for Rhizoctonia disease, using soil from different Pukekohe rotation treatment trial plots, which was either non-inoculated or inoculated with *Rhizoctonia solani* (Path). ANOVA and 4 × 2 factorial ANOVA probability values are included

Rot. Trt.[A]	Reps	Path.	Days to emerge	No. shoots 'nipped'	No. stolons	Diseased stolons (%)	No. symptom-less stolons	No. stolon tips	Tips nipped (%)	No. non-'nipped' stolon tips	No. tubers	No. symptom-less tubers	Total tuber weight (g)	Average tuber weight (g)
2	3	No	9.3 a	0.0 a	6.4 a	12.4 ab	5.4 bcd	6.4 a	3.0 a	6.2 ab	2.9 a	2.8 bc	7.12 c	2.77 ab
	3	Yes	10.0 ab	0.3 a	10.0 c	34.1 bc	6.0 cd	10.2 bc	27.2 bc	6.7 b	2.4 a	2.3 abc	5.11 ab	2.90 ab
3	4	No	9.3 a	0.3 a	6.9 ab	16.2 ab	5.7 bcd	7.5 a	11.8 ab	6.3 ab	2.5 a	2.5 bc	6.38 c	3.26 b
	4	Yes	9.8 a	0.4 a	9.0 bc	47.2 cd	4.4 bc	10.1 c	32.6 c	6.3 ab	2.6 a	1.9 ab	4.59 a	1.91 a
4	4	No	11.3 ab	0.0 a	6.6 a	21.8 ab	5.1 bcd	7.1 a	15.1 ab	6.0 ab	2.8 a	2.6 bc	6.19 bc	2.46 ab
	4	Yes	10.2 ab	0.4 a	6.3 a	51.2 cd	2.7 a	7.6 ab	33.1 c	4.7 a	2.6 a	2.3 abc	4.22 a	2.12 ab
6	4	No	12.4 b	0.1 a	7.0 ab	4.7 a	6.7 d	7.0 a	4.7 a	6.7 b	3.0 a	3.0 c	6.45 c	2.67 ab
	4	Yes	9.5 a	0.1 a	9.2 bc	60.0 d	4.1 ab	10.3 c	37.9 c	6.3 ab	2.5 a	1.4 a	4.49 a	2.27 ab
4 x 2 factorial ANOVA (4 rotation treatments, +/- pathogen)														
P value	Rot. Trt.		0.315	0.524	0.12	0.36	0.029	0.361	0.585	0.27	0.939	0.714	0.236	0.905
	+/- Path.		0.232	0.06	0.003	<0.001	<0.001	<0.001	<0.001	0.424	0.314	0.005	<0.001	0.126
	Rot. × Path.		0.218	0.571	0.168	0.107	0.082	0.306	0.632	0.598	0.811	0.242	0.993	0.286
+ pathogen mean			9.8	0.31	8.5	49.1	4.18	9.49	33.1	5.91	2.53	1.98	4.57	2.24
- pathogen mean			10.7	0.09	6.8	13.9	5.73	7.04	9.0	6.29	2.78	2.71	6.50	2.69

[A]Rotation treatment 2 = alternating onion and potato in 2 year cycles, 3 = mixed rotation ending with potato, 4 = potato monoculture, 6 = mixed rotation ending with onion

In addition to the results presented in Table 6.4, for root dry weight there was no significant difference between any of the treatment means in the overall ANOVA. The only statistically significant effect from the factorial analysis was of pathogen ($P = 0.098$), with plant roots slightly heavier (0.32 g) when inoculated with pathogen than un-inoculated plant roots (0.29 g). For shoot dry weight, only rotation treatment 6 had +/-pathogen group means significantly different at $P<0.05$ in the overall ANOVA, with plant shoots heavier (0.89 g) when inoculated with pathogen than un-inoculated plant shoots (0.64 g). The only significant effect from the factorial analysis was of pathogen ($P = 0.016$), with plant shoots heavier when inoculated with pathogen (0.812 g) than un-inoculated plant shoots (0.681 g).

6.4.3 The effect of crop rotation on inoculated *Rhizoctonia solani* AG 2-1 quantity

All plant growth medium samples from the greenhouse assay tested had quantifiable *R. solani* AG 2-1 DNA (Appendix B.3.3). The ANOVA of qPCR results from the inoculated treatments in the greenhouse assay demonstrated that there was no significant difference ($P = 0.617$) between the group (rotation treatment) means in terms of the concentration of AG 2-1 DNA present. The mean amounts of AG 2-1 DNA in soil were: Rotation 2, 8.57 pg/g; rotation 3, 6.05 pg/g; rotation 4, 6.74 pg/g; and rotation 6, 5.28 pg/g DNA per g soil. AG 2-1 DNA was present in all 19 inoculated plots at harvest.

6.4.4 Relationships between soil microbial community parameters (plus pH), Rhizoctonia disease and plant physical parameters (plus AG 2-1 populations)

The soil community data which had been determined for each of the sampled Pukekohe rotation treatment plots (i.e. bacterial and fungal OTU diversity, richness and evenness, bacterial and fungal culturable populations, as well as soil community metabolism and metabolic diversity) plus pH were tested for relationships to Rhizoctonia disease and plant physical parameters determined in the subsequent greenhouse assay (6.3.5), using regression analysis.

For plants grown in soil without additional *R. solani* inoculum, of the 121 combinations of soil community parameters (or pH) *vs.* Rhizoctonia disease or plant physical parameter, twelve were significantly ($P \leq 0.1$) correlated (Table 6.5). Only three community parameters (bacterial community evenness, fungal diversity and culturable fungal population) significantly correlated with any of the Rhizoctonia disease-related parameters. Of the plant physical parameters which correlated with soil parameters, four negatively correlated with

increasing pH. These were number and total weight of tubers, and dry weights of shoots and roots.

Table 6.5 Regression analyses of soil community parameters (per Pukekohe rotation treatment plot) *vs.* mean Rhizoctonia disease and plant physical parameters from the greenhouse assay (non-inoculated treatments only)

Soil community parameter (or pH)	Rhizoctonia disease or plant physical parameter	*P* value	R^2 value	Correlation
Bacterial community evenness	No. symptomless stolons	0.030	0.334	positive
	No. tubers	0.007	0.468	positive
	Average tuber weight	0.060	0.264	negative
Fungal diversity	Emergence time (days)	0.087	0.224	positive
Fungal CFU	No. symptomless tubers	0.060	0.246	negative
	Shoot dry weight	0.067	0.235	negative
Soil metabolism (AWCD)	Shoot dry weight	0.036	0.295	positive
Soil metabolic diversity	Shoot dry weight	0.081	0.215	positive
pH	No. tubers	0.039	0.287	negative
	Total tuber weight	0.018	0.362	negative
	Shoot dry weight	0.037	0.294	negative
	Root dry weight	0.016	0.370	negative

Regression with results from greenhouse assay experiment without *R. solani* inoculation, only regressions significant at $P \leq 0.1$ included in the table.

For plants grown in soil inoculated with isolate Rs043-2 (AG 2-1 isolate), of the 156 combinations of soil community parameters (or pH) vs. Rhizoctonia disease or plant physical parameters, 25 were significantly ($P \leq 0.1$) correlated (Table 6.6). Of the Rhizoctonia disease parameters determined, increases in the number of 'nipped' shoots correlated with increases in bacterial community richness and diversity, and fungal community richness. Decreases in the number of symptomless stolons correlated with increases in fungal community richness, diversity and evenness. Conversely, decreases in the proportion of stolons with cankers and of stolon tips 'nipped' correlated with increases in the total culturable fungal populations, and increases in number of stolon tips which had not been 'nipped' correlated with increases in bacterial community evenness. *Rhizoctonia solani* AG 2-1 population did not correlate with any direct measure of Rhizoctonia disease, and larger populations at experiment termination correlated with greater total and average tuber weights.

Table 6.6 Regression analyses of soil community parameters (per Pukekohe rotation treatment plot) vs. mean Rhizoctonia disease and plant physical parameters from the greenhouse assay (*Rhizoctonia solani* inoculated treatments only)[+]

Soil community parameter (or pH)	Rhizoctonia disease or plant physical parameter	*P* value	R^2 value	Correlation
Bacterial community richness	No. nipped shoots	0.020	0.376	positive
Bacterial community diversity	No. nipped shoots	0.089	0.222	positive
Bacterial community evenness	No. non-'nipped' tips	0.046	0.292	positive
Fungal community richness	No. nipped shoots	0.076	0.239	positive
	No. symptomless stolons	0.059	0.266	negative
Fungal community diversity	No. symptomless stolons	0.058	0.268	negative
	Average tuber weight	0.074	0.242	positive
Fungal community evenness	No. nipped shoots	0.076	0.239	positive
	No. symptomless stolons	0.059	0.266	negative
Bacterial CFU	No. symptomless tubers	0.025	0.332	positive
Fungal CFU	Diseased stolon (%)	0.002	0.530	negative
	Tips nipped (%)	0.008	0.433	negative
	No. symptomless tubers	0.095	0.200	positive
	Shoot dry weight	0.024	0.335	negative
AG 2-1 population at harvest	Days to emerge	0.045	0.275	negative
	Total tuber weight	0.055	0.254	positive
	Average tuber weight	0.035	0.297	positive
	Shoot dry weight	0.024	0.335	negative
Soil metabolism (AWCD)	No. tubers	0.037	0.293	positive
	No. symptomless tubers	0.028	0.319	positive
Soil metabolic diversity	No. tubers	0.090	0.205	positive
pH	Days to emerge	0.089	0.206	negative
	Shoot dry weight	0.048	0.268	negative
	Root dry weight	0.049	0.266	negative

[+]Regression results from greenhouse assay experiment with *R. solani* inoculation, only regression significant at $P \leq 0.1$ included in the table.

6.4.5 Relationships between soil community parameters plus pH

Relationships between selected soil community parameters, as well as between these parameters and soil pH and final AG 2-1 populations (from greenhouse assay, inoculated pots only), were determined using regression analyses.

No statistically significant relationships between the soil community factors measured and final AG 2-1 population (of inoculated plots) were found. Metabolic soil factors only correlated with culturable bacterial populations, increasing populations correlating with greater total metabolism and metabolic diversity (Table 6.7), and not with any fungal or culture independent soil community measure made. None of the soil community measures correlated with soil pH.

Table 6.7 Regression analyses of soil microbial community parameters for each Pukekohe soil plot, inluding population of *Rhizoctonia solani* AG 2-1 (inoculated pots from greehouse assay only) and soil pH

Soil factor 1	Soil factor 2	*P* value	R^2 value	Correlation
Bacterial diversity	Fungal CFU	0.087	0.225	negative
Bacterial CFU	Soil metabolism AWCD	0.001	0.609	positive
Bacterial CFU	Soil metabolic diversity	0.001	0.556	positive
Fungal CFU	Bacterial CFU	0.052	0.261	positive

Only regression significant at $P \leq 0.1$ included in the table.

