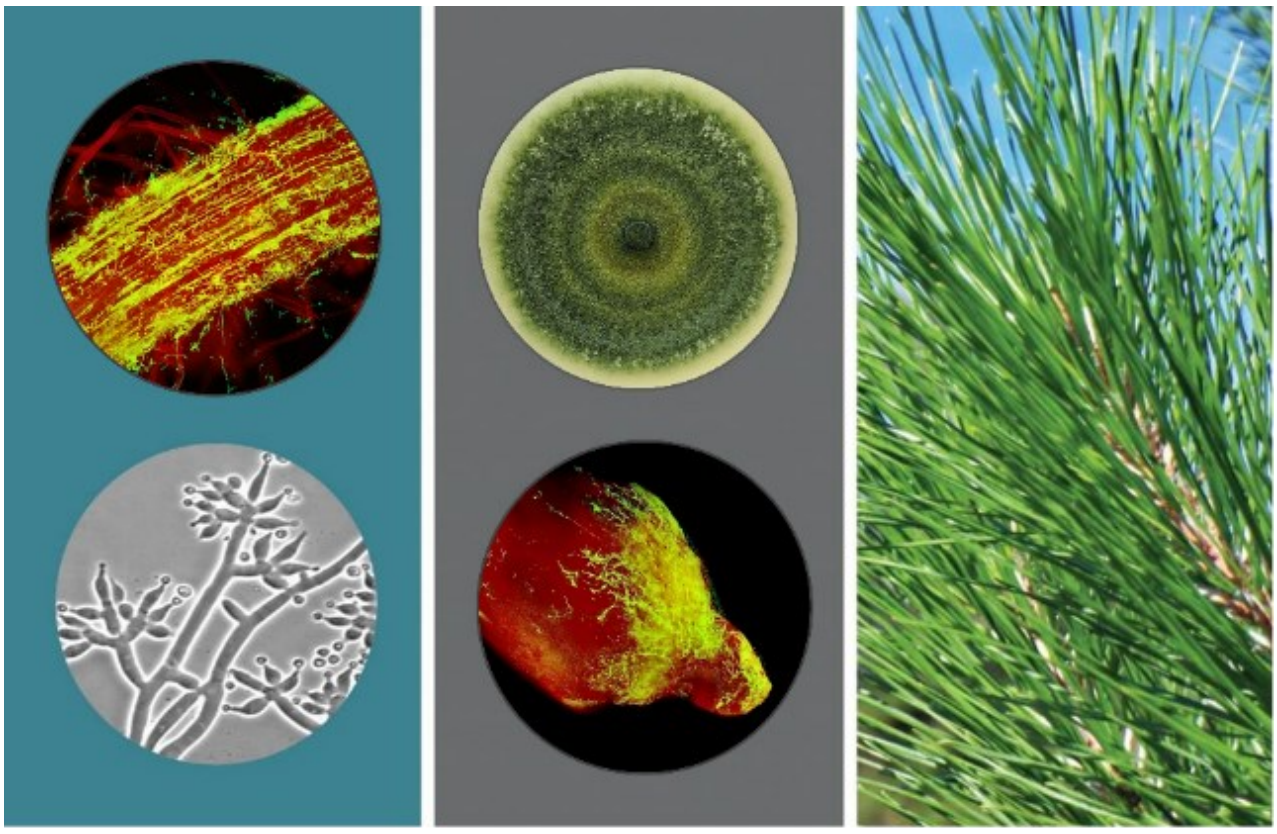
Bio-Protection
Bioprotection science for New Zealand

Bioprotection for foliar diseases and disorders of radiata pine

Project Overview
July 2019

Report prepared for New Zealand Forest Growers Research



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FOREST GROWERS RESEARCH

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EXECUTIVE SUMMARY

Foliar diseases and disorders are the most significant cause of economic loss for the New Zealand forestry industry. In order to alleviate losses caused by existing diseases and to reduce potential impacts of biosecurity threats, ongoing work (initiated in 2012) is being conducted to establish a long-term symbiotic relationship between *Pinus radiata* (radiata pine) and beneficial microbes, in particular, *Trichoderma*. The products of this research project will be *Trichoderma* inoculated radiata pine with significantly enhanced growth and improved foliar health, as well as reduced chemical application in nurseries and plantations.

The *Trichoderma* bioprotectants selected in earlier work (Hill, 2016) continue to provide significant benefits to tree growth and health in New Zealand plantation forests. Important results of recent work include:

- Tree height and trunk diameter (at breast height) were significantly ($P<0.05$) increased by up to 17% and 19% respectively, compared to untreated radiata pine plantation trees, four to 6.5 years after application of *Trichoderma* as a nursery seed-coat.

- Dothistroma needle blight (*Dothistroma septosporum*) severity was significantly ($P<0.05$) reduced by up to 60%, compared to untreated radiata pine plantation trees, 3.5 to 5.5 years after application of *Trichoderma* as a nursery seed-coat. *Trichoderma* seed-treatment reduced but did not eliminate the expression of foliar disease in the field.

- Molecular techniques using species and strain-specific PCR primers confirmed the persistence of an applied bioprotectant *T. atroviride* LU633, in the majority (94%) of root pieces tested, in six radiata pine plantations 3.5 years after tree establishment. In three of the trials measured with high concentrations of root LU633, one recorded significant ($P<0.05$) increases in tree height and trunk diameter three years after tree establishment.

- A pilot trial, where a bioprotectant *Trichoderma* mixture was impregnated into roots, suggested that *Trichoderma* can be inoculated into aged plantation trees and cause a growth response. Efficient and effective methods for introduction of *Trichoderma* into established trees are currently being investigated.

- Effective deployment of bioprotection *Trichoderma* isolates may need to allow for different regional soil temperatures. A study of *Trichoderma* living in natural association with trees in radiata pine plantations in warm and cold regions of New Zealand found that species have adapted to temperature regime. Only three species were common to both regions (*T. atroviride*, *T. spirale* and *T. viridescens*). Both the *Trichoderma* mixtures selected for field validation in 2018 (PR6 and PR3a) will be suitable for deployment in temperate or sub-tropical plantations (the majority of New Zealand's forestland); however, PR3a may be more suitable for colder temperate regions, south of Nelson/Marlborough.