6.5 Discussion

The results from the analysis of the soil samples from the four selected crop rotation treatments demonstrate that, at the time of sampling (post-harvest), there were no significant differences between the pH or total cultural fungal populations across all rotation treatments. There was no significant difference between the three rotation treatments which grew potatoes prior to sampling (treatments 2, 3 and 4) for total cultural bacterial populations, or substrate utilisation parameters. For rotation treatment 6, which grew onion prior to sampling, there were smaller culturable bacterial populations than the other three rotations, and this rotation treatment was significantly different to treatment 3 (the other 'sustainable'/ mixed crop rotation) in several of the substrate utilisation parameters. Together, these data indicate that, for these factors, the crop history had far less influence than the final crop (either crop species itself or the agricultural management practice associated with that crop) on the parameters measured.

The soil community structure, as assessed by culture-independent PCR based analysis (ARISA), demonstrated that the bacterial community structure of any plot (Figure 6.4 a) was often more similar to that of plots from other rotation treatments than to other plots of the same rotation. Also, there was at least 60% similarity between the bacterial communities in all but two of the plots. This indicates that the bacterial community structure in the plots was not strongly influenced either by the cropping history or the identity of the most recent crop. The fungal community analyses (Figure 6.4 b), however, showed that the communities of rotation treatment 6 plots grouped together with 50% similarity, while communities of the other three rotations grouped together (with the exception of one plot) with the same level of similarity, but show no stronger similarities within rotation treatment. This suggests that the fungal community was affected by the identity of the most recent crop (onion in rotation 6, potato in the other three), but was not strongly affected by crop history (three different crop histories for rotations 2, 3 and 4 but no consistent overall differences between their fungal community

profiles). The differences in fungal communities between rotation treatment 6 plots and the others did not have a discernible impact on Rhizoctonia disease expression, as treatment 6 presented the same level of disease as the other rotation treatments for percent tips nipped, number of non-nipped stolon tips and number of shoots nipped, and the same level of disease as treatments 3 and 4 (but not 2) for percent diseased stolons and number of symptomless stolons (Table 6.4).

This similarity between the four rotation treatments sampled in terms of the soil community factors measured is one of the more striking findings from this section of research, as differences in land use can often result in large differences in the structure of soil microbial communities (Garbeva *et al.*, 2004). The data presented here demonstrate that the structure of soil bacterial and fungal communities (as determined by ARISA) was no more affected by eight successive potato crops than by just one (at the Pukekohe rotation trial site). The crop rotation also made no difference to the presence of *R. solani* AG 3 over the four rotation treatments sampled (below limit of detection from all samples). *Rhizoctonia solani* AG 2-1 was detected in all four plots of rotation 3, but not in any other other plots. Furthermore, that rotation treatment made no discernible difference to the expression of Rhizoctonia diseases of potato, as measured in the greenhouse assay, which strongly suggests that whatever the disease suppressive capacity of the Pukekohe site soil, it was not enhanced or diminished by the crop rotation selection, along with its microbial community structure.

To determine if any of the plot to plot variation in plant or disease parameters could be explained by the soil community measurements made, regression analyses between these groups were conducted. Few of the individual plot community measurements correlated with any of the disease expression parameters in the half of the greenhouse experiment which did not receive added *R. solani* inoculum (Table 6.5). The measurements which correlated with decreases in disease related parameters were increasing bacterial community evenness (on number of sympomless stolons), decreasing fungal diversity (on days to emerge) and decreasing culturable fungal populations (on the number of symptomless tubers). Some plant physical parameters correlated with soil community measurements, but soil pH correlated with the most (in the non-inoculated treatments). Decreasing pH correlated with increasing number and total weight of tubers, and dry weight both of shoots and roots. It is not surprising that for the non-inoculated treatments in the greenhouse experiment, there were few correlations between soil community factors and Rhizoctonia disease or plant physical parameters, as there were few overall differences found between the plot microbial communities themselves, or between plant responses to the different rotation soils.

Garbeva *et al*. (2004) indicated a positive correlation between diversity of soil microbial populations and the soil-borne disease suppressive capacity of soils. Regression analysis between soil community factors and treatment plots from the half of the greenhouse assay inoculated with *R. solani* (Table 6.6), gave unexpected correlations. Increasing OTU richness, diversity (of both bacterial and fungal communities) and evenness (fungal community only) correlated with increasing levels of Rhizoctonia disease (numbers of nipped shoots and symptomless stolons). Of the microbial community parameters determined by ARISA, evenness of bacterial community was the only factor to have positive correlation with a reduction in a measure of Rhizoctonia disease (number of stolon tips which were not 'nipped'). These findings suggest that, in general, a soil management strategy aimed at promoting the richness and diversity of bacterial and fungal communities (or of fungal community evenness), would not offer any protection against Rhizoctonia canker diseases of potato, and may even increase disease severity. Conversely, increases in total culturable fungal populations correlated with decreases in three measures of Rhizoctonia disease; proportion of stolons with cankers and of stolon tips 'nipped', as well as increases in the number of symptomless tubers. This suggests that interactions between culturable fungi and the pathogen may have reduced disease severity. If such a relationship exists, then it is unlikely to be one of direct destruction of the pathogen (e.g. mycoparasitism) as there was no relationship between total culturable fungal populations and populations of *R. solani* AG 2-1.

There was no correlation between total population of *R. solani* AG 2-1 (at greenhouse assay harvest) and any direct measure of Rhizoctonia disease of potato. This appears counter-intuitive, but a review by Bonanomi *et al*. (2010) states that several authors have reported the same phenomenon. Plants with greater AG 2-1 populations at experiment termination tended to have greater total tuber and average tuber weights, but less shoot dry weight.

Overall, the Rhizoctonia disease expression in the greenhouse assay was low compared with many of the experiments conducted in previous phases of this study. This suggests that the Pukekohe soil possesses some suppressive capacity towards the *R. solani* isolate used as inoculum in the greenhouse experiment. However, this cannot be stated unequivocally, as treatments using the same soil type as used in the previous greenhouse experiments were not included in the assay carried out here, so direct comparison between the levels of disease cannot be made. It is possible that the variation of inoculation procedure, the quality of the inoculum or some other factor not related to the soil was responsible for the lower disease severity. If the lower disease severity in the assay was a result of the soil, then it remained mostly unaffected by crop rotation, as disease levels were consistent between the four rotation

treatments sampled. There was also a large degree of similarity between rotations for the biotic factors measured. That one, or a combination, of these factors is acting to suppress Rhizoctonia diseases of potato remains a viable hypothesis. Investigating this further would be worthwhile.

6.6 Conclusions

The hypothesis that continual potato culture at the Pukekohe trial site had led to Rhizoctonia disease decline was shown to be false. Since disease severity was low, it is possible that the Pukekohe soil is generally suppressive to Rhizoctonia disease (canker phase), but as no other soil types were tested, further experimentation is required to demonstrate this. The identity of the crop grown prior to sampling made some overall difference to the fungal community structure, but none to the bacterial community structure, and crop history (the previous eight growing seasons) made no difference to either community structure. For the Pukekohe site, decreases in severity of a few of the measures of Rhizoctonia disease correlated with decreases in bacterial and fungal community richness and diversity, and fungal community evenness, and with increases in bacterial community evenness and culturable fungal and bacterial populations. There was no correlation between disease severity and population of AG 2-1 from results of the greenhouse assay.

Chapter 7
General Discussion

7.1 Themes explored

As discussed in Chapter 1, there are many published reports of biological suppression of Rhizoctonia plant diseases, including those of potato. These include suppression achieved via inundative biological control (introduction of single/multiple soil microorganisms suppressing disease), as well as conservation biological control, including disease decline arising as a result of host plant monoculture, and also the induction of suppression from the introduction of exogenous nutrients in the form of organic matter (OM) amendments. In the present study, these three biocontrol alternatives were explored for suppression of Rhizoctonia diseases of potato from a New Zealand agricultural perspective. Microbial strains from three taxa frequently associated with suppression of Rhizoctonia diseases in published studies (*Trichoderma*, fluorescent Pseudomonads and endospore-forming bacteria), isolated from New Zealand potato cropping regions, were tested for their biocontrol potential when applied in an inundative manner in *in vivo* screens. Organic matter amendments were selected, based on published reports of Rhizoctonia disease suppression, along with considerations regarding agricultural economic realities in New Zealand. These OMs were tested for their capacity to suppress Rhizoctonia diseases of potato *in vivo*. Finally, soils from four rotation treatments (including a potato monoculture) at a long-term rotation trial in one of the main potato producing regions in New Zealand, were collected. These soils were analysed to determine if Rhizoctonia disease decline had developed as a result of potato monoculture, and also to determine if differences existed in the capacity of each soil to suppress Rhizoctonia diseases of potato, and whether biological factors of each soil sample were related to levels of disease expression. Exploration of these biocontrol themes highlighted the difficulty in achieving strong and consistent biocontrol of Rhizoctonia diseases of potato. On the basis of the results generated, recommendations for future biocontrol selection strategies are presented, which may prove to be more fruitful than those which were followed in the present study.

7.2 Inundative biological control

The primary aim of the present study was to identify a microbial isolate (or isolates) able to suppress Rhizoctonia diseases of potato *in vivo*, and then to investigate the mechanisms underpinning biological control.

7.2.1 Isolation and characterisation of microbes

The targeted isolation of members of the genera *Trichoderma*, *Bacillus* (and other endospore-forming bacteria) and *Pseudomonas* conducted in Chapter 2 was a reasonable strategy, based on the close relationship that strains from these groups can have with plants, as well as previous reports of suppression of Rhizoctonia diseases, and relative ease of culture (detailed in Chapter 1). This achieved the objective of generating a large collection of microorganisms (345 isolates) which were categorised with respect to their taxa, isolation details, and interactions with *R. solani* in dual culture. Since dual culture suppression of a pathogen is often a poor indication of *in vivo* biocontrol (Deacon, 1991; Fravel, 1988; Weller, 1988), isolates to be tested for *in vivo* suppression of Rhizoctoia diseases of potato were selected for a range of characterisatics, so that potential mechanisms underpinning biocontrol were not limited to direct destruction of the pathogen (production of antifungal compounds or mycoparasitism) but also included other potential mechanisms (spatial/nutrient competition, stimulation of acquired resistance and growth promotion).

While not an original aim of the isolations, an *in vitro* suppression of *R. solani* scale was created for the bacterial isolates, based on the dual culture data. These data were then subjected to statistical analysis to determine if differences existed between the bacterial populations from the different soil locations and sample times. This showed that fluorescent Pseudomonads from the Pukekohe region soil, in general, possessed greater capacity to suppress *R. solani in vitro* than members of the same group from the other soil locations. The Pukekohe soil had come from a potato monoculture, and spontaneous Rhizoctonia disease decline as a result of host plant monoculture has been reported to be, at least in part, due to increases in *R. solani* suppressive Pseudomonad populations (Mendes *et al.*, 2011; Postma *et al.*, 2010a). This formed the basis of one hypothesis tested in Chapter 6 – that monoculture at the Pukekohe site had caused Rhizoctonia disease decline.

It would have been of value to perform a Rhizoctonia disease assay using the four soil samples collected for the isolation experiments, so that the results from the isolations and subsequent *R. solani* dual culture assays could have been analysed with regards to the levels of disease suppression observed in different soils. Unfortunately this was not part of the objective of these experiments, and performing disease assays with newly collected soils from the same areas would have held little meaning, as the isolations would not have been made from the identical soil samples the results could not have been combined.

7.2.2 Rhizoctonia disease bioassay

The results of experiments detailed in Chapter 3 fulfilled the objective of optimising conditions for an *in vivo* greenhouse assay to be used to screen for microbe isolates able to suppress Rhizoctonia diseases of potato. Comparing the Rhizoctonia disease expression over a large (100-fold) difference of initial pathogen inoculum concentrations in the plant growing medium generated interesting results. One *R. solani* isolate tested (R73-13b, AG 3), expressed disease symptoms at a consistent level across the range of inoculum concentrations. For the other isolate (Rs043-2, AG 2-1), however, disease incidence was less at the greater inoculum concentrations. This is consistent with published reports that populations of *R. solani* rarely correlate positively with disease levels (Bonanomi *et al.*, 2010). These results also hold implications for biocontrol of Rhizoctonia diseases of potato. Organisms which are able to decrease the total population of *R. solani* in the soil, for example through mycoparasitism or production of compounds toxic to the pathogen, may not affect expression of disease. This was demonstrated in experiments outlined in Chapter 3, where less pathogen did not equate to less disease. This further justifies the need for potential biocontrol isolates to be screened *in vivo*. The selection process should not be reliant on strong suppression of the pathogen in dual culture, as partial destruction of the pathogen inoculum is unlikely to impact on levels of disease severity, as it may be that there is no inoculum threshold for this pathogen.

7.2.3 Screening of potential BCAs

In Chapter 4, 89 potential biocontrol isolates from the targeted taxa were tested in an inundative manner, for their capacity to suppress Rhizoctonia canker symptoms and disease impacts on potato plant parameters (including number of stolons and tubers, as well as tuber weigh and shoot/root dry weight). This was a much larger number of isolates than most previous biocontrol studies have tested *in vivo* for suppression of Rhizoctonia diseases of potato (Brewer and Larkin, 2005; Grosch *et al.*, 2005; 2006; Lahlali and Hijri, 2010; Tariq *et al.*, 2010). This was to put less emphasis on *in vitro* pre-screening selection of isolates. Few isolates positively affected both a measure of canker symptoms and improvement of plant parameters affected by disease. Of the 22 isolates which demonstrated positive impact on canker disease symptoms or affected plant parameters, 13 were tested in more than one screen, of which only three demonstrated positive effects in more than one assay. Thirteen isolates from the disease suppression screens were re-tested in a minituber system, but these rarely improved the assessed parameters, and often had negative effects on one or more parameter. This lack of convincing biocontrol or reproducibility meant that the investigation into inundative biocontrol was terminated.

The rationale for isolating and screening only members from the genus *Trichoderma*, the fluorescent Pseudomonads and endospore-forming bacteria, based on previous reports of Rhizoctonia disease suppression (Chapter 1, section 1.3.4.3), may not have been the best strategy. Results from this study demonstrated that the strains from the three targeted groups tested did not give strong or consistent biocontrol of Rhizoctonia diseases of potato. Narrowing the selection of isolates may have excluded strains from other groups which could have offered superior biocontrol, such as other members of the γ-Proteobacteria, for example *Lysobacter* spp. (Postma *et al.*, 2010b). Members of groups very different to those tested, such as Actinomycetes or yeasts could also potentially have offered disease suppression (Kulik, 1996). Alternatively, the number of isolates screened *in vivo* (89) may have been insufficient for discovery of a strong BCA. A higher-throughput screening process could compensate for this, as discussed in the following three sections (7.2.3.1, 7.2.3.2 and 7.2.3.3).

The previously published studies tested fewer potential BCAs *in vivo*, and reported stronger, if not always consistent, suppression of Rhizoctonia diseases of potato. However, these studies often applied BCAs using methods which may not be realistic in a New Zealand commercial agricultural setting. This includes using sterile plant growing medium to test BCAs (Lahlali and Hijri, 2010; Tariq *et al.*, 2010), and amending the plant growing medium with nutrient substrate pre-colonised with BCA at high concentrations (Grosch *et al.*, 2006; Lahlali and Hijri, 2010). In the present study, potential BCAs were tested as spore or cell suspensions to non-sterile plant growth media, at concentrations more applicable to the field. However, this attempt to replicate more realistic field conditions within the greenhouse assays is likely to be the reason for poor levels of biocontrol compared with published reports for similar potential BCAs. Potential BCAs could have been applied in basic formulations, including polymer gels such as xanthan gum or alginate (Weller, 1988), rather than aqueous suspensions. This may have improved biocontrol by protecting the organisms from environmental stresses. However, while formulations are necessary to ensure viability and consistency of products, they do not necessarily improve the efficacy of a BCA. Experiments conducted by Brewer and Larkin (2005) tested commercial formulations of the biocontrol products SoilGard® (*Trichoderma virens*, GL-21) and RootShield® (*Trichoderma harzianum*, T-22) alongside aqueous spore suspensions of the strains cultured on PDA, for control of Rhizoctonia diseases of potato. Their results demonstrated that un-formulated spores sometimes achieved the same level of biocontrol as formulated spores, although in some experiments formulated products achieved biocontrol when un-formulated did not. In one experiment, GL-21 aqueous spore suspension reduced black scurf severity when the formulated product did not. This suggests that formulations are unlikely to improve the

performance of a BCA under controlled conditions, and so it is doubtful that formulation of the strains examined in the present study would have improved their efficacy.

The inundative biocontrol results supported the opinion stated by several researchers, that the single isolate inundative approach is insufficient for consistent biological control of Rhizoctonia diseases, and a range of suppressive organisms may be required (Bonanomi *et al.*, 2010; Kuter *et al.*, 1983; Mendes *et al.*, 2011). Multiple isolates may act synergistically due to increased ecological range of activity, as well as by combining several mechanisms of action (Larkin *et al.*, 1998). To incorporate this hypothesis into the screening process, screens could have been based on a factorial design with even numbers of isolates from the three targeted groups in screens. This may have enhanced the likelihood of determining which combinations of isolates were necessary for disease suppression. However, far fewer isolates could be screened by this method compared with the assays described here.

The bioassay system used was sufficient to screen a relatively large number (89) of potential biocontrol agents *in vivo*. Improvements to this assay could make it higher-throughput, reducing total assay time, cost and potentially reduce variability (requiring fewer replicates), so that larger numbers of isolates (or isolate combinations) could be screened over the same period of time. The following sections outline methods that should be adopted for future assessments.

7.2.3.1 Plant propagule choice and disease symptoms assessed

Plants grown from tissue culture plantlets were used in many of the assays which screened the majority of the *Trichoderma*, *Bacillus* and *Pseudomonas* sp. isolates. This was to remove variability introduced by the plant propagule, as all the plants had identical aseptic culture conditions, were physiologically similar and did not carry their own microorganisms (including pathogens). These propagules, however, already possessed above ground shoots at assay initiation they were not susceptible to shoot 'nipping', the early stage of Rhizoctonia diseases of potato, so this symptom could not be assessed. The assays were run for periods when lesions on stolons and early yield impacts could be measured. The total time taken to screen the isolates could have been reduced (along with total cost) if the disease assay had used only plants propagated from tubers, and focused on the shoot nipping symptom. This would reduce the volume of plant growth medium required per plant, because only shoot emergence would have been assessed, rather than effects on stolons and tubers, which require greater volumes of growth medium to support formation. The assays could, therefore, have been conducted in incubators with even greater control and reproducibility of environmental conditions, potentially reducing variability between screens. Due to the very controlled

manner of their production, minitubers or microtubers (Ranalli, 2007; Struik, 2007) would still be the plant propagules of choice, but seed potatoes would also be suitable if graded to be of very similar in size and sprout number upon dormancy breaking. Surface sterilisation of tubers should also be included, to keep variability to a minimum and prevent the introduction of unwanted potato pathogens into the assays. Making these changes to the bioassay would allow a greater number of potential biocontrol isolates to be screened *in vivo*, under more tightly controlled environmental conditions.

7.2.3.2 Pathogen inoculum

Culturing the Rhizoctonia on a nutritionally complex medium (barley grain + V8® juice) could have introduced a source of variability into the assays, in the form of competition from microorganisms present in the plant growth medium for what remained of the introduced substrate. This could explain why the greatest concentrations of inoculum for *R. solani* isolate Rs043-2 gave less disease than the smallest concentrations, as demonstrated in Chapter 3. In future, it would be advisable to culture the pathogen on a less nutritionally complex medium which would be exhausted more fully prior to bioassay inoculation. An example could be the medium of perlite drenched in malt extract peptone, used by Boogert and Luttikholt (2004) in their disease assays. This may also reduce variability within assays.

7.2.3.3 Plant growing medium

The growing medium used in the disease assays was initially a peat:pumice based seed raising mix, with strong disease pressure, followed by a growing medium based on crushed soil as the main component. While this change resulted in less disease in general, it carries with it the issue of inconsistent microflora and physical conditions both between and within assays, as soil is very heterogeneous (Kirk *et al.*, 2004). A soil-based plant growth medium is, however, closer to the environment in which a BCA will have to operate. A compromise between the two would be the preparation of an artificial soil, like the standard mix described in the OECD Guideline 207 (1984), consisting of; 10% sphagnum peat, 30% kaolin clay and 70% quartz sand. Another alternative would be the artificial soil developed by Ellis (2004), designed to replicate a habitat similar to natural soil but lacking factors that complicate experimental work. This consists of the following components from chemical suppliers; sand, kaolinite (1:1 clay), bentonite (2:1 clay), $CaCO_3$ and humic acid. These plant growth media should be physically similar to soil, but sparse in naturally occurring microbial populations, decreasing this source of variability. This type of growing medium would also present the option of inoculation with a homogenous suspension of microflora from soil samples, which should ensure a uniform soil community through the medium, if effects in more biologically realistic

or complex systems are required. Inoculation of an artificial soil with the full or partial microbial community of field soils would also be a useful tool for investigations into naturally occurring disease suppression, a theme explored in Chapter 6. If artificial soils are to be used as bioassay plant growth media, then a variety should be tested and compared with field Rhizoctonia disease results, to select one which presents similar disease pressure levels as those occurring naturally.

7.3 Conservation biological control

Since the single isolate inundative biological control did not present any results which warranted further investigation, the study changed direction to focus on induction of Rhizoctonia disease suppression either by OM amendment, or through crop rotation practice.