- Application of two separate *Trichoderma* bioprotectants (*via* soil bed drenching at setting) to hard-to-root radiata pine clonal cuttings significantly ($P<0.001$) increased root initiation (measured as percentage of cuttings with roots present in 3 or 4 quadrants) 2.5-fold (average of 29.1%), compared to the untreated control (11.9%). Approximately 8 to 10% more cuttings (average of 65.4%) survived to harvest in the *Trichoderma* treatments, compared to the untreated control (56.3%). One *Trichoderma* treatment significantly

(P<0.05) increased plant dry weight, compared to the untreated Control, even though roots and shoots were trimmed during the year as part of standard nursery practice.

- Containerised nursery Douglas-fir *(Pseudotsuga menziesii)* seedling roots were highly compatible to two *Trichoderma* mixtures applied as a potting mixture drench, with *Trichoderma* present in 56% to 84% of root pieces, eleven months after application. Persistence of *Trichoderma* in 18-month old soil bed raised plants was less than or similar to that of the containerised plants, and maintained at least one-quarter colonisation of the roots. Seedling survival was significantly (P<0.05) improved by one *Trichoderma* treatment (81.3%), compared to the untreated control (72.1%) eleven months after application. The *Trichoderma* treatments had large impacts on growth, with highly significant (P<0.001) increases in root collar diameter, height, shoot, root and plant dry weights by approximately 13, 18, 32, 29 and 30% respectively, compared to the untreated Control.

- Selected *Trichoderma* isolates were found to be fast, abundant and persistent colonisers of containerised radiata pine seedlings and cuttings grown under greenhouse or nursery conditions. The most effective isolate, FCC327, had colonised 65% of seedling root pieces eight months after inoculation.

- Standard and confocal fluorescent microscopy, using both Wheat Germ Agglutinin – Alexa Fluor® 488 (WGA-AF488) and Direct Red 80 fluorescent dyes, allowed extremely detailed visualisation and quantification of *Trichoderma* mycelial growth on, and inside, root tissue. Hyphae were observed to have a very close and abundant interaction with individual root cells, root hairs and primary and lateral roots. Generally, the hyphae grew between the cells in the intercellular and middle lamella spaces, although a few examples of intracellular development were observed. During early plant growth, *Trichoderma* had colonised all root structures, including the rhizodermal, sub-epidermal, cortex and primary xylem vessels and callus tissue of cuttings.

- In a study of tree roots from twelve regional New Zealand plantations, sequence-based identifications revealed a diverse population (including at least 57 species) of fungal endophyte species in natural association with radiata pine. This provided important prerequisite information for future examination of the effectiveness of the applied *Trichoderma* bioprotectants and identification of other species that could potentially improve radiata pine health. Some of the species identified were weak or latent plant pathogens and may need to be monitored, particularly if climatic conditions change.

- In three studies of tree roots from nineteen regional New Zealand plantations, sequence-based identifications revealed a diverse population (at least 20 species) of root endophyte *Trichoderma.* Species included: *T. atrobrunneum*, *T. austrokoningii, T. atroviride*, *T. caerulescens, T. citrinoviride*, *T. composticola, T. crassum*, *T. fertile, T .gamsii*, *T. hamatum*, *T. harzianum*, *T. koningii, T. koningiopsis, T. longipile*, *T. polysporum, T. spirale, T. tomentosum, T. trixiae*, T. viridescens, *T. virens* and five currently undescribed taxa *T. sp. 273, 702, 787, 792* and *novaeharzianum*. The impact of these natural *Trichoderma* species on the endophytic development of the applied *Trichoderma* bioprotectants is unknown and requires further research.

- The commercialisation of selected BPRC *Trichoderma* bioprotectants for nursery use is being investigated with a commercial partner.

INTRODUCTION

Foliar pathogens are currently the most significant cause of economic loss for the New Zealand forestry industry and cost the sector over $150 million per annum (Hill, 2016). This research project will reduce these losses by using selected beneficial fungal bioprotectants, which will enhance tree growth and suppress diseases. In addition, these bioprotectants will protect against biosecurity threats from pathogens not yet in New Zealand forest nurseries and plantations. This technology has resulted in reduced pesticide use in nurseries. The anticipated benefit to New Zealand will be >$50 million pa from healthier nurseries and forests and faster tree growth (Hill, 2016).

The project used a novel approach, based on screening endophytes isolated from exceptionally healthy plants, to streamline the selection of beneficial fungal isolates for foliar disease control. Nursery and laboratory assays identified many *Trichoderma* isolates that promoted growth and suppressed disease in radiata pine seedlings, including candidates that reduced the incidence of terminal crook and diplodia canker diseases. The most effective endophyte isolates formed the basis of forestry plantation trials in Nelson and the North Island of New Zealand. Twenty-four trial sites were established between 2012 and 2015 and data indicated that the most effective *Trichoderma* treatments resulted in significant increases in tree growth and health. The most effective isolates were selected from these data, and from other *Trichoderma* work undertaken at BPRC (*eg*. Chirino-Valle *et al.,* 2016), and used in eight large-scale validation trials established in four important forestry regions in 2018. It is anticipated that the most effective treatments will be made available to the industry through a commercial partner.

Dr Robert Hill developed this project in close collaboration with the NZ Forest Owners Association (NZFOA) and the Ministry of Business, Innovation and Employment between 2012 and 2016. Since 2016, the NZFOA, and recently, the NZ Forest Growers Research (FGR), have continued to fund the project with small recurring 12-month contracts. Dr Hill retired from Lincoln University in March 2018, and Dr Helen Whelan took over the project.

BIOPROTECTION PROJECT MILESTONES

The project milestones completed for the period July 2012 to December 2016 (Appendix 1) are summarised as:

1. Targeted *Trichoderma* isolation from healthy radiata pine and other plant species.
2. Optimisation of greenhouse disease screening challenges (including Dothistroma).
3. Greenhouse and field pot trials (to screen endophytes and chemical elicitors using *Diplodia* assay and to test the most effective treatments versus Dothistroma and *Phytophthora pluvialis*).
4. Superior inoculation systems (tissue culture and improved application systems).
5. Nursery trials (test the most effective treatments and determine endophyte persistence).
6. Field trials using young inoculated trees from the nursery.
7. Verification of induced resistance.
8. Controlled environment trials to test the most effective treatments.
9. Forest plantation trials to test the most effective treatments.
10. Biochemical and molecular responses to endophytes and elicitors.

The project milestones and tasks in progress or completed, for the period January 2017 to December 2019, are described in Table 1.