7.3.1 Organic matter amendments

There have been many reports of suppression of Rhizoctonia diseases induced by the addition of organic matter (OM) amendments, although, of the most common soil-borne phytopathogens studied, *R. solani* is the least consistently controlled (Bonanomi *et al.*, 2007). However, when disease suppression has been achieved, it is the biological parameters such as populations of antagonistic microorganisms which were most frequently associated with suppression (Bonanomi *et al.*, 2010), implying that disease suppression has been biological in its nature. The first two experiments in Chapter 5 tested whether selected OM amendments could suppress Rhizoctonia diseases of potato. Since there was a significant linear trend linking increased concentrations of pine bark compost with decreasing Rhizoctonia canker symptoms, this OM was further analysed in a third experiment. Pine bark compost was tested over an 8-fold range of concentrations, to determine if stimulation of soil microbial community by this OM could offer suppression of Rhizoctonia diseases of potato. In the third experiment, the pine bark compost amendments failed to suppress the Rhizoctonia diseases of potato, and therefore deeper analysis of soil microbial community was not conducted. However, analysis of the community metabolic activity and diversity demonstrated that increases in these parameters *per se* were insufficient to suppress disease. This result agrees with the suggestion made above, that biological suppression of Rhizoctonia diseases is reliant upon enhancing populations of a cohort of specific antagonists, rather than an increase in one strain or, in this case, the community as a whole. Several published studies have concluded that pre-inoculation of OM with biocontrol strains improves the consistency of disease suppression of either component alone (Krause *et al.*, 2001; Kwok *et al.*, 1987; Pugliese *et al.*, 2011; Scheuerell *et al.*, 2005). This avenue was not explored in the present research, as the

isolates tested in Chapter 4 had not demonstrated sufficient biological control activity to warrant further study. They had also not been selected for compatibility with the selected OMs. Expecting synergistic effects would have been optimistic. Hence, in Chapter 5 (the OM experiments) it was suggested that future investigations into biocontrol of Rhizoctonia diseases of potato should start with the assumption that BCAs will require a nutrient base when applied in the field, in order to give consistent and effective disease suppression. Therefore, isolations and screenings should be conducted in conjuncture with an economically viable OM, to provide a stronger likelihood of identifying a practical combination of an OM and BCA for use in commercial crop management.

7.3.2 Suppressive soils and crop rotations

There was some evidence from the fluorescent Pseudomonad dual culture data (Chapter 2) that the soil collected from a crop rotation trial site in Pukekohe, New Zealand, may have become suppressive to *R. solani* as a result of potato monoculture. The present study examined whether this was the case by comparing the populations of *R. solani* AG 3 and 2-1 in plots from four rotation treatments using qPCR. In addition, the capacity of soil samples from each of the four rotation treatments to suppress Rhizoctonia diseases and *R. solani* populations was tested in a greenhouse assay. The results of the greenhouse experiment demonstrated that the soils from the four rotation treatments did not differ with respect to the expression of Rhizoctonia canker symptoms, or in their impact on the population of an introduced *R. solani* AG 2-1. The study also found that there had been no build-up of *R. solani* AG 3 or 2-1 in the rotation treatments, even in the treatment which had an 8 year potato monoculture. This was a surprising result, since crop rotations are widely practiced to prevent the build-up of soil-borne phytopathogen populations, including *R. solani* (Garbeva *et al.*, 2008; Larkin *et al.*, 2011; Sweetingham, 1996). The soil at the Pukekohe trial site could be moderately suppressive to Rhizoctonia diseases of potato irrespective of crop history, but no experiment was conducted with soils from different potato cropping regions to compare levels of disease in each soil.

There have been several published reports that the selection of the crop in rotation with potato can impact on the severity of Rhizoctonia diseases, and that crop rotations also affect soil microbial communities (Garbeva *et al.*, 2004; Larkin, 2008). The present study tested what impact the four rotation treatments had on bacterial and fungal soil communities, using culture-dependant enumeration and substrate utilisation profiling, as well as the culture independent PCR-based community fingerprinting technique, ARISA (Ranjard *et al.*, 2001). There was strong similarity between the bacterial communities over the four treatments. The

identity of the crop before harvest influenced the fungal communities, not the rotation history. This lead to the conclusion that eight growing seasons of potato monoculture at the Pukekohe site had not had a greater impact on the soil microbial community than a single potato crop.

It has been indicated that the disease suppressive capacity of soils is frequently correlated with diversity of soil microbial populations (Garbeva *et al.*, 2004). Relationships between the determined community parameters and levels of Rhizoctonia canker symptoms, and *R. solani* soil populations, from the greenhouse assay, were tested using correlation analysis. The results demonstrated that for the soil samples under investigation, increases in bacterial or fungal community richness and diversity correlated with increases in levels of Rhizoctonia disease, the converse of what was expected. These results align with the results from the OM section of the present study, where general increases in microbial community activity were not sufficient to suppress Rhizctonia diseases of potato.

Together, the results outlined in Chapters 5 and 6 indicate that attempts to control Rhizoctonia diseases of potato by enhancing the general activity or diversity of the soil microbial community are very unlikely to generate strong or consistent Rhizoctonia biocontrol. This conclusion is of value to New Zealand potato growers looking for methods alternative or complimentary to synthetic fungicide control for suppression of Rhizoctonia diseases, as simply increasing background microbial community richness and diversity in soil is unlikely to offer control of these diseases.

7.4 Fungicide control

Many of the *in vivo* greenhouse assays in the present study included treatments with the fungicide pencycuron. The New Zealand label registration from Monceren® 250 FS (Bayer CropScience), which was used here, recommends the product only be used for potatoes (AgriMedia Limited, 2008). This fungicide gave strong (often complete) and consistent suppression of Rhizoctonia disease symptoms for the *R. solani* strains used in the assays, even when applied at 12% of the full label rate (Chapter 5). This demonstrates two things; first that the fungicide is still a viable option for potato growers, and second that the assay was able to detect disease suppression. The method of application, thorough mixing through a small volume of plant growth medium, no doubt favoured control, as all pathogen inoculum would have been likely to come into contact with the fungicide. The product label states that the fungicide is only 'slightly mobile in soil layers'. In the field, therefore, where the fungicide is applied only as an application to tubers before planting, *R. solani* inoculum in soil distant to the seed tubers is less likely to be affected. It has been demonstrated in field studies that

fungicide control is less effective when initial inoculum levels are high (Tsror and Peretz-Alon, 2005). Pencycuron has the potential to be applied in conjunction with fungal (or bacterial) BCAs for enhanced Rhizoctonia disease control, because it is a *Rhizoctonia*-specific fungicide. Boogert and Luttikholt (2004) found that co-application of pencycuron with the mycoparasite *Verticillium biguttatum* provided greater reduction of black scurf than either used alone. Disease control strategies which combine chemical fungicides with BCAs have the potential to reduce the concentration of chemical applied, or delay the development of fungicide resistance.

7.5 Final conclusions

The present study has shown that strong, consistent biological control of Rhizoctonia diseases of potato is unlikely to be achieved through the inundative application of single isolate biocontrol microorganisms. Furthermore, these diseases are unlikely to be reduced by general increases in soil microbial richness, diversity or metabolic activity. The study also repeatedly found no correlation between the size of *R. solani* populations and severity of Rhizoctonia diseases of potato. This finding indicates that any biocontrol strategy aimed at destruction of *R. solani* inoculum would have to achieve near complete eradication of the pathogen to ensure disease suppression. It is, therefore, advisable that investigations into biocontrol of these diseases should not focus solely on elimination of the pathogen for disease suppression. Future study of biocontrol of Rhizoctonia diseases of potato should focus on identifying combinations of OM amendments and cohorts of biocontrol isolates which act synergistically. This approach has the greatest potential for achieving worthwhile and practical biological management of these diseases.

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Appendix A

Media and buffers

A.1 Agar and broth

A.1.1 Potato-dextrose agar (PDA)

39.0 g Potato Dextrose Agar (Oxoid)
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity Schott Bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.2 Nutrient Agar (NA)

Nutrient Agar 28.0 g (if Oxoid) or 23.0 g (if Difco)
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.3 Kings Medium 'B' (KB)

15.0 g Agar
15 mL Glycerol
3.0 g Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)
2.0 g Dipotassium hydrogen orthophosphate (K_2HPO_4)
20.0 g Proteose peptone
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.4 *Trichoderma* selective medium (TSM) – Lincoln University

20.0 g Agar
3.0 g Glucose
1.0 g Ammonium nitrate
0.9 g Dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4.3H_2O$)
0.2 g Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)
0.15 g Potassium chloride (KCl)
0.2 g Terrachlor® 75WP fungicide (quintozene 750 g/kg a.i.)
0.15 g Rose Bengal

1 mL chloramphenicol stock solution (a 2.5 mg/mL solution made with absolute (96%) ethanol)

1 mL mixture of following:
1.0 g Iron sulphate (Ferrous sulphate) 7 hydrate (FeSO4.7H2O)
0.65 g Manganous sulphate tetrahydrate (MnSO4.4H2O)
0.9 g Zinc sulphate (ZnSO4.7H2O)
Preparation: dissolve all three ingredients in 1 L dH_2O
1 L dH_2O

Preparation: Combine all ingredients in a 1 L capacity bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.5 ¼ Strength Potato Dextrose Agar

9.8 g Potato Dextrose Agar (Oxoid)
15.0 g Agar
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.6 Luria Bertani (LB) Broth

25.0 g of Luria Broth (Sigma)
1 L dH_2O
Preparation: Combine ingredients in 1 L capacity glass beaker, still thoroughly until all powder dissolved, dispense into required containers (e.g. 18 mL capacity Univeral Bottles) and autoclave at 121 °C, 15 psi, for 15 min.

A.1.7 Minimal Media for Slants

15.0 g malt extract
15.0 g Agar
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity bottle and autoclave at 121 °C, 15 psi, for 15 min.

A.1.8 Physiological peptone solution (PPS)

8.5 g NaCl
1.0 g Bacteriological Peptone
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity Schott Bottle and autoclave at 121°C, 15 psi, for 15 min.

A.1.9 Water Agar (WA)

15.0 g Agar
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity Schott Bottle and autoclave at 121°C, 15 psi, for 15 min.

A.1.10 PDA:NA 1:1 (v/v)

19.5 g Potato Dextrose Agar (Oxoid)
14.0 g Nutrient Agar (Oxoid)
1 L dH_2O
Preparation: Combine all ingredients in a 1 L capacity Schott Bottle and autoclave at 121°C, 15 psi, for 15 min.

A.1.11 0.1% physiological agar

1 g Agar

8.5 g NaCl
1 L dH2O
Preparation: Combine all ingredients in a 1 L capacity Schott Bottle and autoclave at 121°C, 15 psi, for 15 min.

A.1.12 Fungal enumeration agar

39 g Potato dextrose agar (Oxoid)
2 mL Triton X-100 (Scintron®)
100 µL of 100 mg/mL chloramphenicol (Sigma life science) stock solution (in 96% Ethanol)
300 L of 100 mg/mL streptomycin sulphate (Sigma-Aldrich) stock solution (in RO water)
1 L dH_2O
Preparation: Combine agar, Triton and water in a 1 L Schott Bottle and autoclave at 121 °C, 15 psi, for 15 min. Cool to 60°C in a water bath. Add chloramphenicol stock to bottle, as well as streptomycin stock (filtered through 0.2 µm sterile luer-lock nylon membrane filter), shake end-over-end to mix and immediately pour into standard petri plates. Store at 4°C until required.

A.2 Inoculum Media

A.2.1 Barley grain + V8 juice®

50 g Barley grain
50 mL Original V-8® juice (Campbell's Soups Australia, Australia)
Preparation: Combine all ingredients in a 250 mL capacity Erlenmeyer flask, plug flask neck with cotton wool, cap with tinfoil and autoclave at 121 °C, 15 psi, for 15 min.

A.2.2 Wheat bran + RO water

25 g Wheat bran
50 mL Reverse osmosis water
Preparation: Combine all ingredients in a 250 mL capacity Erlenmeyer flask, plug flask neck with cotton wool, cap with tinfoil and autoclave at 121 °C, 15 psi, for 15 min.

A.2.3 Bran:peat medium

25 mL wheat bran (loose)
25 mL sphagnum peat (loose)
25 mL dH_2O
Preparation: Combine all ingredients in a 250 mL capacity conical flask, plug neck loosely with cotton wool and cap with tinfoil. Autoclave at 121°C, 15 psi, for 15 min.

A.3 Plant growth media

A.3.1 Bioassay potting mix

Sphagnum peat (New Zealand Growing Media, Winton, NZ)
Bulk pumice 1-3 mm grade (Egmont Commercial, Christchurch, NZ) 3:2 (v/v), with 200 g Osmocote Extract Mini® N:P:K (16:3.5:0.1) + Trace Elements (Scotts, NSW, Australia
Dolomite lime (Golden Bay Dolomite, Golden Bay, NZ)
Hydraflo2® wetting agent (Scotts, NSW, Australia)

Preparation: Mix peat and pumice in a 3:2 (v/v) ratio, with 200 g Osmocote, 400 g lime and 100 g wetting agent per 100 L.

A.3.2 John Innes shrub mix

7 parts Wakanui silt loam soil passed through crusher
3 parts sphagnum peat
2 parts pumice (1-3 mm grade)
1.2 g/L blood and bone
1.2 g/L superphosphate
0.3 g/L potassium sulphate
2.0 g/L Agricultural lime
3.5 g/L dolomite lime
Preparation: Combine all ingredients and turn three times by shovel to mix thoroughly, store in shade and add water if required to prevent mix becoming dry.

A.3.3 Soil:pumice mix

7 parts Wakanui silt loam soil passed through crusher
3 parts pumice (1-3 mm grade)
1.2 g/L blood and bone
1.2 g/L superphosphate
0.3 g/L potassium sulphate
2.0 g/L Agricultural lime
3.5 g/L dolomite lime
Preparation: Combine all ingredients and turn three times by shovel to mix thoroughly, store in shade and add water if required to prevent mix becoming dry.

A.4 Buffers

A.4.1 Soil extraction buffer

100 mL of 1M Tris-HCl
20 mL of 5M NaCl
100 mL of 0.5 M EDTA
100 mL of 10% SDS solution
Preparation: Combine Tris-HCl, NaCl and EDTA solutions in a measuring cylinder, make up to 800 mL with ddH_2O. Add SDS solution. Check solution pH, adjust to pH6.4 with cHCl. Make up to 1 L with ddH_2O.

A.4.2 Ammonium acetate (10 M)

77 g Ammonium acetate
100 mL H_2O
Preparation: Dissolve ammonium acetate in 70 mL of H_2O at room temperature. Adjust volume to 100 mL with H_2O. Sterilise the solution by passing it through a 0.22 µm filter. Store solution in tightly sealed bottle at or below room temperature.

Appendix B

Additional tables and figures

B.1 Isolate selection

B.1.1 Isolates from Bio-Protection Research Centre Culture Collection, Lincoln selected to be screened for *in vivo* suppression of Rhizoctonia diseases of potato

Table 7.1 Isolates from Bio-Protection Research Centre Culture Collection selected for *in vivo* screening

Identifier	Isolation location	Identity	Selection Rationale
LU132	Pukekohe, Auckland, NZ	*Trichoderma atroviride*	Biocontrol of *Sclerotium cepivorum*
LU144	UV mutant of LU132		Biocontol of *Rhizoctonia* sp.
LU298	Tauranga, NZ		Biocontrol of *Botrytis cinerea*, *Sclerotinia sclerotiorum*
LU569	Wellington, NZ	*T. virens*	Biocontrol of *Ciborinia camelliae*
LU151	NZ	*T. harzianum*	Biocontrol of *Phytophthera* sp.
LU713	Auckland, NZ	*T. koningiopsis*	Biocontrol of *Phytophthora cactorum*
LU740	Wainui, Auckland, NZ	*T. hamatum*	
LU593	Canterbury, NZ		Biocontrol of *Sclerotinia sclerotiorum*
LU140	Pukekohe, Auckland, NZ	*T. atroviride*	Biocontrol of *Sclerotium cepivorum*
LU547	Christchurch, NZ	*T. virens*	Biocontrol of several fungal plant pathogens
LU549			
LU806	LU132 protoplast fusion	*T. atroviride*	Cold tolerant
LU297	Auckland, NZ	*Trichoderma* sp.	Growth promotion of several plant species
LU753	Canterbury, NZ		
LU1370	Christchurch, NZ		
LU540	NZ	*T. virens*	
LU996	Canterbury, NZ	*Trichoderma* sp.	Potential plant growth promoter
SS1635	Brassica seed	*Bacillus* sp.	Strong antibiotic producer and potential BCA of plant pathogens
SS1708			
SS1902			
SS573		Bacterium	
LU1187	Canterbury, NZ	*Paenibacillus polymyxa*	Suppressive to *Aphanomyces euteiches*

B.2 Screening results

B.2.1 Full screening results tables

Table 7.2 Screen 1 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell suspensions as treatements, with target 1×10^6 CFU/g plant growth medium

Treatment	Applied conc^n (CFU/g)	Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.0 a	0.0 a	9.4 a	9.4 a	1.99 d	0.21 c	4.7 a	4.61 a
Pathogen control		48.2 e	76.1 cd	3.9 d	0.8 bcd	1.14 ab	0.11 a	1.4 c	3.09 ab
Appl^n solution (LB:PPS)		36.9 cde	47.7 b	4.0 cd	1.5 bc	0.85 a	0.08 a	1.7 bc	0.86 cde
RSP0002	1.00×10^6	49.8 e	84.6 cdef	3.1 d	0.2 de	0.86 a	0.10 a	0.7 c	0.53 efg
RSP0017	7.21×10^5	44.6 de	87.8 def	5.3 bcd	0.7 bcde	1.19 abc	0.13 ab	1.4 bc	0.33 fg
RSP0144	7.29×10^5	30.5 cde	65.0 bc	3.9 cd	0.7 bcde	1.07 ab	0.13 ab	1.9 bc	0.61 def
RSP0170	5.43×10^5	33.8 cde	94.3 ef	5.5 bcd	0.4 cde	1.24 abc	0.13 ab	1.6 bc	0.40 efg
RSP0268	8.53×10^5	13.6 abc	79.4 cde	7.3 abc	1.8 bc	1.85 bcd	0.15 abc	2.8 ab	0.82 cd
RSP0362	7.05×10^5	38.1 cde	91.8 def	5.4 cd	0.2 de	1.43 abcd	0.14 abc	1.4 bc	0.62 def
RSP2004	1.85×10^6	19.9 abcd	100.2 f	6.2 abcd	0.0 e	1.37 abcd	0.14 abc	1.6 bc	0.56 def
RSP2020	1.07×10^6	35.5 cde	101.8 f	3.7 cd	0.0 e	0.77 a	0.09 a	0.6 c	0.72 def
RSP2042	1.36×10^6	27.9 bcde	98.6 ef	5.6 bcd	0.0 e	1.13 ab	0.10 a	1.9 c	0.55 bc
RSP2086	2.02×10^6	36.8 cde	104.0 f	4.9 cd	0.0 e	0.80 a	0.08 a	1.4 c	0.66 def
RSP2139	8.45×10^5	48.6 de	47.9 b	4.3 cd	1.8 b	0.79 a	0.08 a	0.9 c	0.24 g
RSP2183	9.30×10^6	48.3 de	87.6 def	3.8 cd	0.7 bcde	1.15 ab	0.12 a	2.1 bc	1.89 bc

Table 7.3 Screen 2 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from minitubers in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected spore suspensions as treatements, with target 1×10^6 spores/g plant growth medium

Treatment	Applied conc^n (spores/g)	Emerging shoots nipped (%)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.0 a	0.5 a	7.9 a	7.8 a	1.86 a	0.22 a	4.4 a	4.18 a
Pathogen control		65.1 bc	79.8 cd	0.9 de	0.2 cd	0.23 de	0.04 d	0.5 c	0.22 bc
RSPT113	1.00×10^6	67.5 bc	42.5 b	1.8 bcde	0.9 bc	0.44 cde	0.04 cd	0.2 bc	0.03 bc
RSPT105	1.00×10^6	59.2 bc	100.0 e	2.1 bcde	0.4 bcd	0.33 cde	0.07 bcd	0.5 bc	0.05 bc
RSPT124	7.21×10^5	81.1 c	69.2 c	1.1 bcde	0.6 bcd	0.34 cde	0.08 bcd	0.2 bc	0.27 bc
RSPT119	1.00×10^6	63.9 bc	50.0 b	1.9 bcd	0.5 bcd	0.70 bcd	0.10 bcd	0.7 bc	0.14 bc
RSPT028	1.00×10^6	55.9 bc	72.6 c	2.7 b	0.5 bcd	0.73 bcd	0.11 bc	0.3 bc	0.16 bc
RSPT037	1.00×10^6	49.3 b	96.7 e	2.3 bcd	0.6 bcd	0.71 bcd	0.07 bcd	0.9 bc	0.18 bc
RSPT085	6.98×10^5	59.2 bc	100.0 e	0.7 cde	0.0 d	0.29 de	0.04 cd	0.2 bc	0.01 bc
RSPT122	1.00×10^6	56.4 bc	43.4 b	2.2 bcd	1.0 b	0.76 bc	0.07 bcd	0.9 bc	0.16 bc
LU806	1.00×10^6	80.2 c	2.9 a	0.1 e	0.1 d	0.04 e	0.03 d	0.0 c	0.00 c
RSPT106	9.77×10^5	45.2 b	90.0 de	2.6 b	0.3 bcd	1.19 b	0.14 b	0.7 b	0.70 b
RSPT116	1.00×10^6	46.4 b	73.2 c	2.6 bc	0.7 bcd	0.94 bc	0.10 bc	0.5 bc	0.18 bc
RSPT125	7.21×10^5	60.1 bc	4.2 a	0.8 bcde	0.2 bcd	0.43 cde	0.05 cd	0.1 bc	0.01 bc

Table 7.4 Screen 3 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 CFU or spores/g plant growth medium