Table 1. Milestones and tasks for the period 1 January 2017 to 31 December 2019

Task No.	Activity		
	2017	**2018**	**2019**
	Milestone 1 – Production of *Trichoderma* inoculum		
1.1	Production of *Trichoderma* inoculum for treatment of plant material for tasks 2.2, 3.1 and 3.2	Production of *Trichoderma* inoculum for treatment of plant material for tasks 2.2, 3.4, 4.1 and 5.1	Production of *Trichoderma* inoculum for treatment of plant material for tasks 2.1, 3.4 and 5.1
	Milestone 2 – Colonisation and persistence of *Trichoderma*		
2.1	Monitoring establishment and long-term persistence of a model *Trichoderma* isolate LU633 in plantation trials using qPCR	Isolation and characterisation of cold and warm tolerant *Trichoderma* isolates	Impact of nursery chemicals on colonisation and persistence of *Trichoderma* inoculants (in progress)
2.2	Examining root colonisation by *Trichoderma* following inoculation of seedlings under controlled conditions	Examining root colonisation and persistence by *Trichoderma* following inoculation of cuttings under controlled conditions	
	Milestone 3 – Nursery and forest plantation trials		
3.1	Inoculation of *P. radiata* seedlings and/or cuttings with selected *Trichoderma* treatments	Assess root establishment and cutting growth in hard-to-root seed lots treated with *Trichoderma*	Assess root establishment and cutting growth in hard-to-root seed lots treated with *Trichoderma*
3.2	Establish validation trials for most-effective treatments to date for regions	Establish validation trials for most effective treatments to date for regions	Large validation trials for most effective treatments to date in four main regions
3.3	Assess plant growth (tree height, stem diameter) in nursery and plantation trials and treatment effect on tree stability (wind-throw)	Assess plant growth (tree height, stem diameter) and disease in plantation trials and treatment effect on tree stability (wind-throw)	Assess plant growth (tree height, stem diameter) and disease in plantation trials
	Assess disease in established field trials to determine the effect of *Trichoderma* inoculation on foliar disease expression		
3.4	Assess feasibility of treating older trees with *Trichoderma* root endophytes to mitigate disease problems	Assess feasibility of treating older trees with *Trichoderma* root endophytes to mitigate disease problems	Assess feasibility of treating older trees with *Trichoderma* root endophytes to mitigate disease problems
	Milestone 4 - Other forestry species disease issues		
4.1	Assess potential for *Trichoderma* root endophyte treatments to control cypress canker	Assess potential for *Trichoderma* root endophyte treatments to control cypress canker	Assess potential for *Trichoderma* root endophyte treatments to control cypress canker
4.2	Assess potential for *Trichoderma* root endophyte treatments to improve growth and control Swiss needle cast in Douglas fir	Assess potential for *Trichoderma* root endophyte treatments to improve growth and control Swiss needle cast in Douglas fir	Assess potential for *Trichoderma* root endophyte treatments to improve growth and control Swiss needle cast in Douglas fir
	Milestone 5 - Microbiome		
5.1	Isolation and characterisation of fungal partners in the *P. radiata* root microbiome		

2.1 Milestone 1 – Production of *Trichoderma* inoculum

Production of *Trichoderma* inoculum has mainly been by solid-state fermentation methods, including agar, brown rice and peat/wheat substrate media (refer to Section 5.4 of Hill 2016 report). The method chosen was dependent on the number of spores required and the production time available. Agar media methods were used for laboratory experimentation and small-scale greenhouse trials, while brown rice and peat/wheat media methods were used for large-scale greenhouse and field trials.

Recently, the peat/wheat method was modified with the elimination of the final drying and harvest (via a Mycoharvester) steps. Spore extraction was by addition of water to the media and using a sieve to separate the spore solution from the media. Spore inoculum generated using this method will be incorporated into the seed-coat application of *Trichoderma* at PF Olsen Ltd Waiuku Nursery in September 2019.

Trichoderma inoculum has been supplied to PF Olsen Ltd Waiuku and Seddon, Southern Cypresses, Appletons, Timberlands Te Ngae and Proseed Nurseries, for seed-coat and/or drench application to seed (approximately 13 million) or cuttings or ramets, between 2017 and mid-2019. Recently, Rotorua Forest Nursery expressed interest in *Trichoderma* trial work.

2.2 Milestone 2 – Colonisation and persistence of *Trichoderma*

A major question arising from the programme is whether the applied *Trichoderma* isolates can persist under nursery and field conditions to provide long-term growth benefits and protection from disease. Colonisation and persistence of applied *Trichoderma* in the short- and medium-term were investigated in the following studies.

2.2.1 Establishment and persistence of a model T*richoderma* LU633 in plantation trials

Selected *Trichoderma* isolates were found to be fast, abundant and persistent colonisers of containerised seedlings and cuttings grown under nursery/controlled conditions (see Section 2.2.3). These isolates were introduced into numerous bioprotection trials established in the main radiata pine growing regions between 2012 and 2015. However, it was not known whether these isolates persisted under field conditions once the inoculated seedlings or cuttings were planted.

A sensitive, species and strain-specific real-time polymerase chain reaction (qPCR) assay was used successfully to determine the persistence of one of the *Trichoderma* bioprotectants, *T. atroviride* LU633, in radiata pine roots, sampled from six trials established 3.5 years prior to sampling (Hill *et al.,* 2017). This isolate was applied as a quarter part of a seed-coat *Trichoderma* mixture in the nursery.

Key results:

- The majority of samples (47 out of 52; 94%) showed presence of *T. atroviride* LU633 in the singleplex reaction qPCR assay using LU633 primers and probe, with nine samples from the Golden Downs (Kohatu block), Harakeke and Waipaoa plantations having abundant LU633 DNA material, according to cycle threshold (Ct) values (Figure 1).

- Radiata pine root samples treated with *T. atroviride* LU633 were shown to have LU633 DNA levels ranging from 0 to 2.6 ng/µL with the highest levels in the Golden Downs (Kohatu block) plantation at an average of 0.39 ng/µL (Figure 1).