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	Total no. of stolons	No. of symptom-less stolons	Shoot dry weight (g)$^+$	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control		0.4 a	4.6 a	4.4 a	4.18	0.95 abc	4.0 a	9.01 a
Pathogen Control		91.0 de	2.7 cde	0.3 d	5.06	0.94 abc	0.3 cd	0.29 b
Appl^n solution (LB:PPS)		72.3 bc	3.7 abc	1.2 b	3.60	0.62 ef	0.2 cd	0.04 b
RSP0268	1.01×10^6	94.7 de	4.0 ab	0.2 d	5.53	0.96 abc	0.3 cd	0.09 b
RSPT5080	1.00×10^6	81.6 cde	2.7 cde	0.6 bcd	5.34	0.95 abc	0.7 bc	0.67 b
RSP2002	6.59×10^5	88.3 cde	2.7 cde	0.4 cd	4.70	0.88 bcd	0.7 bc	0.17 b
RSP2009	1.63×10^6	60.8 b	2.4 cde	1.2 bc	3.68	0.72 def	0.0 d	0.00 b
RSP2014	3.10×10^5	85.6 cde	3.3 abc	0.5 bcd	5.13	1.10 a	0.7 cd	0.17 b
RSP2015	1.09×10^6	93.0 de	2.9 bcde	0.3 d	5.43	0.97 abc	0.4 bcd	0.14 b
RSP2110	3.88×10^5	78.7 cd	2.8 abc	0.6 bcd	4.16	0.73 def	0.4 bcd	0.03 b
RSPT018	1.00×10^6	83.4 cde	2.2 cde	0.5 cd	5.14	0.93 abc	0.4 cd	0.10 b
RSPT037	1.00×10^6	87.7 cde	3.3 abc	0.5 cd	4.54	0.85 cd	0.4 cd	0.36 b
RSPT084	1.00×10^6	98.2 e	2.9 abc	0.1 d	5.79	0.11 ab	0.9 b	0.46 b
RSPT5030	1.00×10^6	98.4 e	3.0 abcd	0.1 d	5.12	0.99 abc	0.2 cd	0.13 b
RSP0362	7.05×10^5	91.7 de	2.6 cde	0.2 d	5.03	0.82 cde	0.1 cd	0.01 b
LU 132	1.00×10^6	85.9 cde	1.9 e	0.4 bcd	3.84	0.57 f	0.1 cd	0.17 b
LU 140	1.00×10^6	92.7 de	2.1 de	0.2 d	4.66	0.85 cd	0.3 cd	0.02 b

$^+$No differences ($P < 0.05$) between any of the shoot dry weight treatment means

Table 7.5 Screen 4 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1 × 10^6 CFU or spores/g plant growth medium

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	No. of Stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)
No pathogen control		1.0 a	2.4 a	1.78 abcde	0.39 abcde	2.5 a
Pathogen control		62.9 cdefg	0.5 cdef	1.86 bcde	0.35 de	1.6 ab
Appl^n solution (LB:PPS)		80.7 fghi	0.5 cdefg	1.77 abcde	0.33 e	2.3 ab
RSP2002	5.43×10^4	39.6 b	0.8 bcde	1.89 abcde	0.37 bcde	1.6 abc
RSP2009	1.55×10^6	55.3 bcdef	0.9 bcdef	2.06 e	0.45 a	2.3 ab
RSP2013	8.14×10^5	39.7 b	1.4 b	1.62 abc	0.35 cde	2.3 ab
RSP2014	1.09×10^6	63.3 bcdefgh	0.5 bcdefg	1.66 abcde	0.33 e	1.5 ab
RSP2016	6.16×10^5	90.1 hi	0.4 defg	1.57 ab	0.36 cde	2.6 a
RSP2023	2.56×10^6	43.2 bcd	1.0 bcdef	1.50 a	0.37 bcde	2 ab
RSP2024	8.53×10^4	68.5 defghi	0.7 bcdefg	1.91 abcde	0.40 abcde	1.9 abc
RSP2026	1.47×10^6	67.3 cdefghi	1.0 bcdef	1.79 abcde	0.35 cde	2.4 ab
RSP2027	0.00	51.6 bcde	0.5 bcdefg	1.95 bcde	0.37 abcde	1.1 bcd
RSP2053	4.88×10^5	46.3 bcd	0.3 defg	1.60 ab	0.33 e	0.7 cd
RSP2057	1.71×10^6	93.9 i	0.1 g	2.01 cde	0.37 abcde	1.4 abc
RSP2067	1.02×10^6	67.0 bcdefghi	0.2 efg	2.03 cde	0.19 abcde	0.7 d
RSP2071	2.48×10^6	40.9 bc	1.6 bc	1.85 abcde	0.35 cde	2.4 ab
RSP2089	1.82×10^6	70.0 defghi	0.6 bcdefg	1.75 abcde	0.35 cde	1.9 ab
RSP2116	1.67×10^6	75.2 efghi	0.7 bcdefg	1.84 abcde	0.43 abc	2.3 ab
RSPT001	1.00×10^6	84.6 ghi	0.2 efg	1.64 abcd	0.36 cde	1.4 abc
RSPT005	1.00×10^6	81.3 fghi	0.4 defg	1.82 abcde	0.41 abcd	1.6 ab
RSPT007	1.00×10^6	49.0 bcde	0.9 bcd	1.69 abcde	0.37 abcde	1.7 ab
RSPT029	1.00×10^6	80.0 fghi	0.4 cdefg	1.79 abcde	0.42 abc	2.2 ab
RSPT097	1.00×10^6	68.3 cdefghi	0.6 cdefg	1.76 abcde	0.39 abcde	1.7 ab
RSPT107	1.00×10^6	80.5 fghi	0.6 bcderfg	1.87 abcde	0.38 abcde	2.3 ab
RSPT110	1.00×10^6	90.0 hi	0.2 fg	1.83 abcde	0.42 abc	1.8 ab

Table 7.6 Screen 5 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1 × 10^6 CFU or spores/g plant growth medium

Treatment	Applied conc^n (spores or CFU /g)	Diseased stolons (%)	No. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)$^+$	No. of tubers	Total tuber weight (g)
No pathogen control		1.9 a	5.0 fg	4.9 a	4.16 a	0.68	4.5 a	13.63 a
Pathogen control		95.8 de	3.2 abc	0.2 bcd	5.74 cdef	0.73	0.6 cd	0.36 d
Appl^n solution (PPS)		97.7 de	3.6 abcde	0.2 bcd	5.50 bcdef	0.69	0.6 bcd	0.57 d
LU 1187	3.33×10^5	98.4 e	4.4 defg	0.1 cd	6.18 fg	0.76	0.9 bcd	0.52 d
SS 573	5.81×10^5	94.2 bcde	4.1 cdefg	0.3 bcd	5.65 bcdef	0.66	0.6 bcd	0.15 d
SS 1635	7.37×10^5	93.7 bcde	3.6 abcde	0.4 bc	5.30 bcde	0.71	1.2 bcd	0.63 d
SS 1708	9.57×10^5	90.2 bcd	3.9 bcdefg	0.4 bc	5.82 cdef	0.74	1.3 b	1.71 d
SS 1902	9.42×10^5	98.1 de	3.7 abcde	0.4 bcd	5.27 bcde	0.67	0.6 bcd	0.56 d
RSP 2072	8.45×10^5	89.2 bc	4.9 defg	0.6 b	5.03 b	0.62	0.9 bcd	1.79 d
RSP 2090	7.71×10^5	87.2 b	3.8 abcdefg	0.5 bc	5.18 bc	0.58	1.3 bc	3.62 c
RSP 2094	6.67×10^5	94.2 bcde	2.9 abc	0.2 bcd	5.87 cdef	0.64	0.8 bcd	0.22 d
RSP 2110	1.07×10^6	100.0 e	4.2 abcdefg	0.0 d	5.62 bcdef	0.72	1.3 bcd	1.62 d
RSP 2120	1.43×10^6	100.0 e	3.4 abcd	0.0 d	5.78 cdef	0.69	0.5 bcd	0.59 d
RSP 2158	6.98×10^5	98.0 de	2.9 abc	0.1 cd	5.73 bcdef	0.77	0.7 bcd	0.18 d
RSP 2013	8.53×10^5	93.7 bcde	3.4 abc	0.3 bcd	6.87 g	0.83	0.2 d	0.05 d
LU 144	1.00×10^6	97.2 cde	2.7 ab	0.1 cd	6.17 fg	0.74	0.7 bcd	0.89 d
RSP 5182	1.00×10^6	100.0 e	3.9 abcdefg	0.0 d	5.48 bcdef	0.69	0.4 bcd	0.44 d
RSPT 003	1.00×10^6	96.7 cde	2.5 a	0.1 cd	5.95 ef	0.77	0.7 bcd	0.18 d
RSPT 031	1.00×10^6	96.3 cde	4.6 efg	0.2 bcd	5.88 cdef	0.72	1.0 bc	0.37 d
RSPT 036	1.00×10^6	94.7 bcde	4.1 cdefg	0.2 bcd	5.19 bcd	0.69	1.0 bc	1.77 d
RSPT 060	1.00×10^6	93.6 bcde	3.5 abcde	0.5 bcd	5.62 bcdef	0.77	0.9 bcd	0.68 d
RSPT 079	1.00×10^6	98.0 de	3.4 abcdef	0.4 bcd	5.90 cdef	0.81	0.8 bcd	0.18 d
RSPT 093	1.00×10^6	100.0 e	3.4 abc	0.1 cd	5.96 ef	0.75	0.7 bcd	1.07 d

$^+$No differences ($P < 0.05$) between any of the root dry weight treatment means

Table 7.7 Screen 6 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in peat mix or soil mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected cell/spore suspensions as treatements, with target 1×10^6 CFU or spores/mL plant growth medium

Treatment	Plant growth medium	Applied conc^n (spores or CFU /mL)	Diseased stolons (%)	No. of stolons	No. of symptom-less stolons	Shoot dry weight (g)	Root dry weight (g)	No. of tubers	Total tuber weight (g)
No pathogen control	Peat mix		0.6 a	10.0 a	9.9 a	3.03 ab	0.74 c	6.6 a	8.54 a
Pathogen control			97.0 fg	4.8 de	0.2 h	3.99 gh	0.78 ab	0.4 ef	0.17 g
Appl^n solution (PPS)			100.0 fg	5.1 cde	0.0 h	3.93 efgh	0.70 cd	0.3 def	0.0 g
Pencycuron			0.0 a	9.0 ab	9.0 ab	2.92 a	0.70 c	7.1 a	8.12 a
SS 1708		1.13×10^6	100.0 fg	4.3 cde	0.0 g	4.02 fghi	0.69 cd	0.9 def	0.14 g
RSP 2090		1.19×10^6	100.0 fg	5.4 cde	0.0 h	4.29 hi	0.74 abc	0.6 def	0.08 g
RSP 2083		1.00×10^6	96.0 efg	5.3 cde	0.2 gh	4.26 hi	0.76 abc	0.8 d	0.81 fg
RSP 2125		9.44×10^5	100.0 fg	5.9 bcde	0.0 h	4.09 ghi	0.76 abc	0.9 de	0.55 g
RSP 2193		1.11×10^5	90.2 def	5.0 cde	0.5 fgh	4.07 ghi	0.73 bc	0.2 def	0.18 g
RSPT 124		1.00×10^6	100.0 g	5.9 bcde	0.0 h	3.66 def	0.71 c	0.3 def	0.02 g
RSPT 106		1.00×10^6	95.0 efg	4.6 cde	0.1 h	4.54 i	0.82 a	0.1 f	0.03 g
LU 540		1.00×10^6	100.0 fg	6.0 bcde	0.0 h	3.82 efg	0.70 c	0.9 de	0.47 g
LU 713		1.00×10^6	92.5 efg	3.8 e	0.2 gh	3.94 efgh	0.75 abc	0.3 def	0.10 g
LU 740		1.00×10^6	99.6 fg	5.9 bcde	0.0 h	4.34 i	0.76 abc	0.6 def	0.28 g
No pathogen control	Soil mix		4.5 a	7.3 abcd	7.1 b	3.20 abc	0.57 ef	6.7 a	7.52 ab
Pathogen control			80.6 cd	6.2 bcde	1.7 d	3.32 bcd	0.56 efg	4.3 ab	4.72 cd
Pencycyron			1.1 a	7.5 abcd	7.4 b	3.11 ab	0.47 g	5.7 ab	5.44 c
SS 1708		1.06×10^6	76.3 c	6.6 bcde	2.3 de	3.33 bcd	0.47 g	4.6 b	2.50 ef
RSP 2090		9.63×10^5	88.1 de	6.9 bcde	1.0 def	3.57 cde	0.53 fg	2.7 c	3.21 de
RSPT 124		1.00×10^6	86.9 de	7.5 abcd	0.9 efg	3.93 efgh	0.62 de	1.8 c	1.10 fg
RSPT 106		1.00×10^6	59.9 b	7.1 bcde	3.3 c	3.10 ab	0.50 fg	5.4 ab	4.47 cd