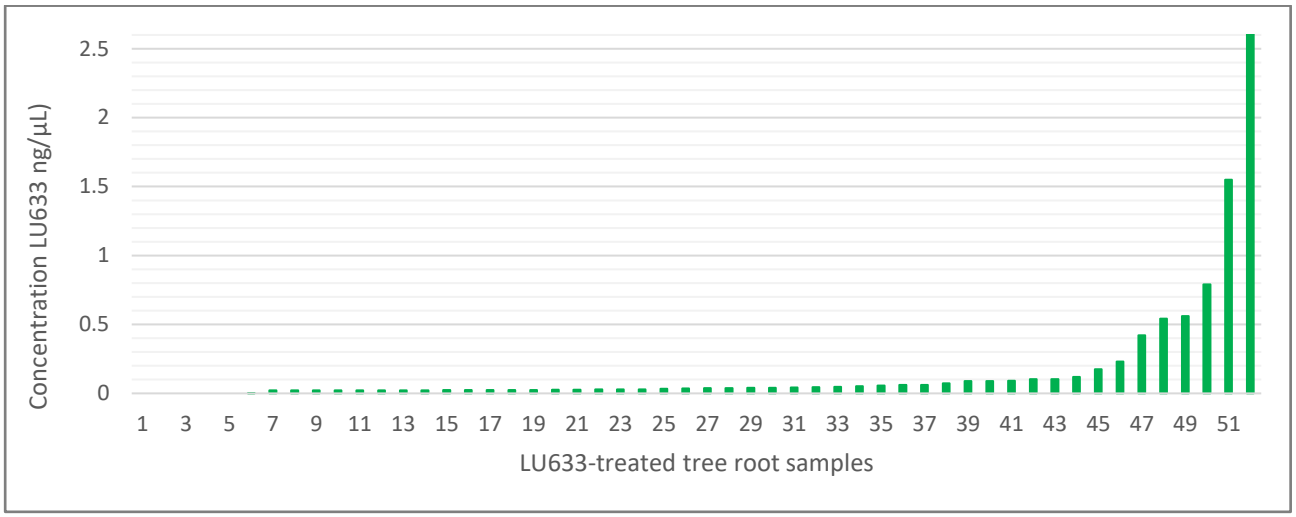

Figure 1: Concentration of *T. atroviride* LU633 in radiata pine root pieces sampled from seed-coated *Trichoderma* inoculated trees in six plantation trials.

Presence of *T. atroviride* LU633 in roots implies the provision of growth benefits and protection from disease. Tree height and trunk diameter at breast height, in the Harakeke trial, were significantly increased by 12% and 17% respectively in the *Trichoderma* treatment that included LU633, compared to the untreated Control. However, no increases in tree growth were found in the Kohatu and Waipaoa trials, one- and three-year after trial establishment, respectively, compared to the untreated Control. The effect of *T. atroviride* LU633 on disease suppression has not been determined in the Kohatu, Harakeke and Waipaoa trials due to lack of disease at assessment times. The Kohatu and Harakeke trials continue to be monitored.

As the tree matures, the bioprotectant may be exposed to biological and environmental change, including population changes of root microorganism in the rhizoplane, rhizosphere and root, tree resource competition and climate change, particularly at canopy closure. Further monitoring work is required to determine if biological and environmental changes affect the persistence, and potentially the effectiveness, of *T. atroviride* LU633, and therefore, the long-term bioprotection of plantations as they mature.

This study used a qPCR assay for direct detection of *T. atroviride* LU633 present in treated tree roots in the 2012 to 2105 plantation trials. Further research effort should focus on the development of additional strain-specific assays for direct detection of *Trichoderma* species used in the 2018 validation plantation trials (namely, FCC55, *T. harzianum,* FCC318, *T. atrobrunneum,* FCC327, *T. harzianum,* FCC340, *T. harzianum,* FCC13, *T. asperellum,* FCC14, *T. atroviride,* FCC15, *T. atroviride* and FCC180, *T. crassum*).

2.2.2 Isolation and characterisation of cold and warm tolerant *Trichoderma* isolates

The research programme has selected a range of beneficial *Trichoderma* isolates for use in New Zealand plantation forestry. An important question about the deployment of these isolates is how effective they are in different abiotic and biotic environments, potentially affecting the long-term bioprotection of the plantation. In New Zealand, radiata pine has been established in most regions, ranging from sub-tropical zones in the north to more temperate zones further south. However, low temperatures in winter, or hot temperatures in summer, may cause problems for biological control by influencing the activity of the bioprotection agents. Information on naturally occurring *Trichoderma* species in different temperature environments may indicate the preference these species have to the conditions. This information can then be used for deployment decisions of bioprotection *Trichoderma* isolates.

A study characterising the *Trichoderma* species isolated from surface-sterilised roots of radiata pine collected from five warm (Northland) and four cold (South Canterbury and South Otago) forests was undertaken in 2018 (Whelan, 2018). In total, 112 trees were sampled, 1790 root pieces were tested, and 133 *Trichoderma* isolates were isolated and cultured (Figure 2). *Trichoderma* species were identified using sequence polymerase chain reaction (PCR) primers and direct sequencing of the *tef1*α barcoding region.

Key results were:

1. a diverse population of *Trichoderma* species were found in natural association with plantation radiata pine trees including at least thirteen named (*T austrokoningii, T. atroviride, T. caerulescens, T. composticola, T. crassum, T. fertile, T. hamatum, T. harzianum, T. koningii, T. polysporum, T. spirale, T. viride, T. viridescens*), and two undescribed taxa (*T. sp. 273* and *T. sp. 787*). This information adds to a previous study done by Cummings and Hill (2016), who identified the following species: *T. atrobrunneum*, *T. austrokoningii, T. atroviride*, *T. citrinoviride*, *T. crassum*, *T. hamatum*, *T. harzianum*, *T. longipile*, *T. polysporum, T. spirale, T. tomentosum, T. trixiae*, *T. virens*, and three currently undescribed taxa (*T. sp. 702, 792 and novaeharzianum).*

2. Species diversity was similar, with eight or ten species found in the cold and warm forests respectively. However, species richness in each forest was considerably different, with high levels found in warm forests (six species), compared to low levels in cold forests (often one or two species).

3. *Trichoderma* species appeared to have adapted to the warm and cold environments in this study with only three species common to both regions (*T. atroviride*, *T. spirale* and *T. viridescens*).