Table 7.8 Screen 7 full results, mean values for Rhizoctonia disease and physical plant parameters for potato plants grown from tissue-cultured plantlets in soil mix, inoculated with *Rhizoctonia solani* isolate Rs043-2 and selected spore suspensions as treatements, with target 1×10^6 spores/mL plant growth medium

Treatment	Applied conc^n (spores/mL)	Diseased stolons (%)	No. of Stolons	No. of symptom-less stolons	Shoot dry weight (g)$^+$	Root dry weight (g)$^+$	No. of tubers	Total tuber weight (g)
No pathogen control		0.2 a	4.89 bc	4.9 a	3.95	0.38	3.28 ab	6.05 bcde
Pathogen control		66.2 defg	4.05 bcd	1.3 d	4.12	0.42	2.26 cd	4.08 def
Pencycuron		5.3 a	7.20 a	6.8 a	3.57	0.4	4.20 a	10.08 a
LU 132	1.00×10^6	45.3 bcd	5.00 bc	2.7 bc	3.94	0.36	2.60 bcd	7.09 abc
LU 1370	1.00×10^6	67.3 defg	5.33 ab	2.1 bcd	3.74	0.40	2.00 cd	2.55 f
LU 151	1.00×10^6	66.1 cdefg	3.90 bcd	1.4 bcd	4.06	0.42	2.70 bcd	5.34 bcdef
LU 297	1.00×10^6	66.3 cdefg	4.87 bcd	1.8 bcd	4.12	0.44	2.75 abcd	4.41 bcdef
LU 298	1.00×10^6	78.1 g	3.67 bcd	1.2 d	4.43	0.37	2.22 bcd	3.12 ef
LU 540	1.00×10^6	53.1 bcdefg	5.40 ab	2.5 bc	4.71	0.46	2.30 bcd	7.93 ab
LU 547	1.00×10^6	55.5 bcdefg	5.00 bc	2.0 bcd	5.12	0.56	2.83 abcd	5.63 bcdef
LU 549	1.00×10^6	37.2 b	4.60 bcd	2.5 bc	3.66	0.36	2.20 bcd	7.21 abc
LU 593	1.00×10^6	75.8 fg	3.80 bcd	1.2 d	4.82	0.47	2.40 bcd	5.52 bcdef
LU 753	1.00×10^6	73.1 efg	3.89 bcd	1.5 cd	4.24	0.37	2.00 cd	6.69 abcde
LU 806	1.00×10^6	60.4 bcdefg	4.40 bcd	1.8 cd	4.75	0.49	2.50 bcd	4.24 bcdef
RSP 5075	1.00×10^6	52.3 bcdefg	3.00 d	2.1 cd	4.23	0.40	1.40 d	2.44 f
RSP 5163	1.00×10^6	53.7 bcdefg	3.30 cd	1.2 cd	5.06	0.48	1.60 d	3.02 ef
RSPT 037	1.00×10^6	60.6 bcdefg	3.80 bcd	1.4 cd	4.08	0.42	2.50 bcd	7.34 abc
RSPT 084	8.25×10^5	66.3 defg	4.50 bcd	1.4 cd	4.33	0.42	2.60 bcd	4.44 bcdef
RSPT 085	1.00×10^6	61.7 bcdefg	3.90 bcd	1.4 cd	4.03	0.36	1.90 cd	4.46 bcdef
RSPT 106	1.00×10^6	39.2 bc	3.44 cd	2.1 bcd	4.11	0.38	2.78 abcd	4.28 bcdef
RSPT 119	1.00×10^6	55.4 bcdefg	4.67 bcd	2.0 bcd	4.51	0.43	2.50 bcd	2.13 f
RSPT 122	1.00×10^6	50.2 bcdef	4.40 bcd	2.4 bc	3.82	0.36	3.20 abc	3.91 cdef
LU 144	1.00×10^6	35.7 b	4.80 bcd	2.9 b	3.39	0.35	2.00 cd	7.20 abc
RSP 5182	1.00×10^6	47.9 bcde	3.89 bcd	2.3 bcd	4.05	0.39	2.78 abcd	3.93 bcdef
LU 569	1.00×10^6	74.4 fg	4.30 bcd	1.4 cd	4.14	0.46	2.40 bcd	7.69 abc

$^+$No significant differences ($P < 0.05$) between shoot dry weight means or root dry weight means

B.2.2 Dual plate sclerotial suppression

Table 7.9 Mean sclerotial suppression scores from agar plate assays (for bacterial isolates) assessed on a 0 to 3 scale: 0 = no sclerotia, 1 = light coverage, 2 = medium coverage, 3 = heavy coverage (similar to experimental controls)

Identifier	Identity	*R. solani* isolate	
		Rs043-2	Rs018-2
RSP2015	*Bacillus* sp.	2.5	2
RSP2067		3	3
SS 1635		1	1
SS 1708		1	1
SS1902		1	1
RSP2071	*Bacillus methylotrophicus*	1	0.5
RSP0170	*Bacillus mycoides*	2	1
RSP2002		1.5	2
RSP2009		3	3
RSP2013		1	1
RSP2072		3	3
RSP2027	*Bacillus soli*	3	3
RSP0002	*Bacillus thuringiensis*	3	3
RSP0017		3	2
RSP0144		3	2
RSP0268		2	2
RSP2016		3	3
RSP2158		3	2
RSP2024	*Brevibacillus laterosporus*	3	2.5
RSP2090	Endospore bacterium	3	3
RSP2026	*Flavobacterium* sp.	2	3
RSP2004		3	3
RSP2020		3	3
RSP2053		3	3
RSP2094		3	3
RSP2193		2	2
RSP2139	*Paenibacillus peoriae*	3	1
LU1187	*Paenibacillus polymyxa*	3	2
RSP0362	*Paenibacillus* sp.	3	2.5
RSP2057	*Pseudomonas* sp.	2	1.5
RSP2086		3	2
RSP2110		0.5	0
RSP2083	*Pseudomonas brassicacearum*	2	3
RSP2023	*Pseudomonas jessenii*	2	1.5
RSP2042		3	3
RSP2014	*Pseudomonas koreensis*	2	1.5
RSP2125		1.5	0.5
RSP2116	*Pseudomonas lurida*	1	0
RSP2120		1	0
RSP2089	*Pseudomonas mohnii*	3	3
RSP2183	*Pseudomonas reinekei*	0	0
SS 573	Bacterium	1	0

B.3 Real-Time Quantitative PCR Data

AG 3 and AG 2-1 standards were created by extraction of DNA from pure fungal cultures. DNA concentrations were measured by NanoDrop (Thermo Scientific) spectrophotometry, and then serially diluted. The concentration of standards down to 10 ng/µL of DNA was confirmed by NanoDrop (personal communication, Sandi Keenan, Plant and Food Research Ltd, Lincoln).

B.3.1 AG 3 qPCR data from Pukekohe trial soil samples

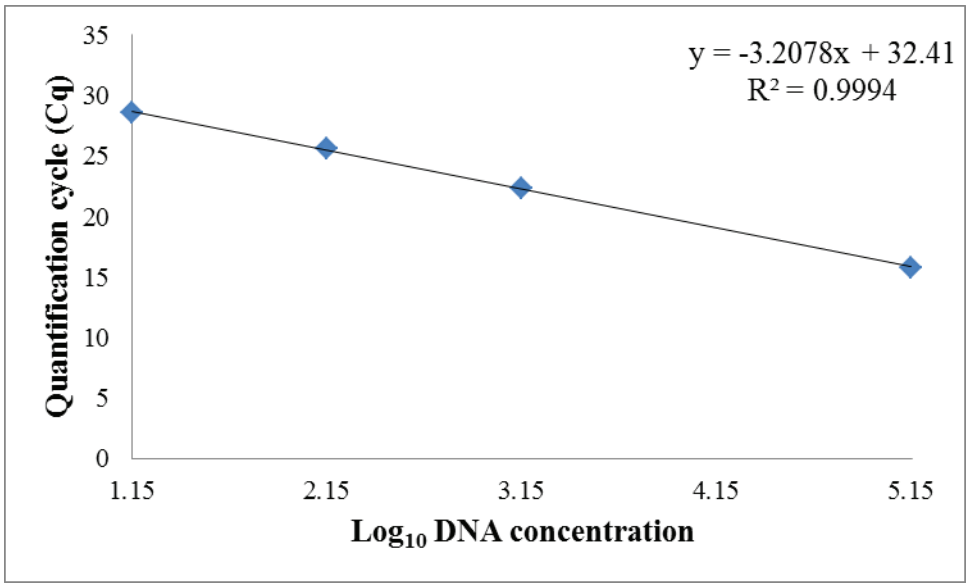

Figure 7.1 Calibration curve of *Rhizoctonia solani* AG 3 standards with slope, intercept and r^2, mass of template DNA in standards was 142, 1.42, 0.142 and 0.0142 pg DNA.

PCR amplification efficiency = $10^{-1/slope}$-1, as detailed by Bustin *et al.* (2009), calculated as 1.0499.