The growth response of the regional isolates to temperature (2, 7, 12, 17, 22 and 27°C) was characterised by measurement of colony mycelial growth on potato dextrose agar plates and compared to a selection of beneficial *Trichoderma* isolates from the New Zealand *Trichoderma* plantation trials (PR3a mixture: FCC13, FCC14, FCC15 and FCC180; PR6 mixture: FCC55, FCC318, FCC327 and FCC340). Isolates had significantly ($P<0.05$) different growth rates at the temperatures studied, both within and between isolate groupings, demonstrating the large variation of isolate and species response to temperature. Incubation at a prolonged low temperature (2°C) found that all cold region isolates, except one (*T. spirale*), grew after five

months incubation (Appendix 2). Only five of the warm region isolates grew, albeit very slowly, and no growth was observed in the beneficial isolates (Appendix 2). Production of spores in *T. sp. 787* and *T. fertile,* in a 2°C growing regime, appear to be a strategy for survival in extremely cold (sub-zero) environments, for example, those found in the South Canterbury region. Prolonged low temperatures generally did not affect isolate viability, with all except two isolates, producing spores after reintroduction to warm temperatures (Appendix 2).

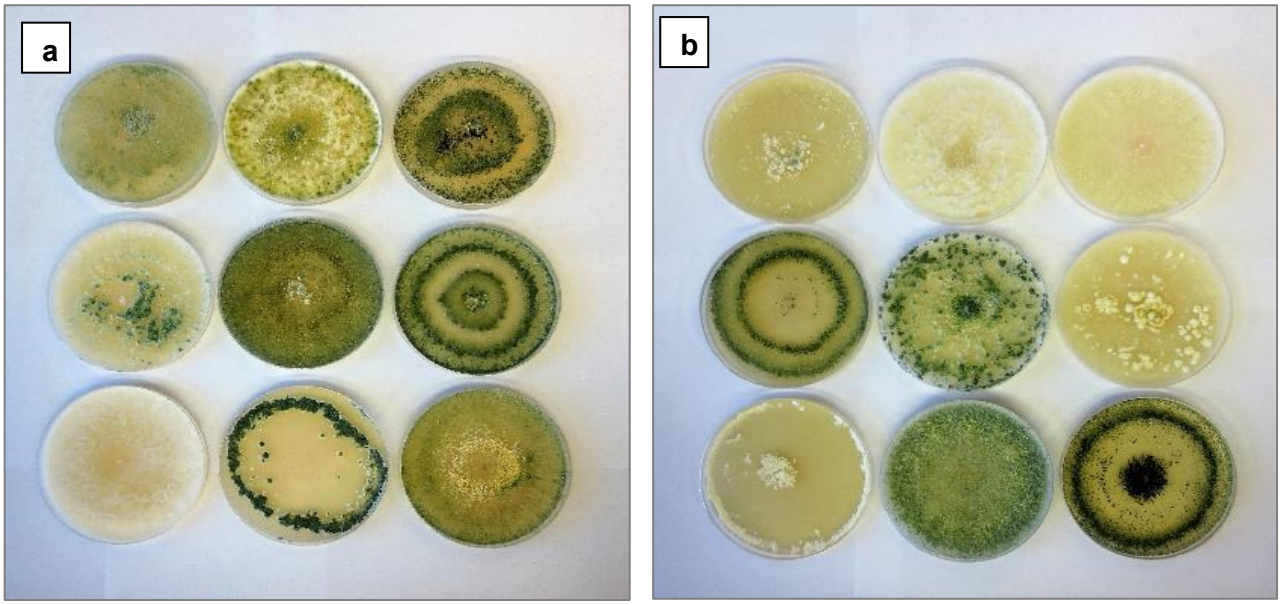

Figure 2: *Trichoderma* colonies grown on MYE agar, sub-cultured from surface-sterilised radiata pine root pieces, sampled from plantations in a) warm and b) cold regions of New Zealand.

Targeted deployment of current beneficial bioprotection mixtures to specific temperature zones may be required for improved performance and persistence, with PR6 and PR3a mixtures suitable for central temperate and sub-tropical regions (the majority of New Zealand forestland) and PR3a mixture also suitable for colder temperate regions of New Zealand (south of Nelson/Marlborough). Additional isolation and screening work of local isolates may be required to ensure effective bioprotectant selections are made with potential increases in temperature range, due to climate change in the future.

2.2.3 *Trichoderma* root colonisation of seedlings and cuttings under controlled conditions

A series of greenhouse studies between 2016 and 2018 investigated the ability of *Trichoderma* isolates to colonise and persist in radiata pine seedlings and cuttings (Hill *et al*., 2016, Whelan and Hill, 2016a and b, Whelan and Hill, 2017 and Whelan 2019). Isolates, including LU132, LU140, LU297, LU584, LU633, LU668, LU753, FCC13, FCC14, FCC320 and FCC327, were selected from a core set of endophytic isolates used in the forestry bioprotection research. Colonisation was determined by direct re-isolation of *Trichoderma* cultures on isolation media and examination of fluorescently labelled root issue using fluorescent microscopy. Emphasis was placed on the visualisation of the root colonisation process to provide impactful images for media presentations.

Key Results:

- All isolates tested had colonised roots ten days after seedling and *Trichoderma* inoculation of the potting mixture. It is possible the roots were colonised immediately after the radicle (primary root) emerged from the seed micropyle.

- All isolates, except LU297 and FCC320, colonised at least 50% of root pieces seven days after seedling emergence. The strongest colonisers were LU132, LU140, LU633, LU753, FCC13 and FCC327. In cuttings, isolates LU633, FCC327, FCC13 and FCC14 were strong colonisers seven months after setting and inoculation.

- *Trichoderma* colonisation was higher in the taproot, compared to lateral root tissue, during the first eight months of seedling growth.

- Eight months after seeding, isolate FCC327 was the strongest coloniser of roots, with 65% and 100% of root pieces colonised, according to the direct-isolation and fluorescent labelling techniques respectively.

- In cuttings, *Trichoderma* was found abundantly in three root tissue types, callus, roots and needles, five months after setting and potting mixture inoculation, with colonisation highest in the callus tissue (at least 80% of pieces colonised). At seven months, colonisation levels had declined in callus and needle tissue, while levels were maintained, or increased in roots.

- The colonisation of seedling roots with the applied *Trichoderma atroviride* LU633 isolate was verified using a species and strain-specific real-time polymerase chain reaction (qPCR) assay.

Fluorescent microscopy (using both standard and confocal microscopes) allowed extremely detailed visualisation and quantification of *Trichoderma* mycelial growth on, and inside, root tissue. Treatment of root tissue with Wheat Germ Agglutinin – Alexa Fluor® 488 (WGA-AF488) and Direct Red 80 fluorescent dyes resulted in bright green and red imagery of fungal and plant cell wall chitin respectively (Figure 3).

The colonisation of radiata pine roots by *Trichoderma* was described as:

1. A very close and abundant interaction of fungal hyphae and individual root cells (Figure 3a,b), root hairs (Figure 3c), primary and lateral roots (Figure 4). Hyphae generally grew between the cells in the intercellular and middle lamella spaces (Figure 5a), although a few examples of intracellular hyphal development were observed (Figure 5b). *Trichoderma* may find the path between the plant cell walls to be of least physical resistance and/or not have the ability to successfully colonise inside the cell on a frequent basis.

2. Cutting callus cells were highly colonised with hyphae, but there was no structured growth pattern of the hyphae due to the callus parenchyma cells being unstructured.

3. Roots were initially colonised by hyphae growing parallel along the main axis of the root with minimal branching. As the roots aged, hyphae were observed growing in parallel

along and in transverse to, the main axis of the root and without any particular orientation (Figure 4). In addition, hyphae were often highly branched.

4. Approximately half of the root tips observed at 2.5 and five months after seeding were colonised with *Trichoderma* hyphae. Some roots were highly colonised at the tip end (Figure 6). At three months after seeding and inoculation, hyphae had colonised the rhizodermal, sub-epidermal, cortex (Figure 7a) and primary xylem vessel (Figure 7b) cells.

5. Mature differentiated root cells were often highly colonised with localised dense mats of hyphae that displayed intense fluorescent signals. There appeared to be no apparent signs of necrosis or physical constriction to the growth of the plant cells caused by fungal hyphae in any of the plants studied.

6. Visualisation of fungal hyphae in the centre of the older root pieces was difficult due to the high natural fluorescence ("auto-fluorescence") of the primary and secondary xylem cell wall pigments.

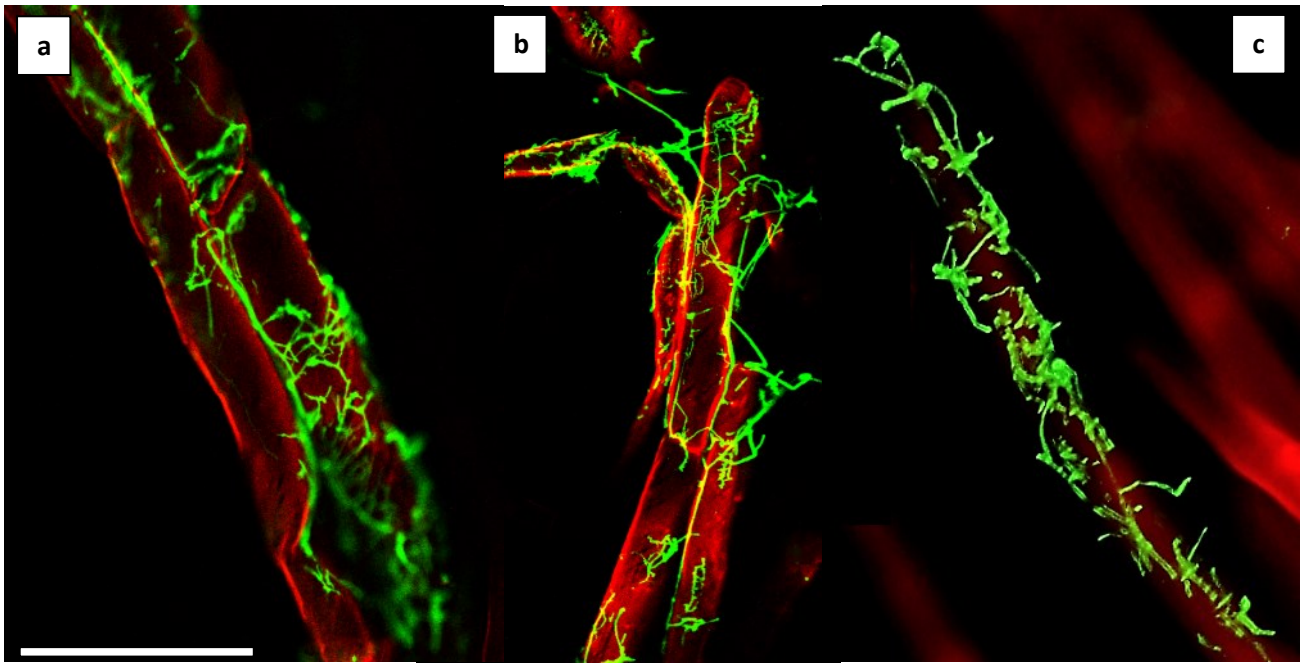

Figure 3: *Trichoderma* hyphae (green) closely associated with individual radiata pine root cells (red; a, b) and an unsterilised root hair (c). Bar: 50μm.