Table 7.10 Results for *Rhizoctonia solani* AG 3 qPCR analysis of Pukekohe trial plots, including quantification cycle (C_q) value, calculated quantity of DNA per sample plus mean and standard deviation (SD) for DNA quantity per sample

Sample Name	C_q	Quantity (pg DNA)	Quantity mean (pg DNA)$^+$	Quantity SD
Water	36.74320602	0		
Water	Undetermined	Undetermined	0	N/A
Plot 2	Undetermined	Undetermined		
Plot 2	Undetermined	Undetermined	0	N/A
Plot 3	36.30620956	6.09873E-05		
Plot 3	Undetermined	Undetermined	0	N/A
Plot 4	Undetermined	Undetermined		
Plot 4	Undetermined	Undetermined	0	N/A
Plot 5	36.45093918	5.49694E-05		
Plot 5	Undetermined	Undetermined	0	N/A
Plot 8	Undetermined	Undetermined		
Plot 8	36.22825241	6.44973E-05	0	N/A
Plot 9	Undetermined	Undetermined		
Plot 9	34.62841415	0.000203365	0	N/A
Plot 11	Undetermined	Undetermined		
Plot 11	Undetermined	Undetermined	0	N/A
Plot 12	36.86979675	4.06955E-05		
Plot 12	Undetermined	Undetermined	0	N/A
Plot 13	36.81325912	4.2381E-05		
Plot 13	36.03949356	7.38559E-05	C_q > LOD	C_q > LOD
Plot 14	Undetermined	Undetermined		
Plot 14	36.62240982	4.86034E-05	0	N/A
Plot 15	Undetermined	Undetermined		
Plot 15	Undetermined	Undetermined	0	N/A
Plot 16	Undetermined	Undetermined		
Plot 16	Undetermined	Undetermined	0	N/A
Plot 17	Undetermined	Undetermined		
Plot 17	Undetermined	Undetermined	0	N/A
Plot 19	Undetermined	Undetermined		
Plot 19	36.5153923	5.24842E-05	0	N/A
Plot 22	36.27754593	6.22551E-05		
Plot 22	35.95970917	7.82091E-05	C_q > LOD	C_q > LOD
Plot 24	Undetermined	Undetermined		
Plot 24	Undetermined	Undetermined	0	N/A

$^+$As one replicate of water control gave a C_q value of 36.7, a limit of detection (LOD) was set at Cq = 36. Only samples where C_q < LOD for both replicates were considered to have true detection of AG 3 DNA.

B.3.2 AG 2-1 qPCR data from Pukekohe trial soil samples

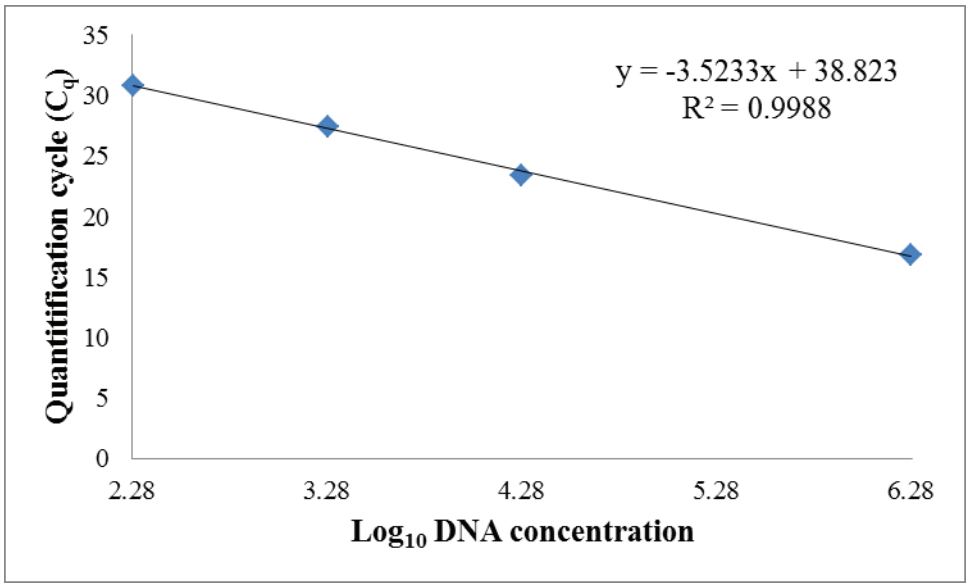

Figure 7.2 Calibration curve of *Rhizoctonia solani* AG 2-1 standards with slope, intercept and r^2, mass of template DNA in standards was 1.92, 1.92×10^{-2}, 1.92×10^{-3} and 1.92×10^{-4} pg DNA

PCR amplification efficiency = 0.922.

Table 7.11 Results for *Rhizoctonia solani* AG 2-1 qPCR analysis of Pukekohe trial plots, including quantification cycle (C_q) value, calculated quantity of DNA per sample plus mean and standard deviation (SD) for DNA quantity per plot

Sample Name	C_q	Quantity (pg DNA)	Quantity mean (pg DNA)$^+$	Quantity SD
Water	Undetermined	Undetermined		
Water	Undetermined	Undetermined	0	N/A
Plot 2	36.90377045	3.50428E-06		
Plot 2	34.91073608	1.2891E-05	8.19765E-06[A]	4.69337E-06
Plot 3	Undetermined	Undetermined		
Plot 3	Undetermined	Undetermined	0	N/A
Plot 4	Undetermined	Undetermined		
Plot 4	Undetermined	Undetermined	0	N/A
Plot 5	Undetermined	Undetermined		
Plot 5	Undetermined	Undetermined	0	N/A
Plot 8	35.37225342	9.5344E-06		
Plot 8	35.51543427	8.68268E-06	9.10854E-06 [A]	4.25859E-07
Plot 9	35.75098038	7.44386E-06		
Plot 9	Undetermined	Undetermined	0	N/A
Plot 11	Undetermined	Undetermined		
Plot 11	Undetermined	Undetermined	0	N/A
Plot 12	38.37695313	1.33801E-06		
Plot 12	Undetermined	Undetermined	0	N/A
Plot 13	Undetermined	Undetermined		
Plot 13	Undetermined	Undetermined	0	N/A
Plot 14	Undetermined	Undetermined		
Plot 14	Undetermined	Undetermined	0	N/A
Plot 15	36.61934662	4.22015E-06		
Plot 15	36.5336647	4.4632E-06	4.34167E-06 [A]	1.21529E-07
Plot 16	Undetermined	Undetermined		
Plot 16	Undetermined	Undetermined	0	N/A
Plot 17	36.25614166	5.35078E-06		
Plot 17	34.45550537	1.73579E-05	1.13543E-05 [A]	6.00355E-06
Plot 19	Undetermined	Undetermined		
Plot 19	Undetermined	Undetermined	0	N/A
Plot 22	35.91032791	6.70765E-06		
Plot 22	35.33633804	9.76084E-06	8.23425E-06 [A]	1.5266E-06
Plot 24	Undetermined	Undetermined		
Plot 24	Undetermined	Undetermined	0	N/A

+Mean value calculated if both plot replicates provided a determined C_q. As neither water replicate gave a C_q value, no limit of detection was set.

[A]Values fall outside of the standard curve, and so absolute quantity of DNA cannot be determined.

B.3.3 AG 2-1 qPCR data from Pukekohe soil greenhouse disease assay

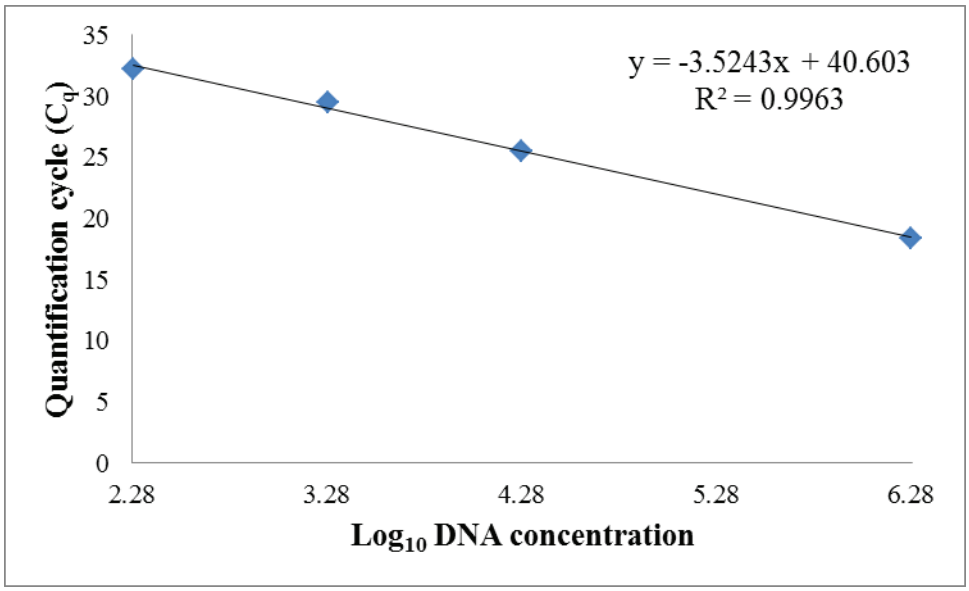

Figure 7.3 Calibration curve of *Rhizoctonia solani* AG 2-1 standards with slope, intercept and r^2, mass of template DNA in standards was 1.92, 1.92×10^{-2}, 1.92×10^{-3} and 1.92×10^{-4} pg DNA

PCR amplification efficiency = 0.923.

Table 7.12 Results for *Rhizoctonia solani* AG 2-1 qPCR analysis of Pukekohe soil greenhouse disease assay, including quantification cycle (C_q) value, calculated quantity of DNA per sample plus mean and standard deviation (SD) for DNA quantity per plot

Sample Name	C_q	Quantity (pg DNA)	Quantity mean (pg DNA)$^+$	Quantity SD
Water	Undetermined	0		
Water	Undetermined	0	0	N/A
Plot 2	28.94010162	0.002038401		
Plot 2	28.82536507	0.002197082	0.002117742	7.93408E-05
Plot 3	29.5643959	0.001355641		
Plot 3	29.43620872	0.00147407	0.001414855	5.92145E-05
Plot 4	27.91941833	0.0039711		
Plot 4	28.73789978	0.002326295	0.003148698	0.000822403
Plot 5	27.932024	0.003938528		
Plot 5	27.95082664	0.00389044	0.003914484	2.40443E-05
Plot 8	27.66910362	0.004676687		
Plot 8	27.64811516	0.00474126	0.004708973	3.22866E-05
Plot 9	27.29111671	0.005986775		
Plot 9	27.48420334	0.005277207	0.005631991	0.000354784
Plot 11	28.86472893	0.002141296		
Plot 11	28.61606216	0.002519049	0.002330172	0.000188876
Plot 12	28.21530533	0.003273047		
Plot 12	28.15021706	0.00341524	0.003344143	7.10964E-05
Plot 13	27.09556961	0.006802677		
Plot 13	26.9943409	0.007267813	0.007035245	0.000232568
Plot 14	28.32759094	0.003041522		
Plot 14	28.24924469	0.003201267	0.003121394	7.98724E-05
Plot 15	28.352705	0.002992022		
Plot 15	28.42509842	0.002853796	0.002922909	6.91129E-05
Plot 16	28.86343765	0.002143104		
Plot 16	28.66718864	0.002436292	0.002289698	0.000146594
Plot 17	28.65478325	0.002456119		
Plot 17	28.79358101	0.002243185	0.002349652	0.000106467
Plot 19	29.10816574	0.001826421		
Plot 19	28.89467621	0.002099806	0.001963114	0.000136692
Plot 22	27.77106667	0.004375281		
Plot 22	27.95005798	0.003892394	0.004133838	0.000241444
Plot 24	29.07152557	0.001870672		
Plot 24	29.02673912	0.00192622	0.001898446	2.7774E-05

$^+$Mean value calculated if both plot replicates provided a determined C_q. As neither water replicate gave a C_q value, no limit of detection was set.