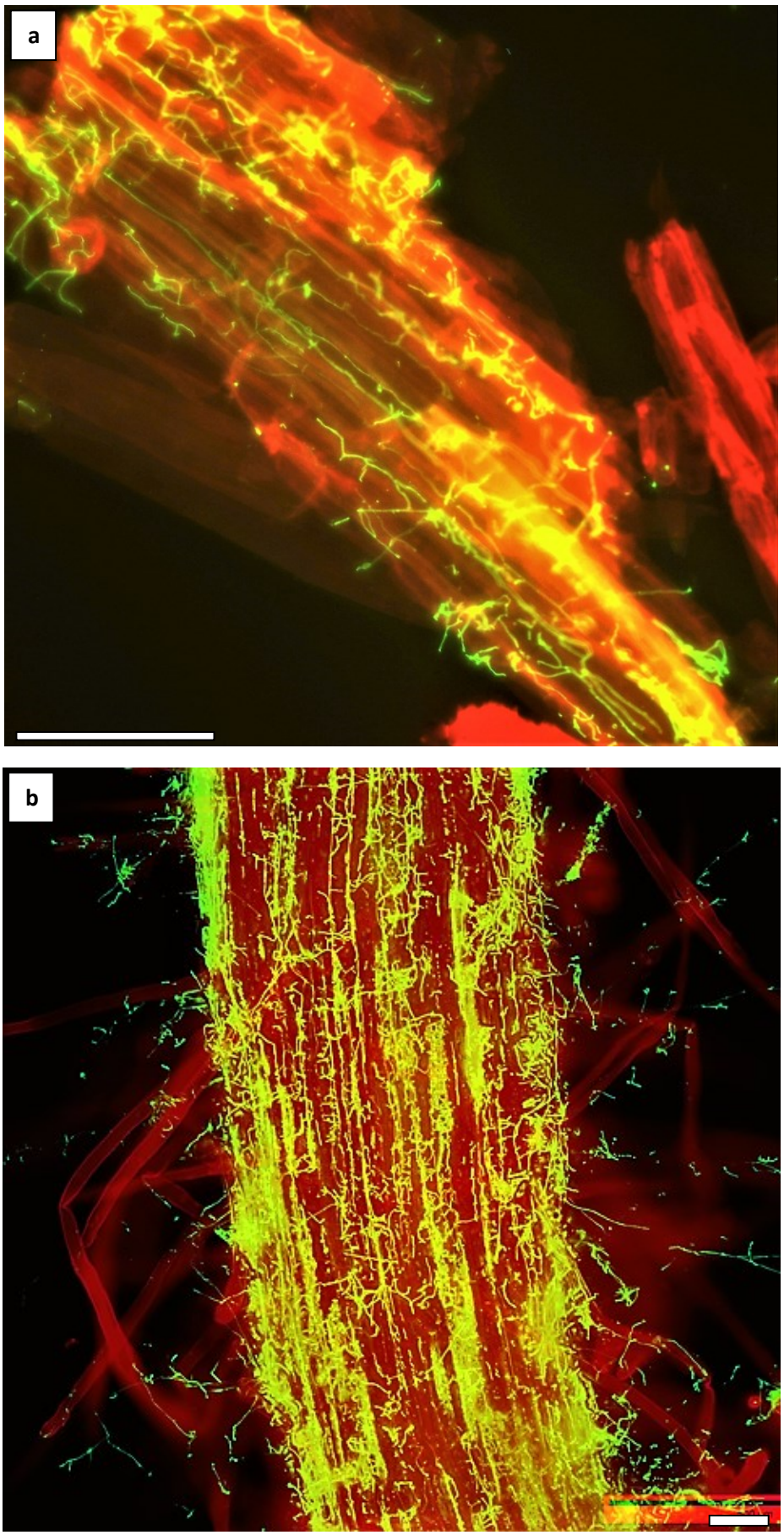

Figure 4: *Trichoderma* hyphae (green/yellow) in root pieces of radiata pine (red) observed by a) confocal and b) standard fluorescent microscopy. Bars: 100µm.

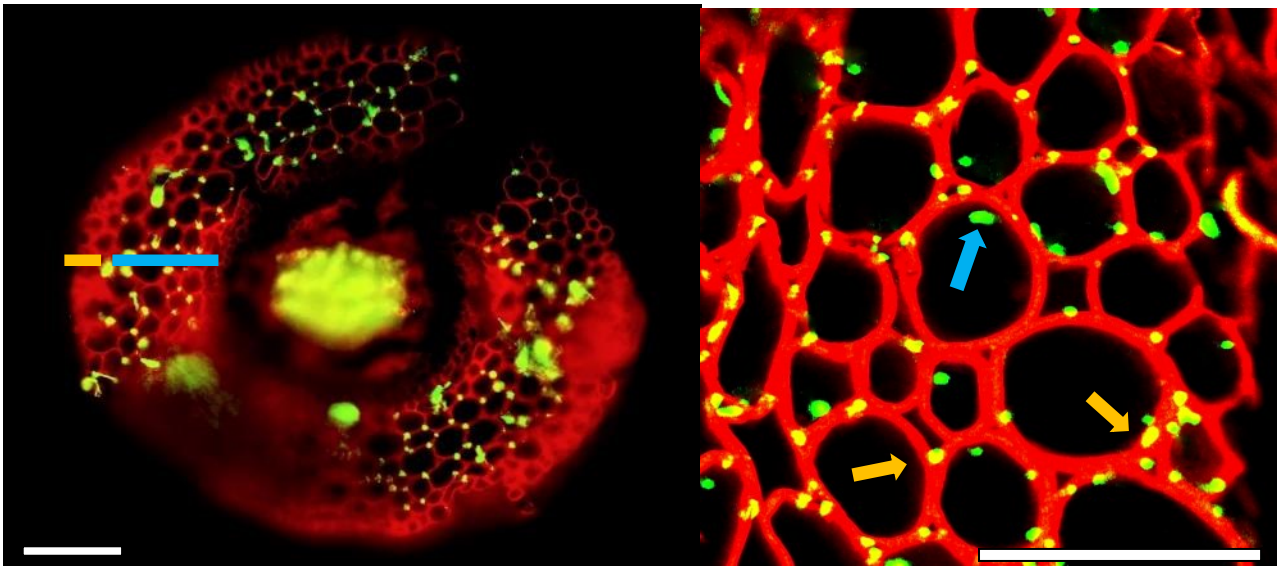

Figure 5: *Trichoderma* hyphae (green) in cross-sectional pieces of radiata pine roots (red) 2.5 months after planting and inoculation. Hyphae observed in the rhizodermal and sub-epidermal (orange bar) and cortex (blue bar) cells and the intercellular and middle lamella spaces (orange arrows) and intracellular (blue arrow) spaces. Bars: 100μm.

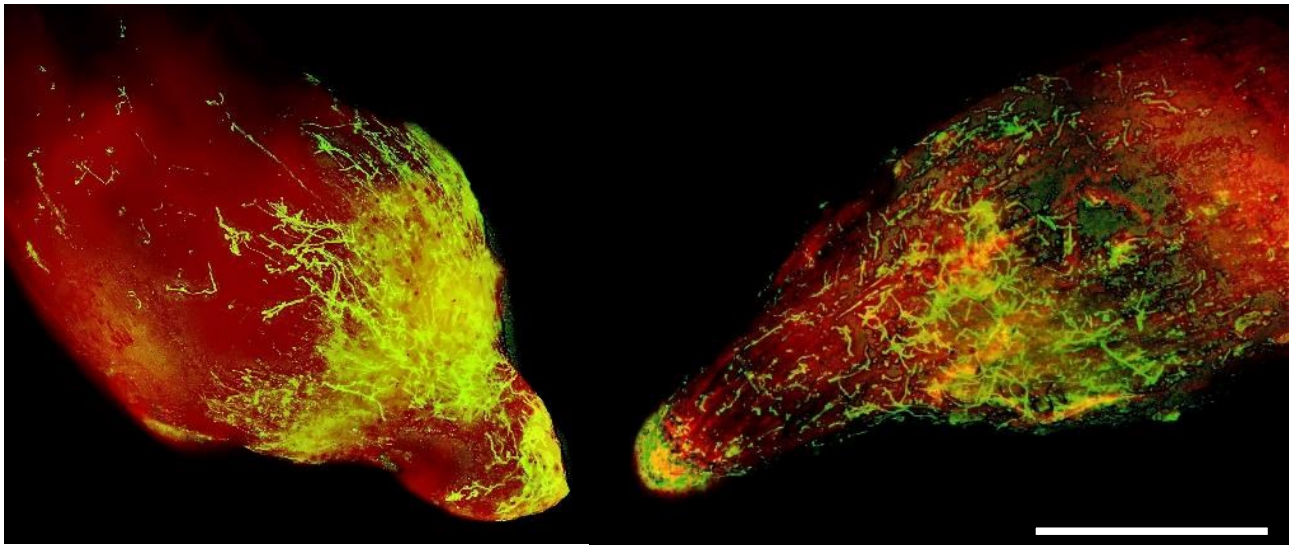

Figure 6: *Trichoderma* hyphae (green/yellow) in surface-sterilised root tips of radiata pine (red) 2.5 months after planting and inoculation. Bar: 500μm.

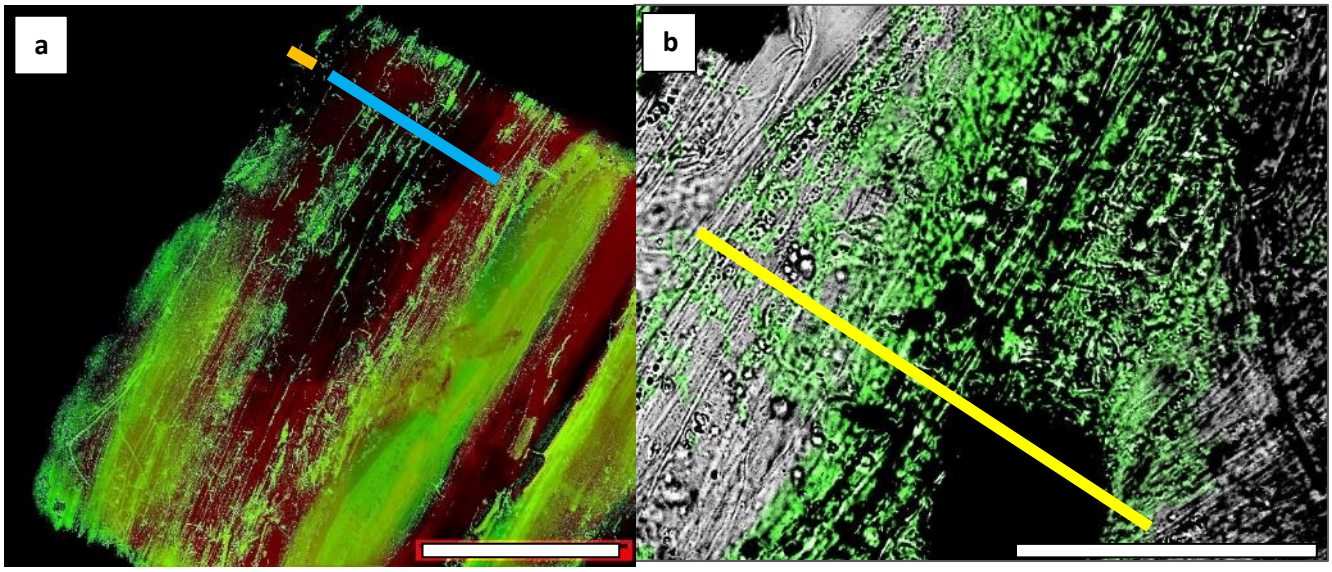

Figure 7: *Trichoderma* hyphae (green) in longitudinal cross-sections of radiata pine roots (red or grey) 2.5 months after planting and inoculation. Hyphae observed in the rhizodermal and sub-epidermal (orange bar), cortex (blue bar) and primary xylem vessel (yellow bar) cells. White bars: 100µm.

2.3 Milestone 3 – Nursery and forest plantation trials in radiata pine

2.3.1 Effect of *Trichoderma* on rooting of hard-to-root clones

New Zealand forestry production is increasingly reliant on the use of high-quality clonal stock for improved crop quality and productivity, in preference to open and crossed pollinated material. However, clonal stock may have poor root initiation and be susceptible to early 'damping off' disease in the nursery, resulting in high production costs per cutting and low numbers of cuttings available for deployment.

The effect of *Trichoderma* root bioprotectants (applied as a soil-bed drench at setting) on cutting survival, root initiation and growth of an important radiata pine clone (Clone 57) was assessed in a pilot trial at Timberland's Te Ngae Nursery, Rotorua in 2017 (Whelan, 2018k). The trial was established considerably later than standard nursery practice (due to the principle researcher being unavailable) and the number of plants with commercially acceptable root scores at harvest was low, at 30% or less. However, the two T*richoderma* treatments (isolate FCC327 and PR6 mixture), significantly ($P<0.001$) increased root initiation (percentage of cuttings with roots present in 3 or 4 quadrants) 2.5-fold (average of 29.1%), compared to the untreated Control (11.9%). In addition, approximately 8 to 10% more cuttings (average of 65.4%) survived to harvest in the *Trichoderma* treatments, compared to the untreated Control (56.3%). *Trichoderma* did not affect root collar diameter or root/shoot ratio. However, treatment with FCC327 resulted in the root, shoot and plant dry weights being significantly ($P<0.05$) greater, compared to the untreated Control, even though roots and shoots were trimmed during the year as part of standard nursery practice.

Table 2: Cutting survival, growth parameters and root scores in the 2017 Te Ngae nursery trial harvested on 25 July 2018.

Treatment	% survival		Root Collar diameter (mm)	Mean Dry Weight (g) [a]			Root/ shoot ratio	No. of plants with Root Score 3 or 4 (%)
	30 Nov 2017	25 July 2018		Root	Shoot [b]	Total plant		
Trichoderma FCC327	99.4 a	66.3 a	6.3 a	1.81 a	13.6 a	15.5 a	0.133 a	28.1 a
Trichoderma PR6	100.0 a	64.4 a	6.3 a	1.66 ab	12.8 ab	14.4 ab	0.132 a	30.0 a
Untreated Control	96.9 b	56.3 b	6.4 a	1.55 b	12.4 b	14.0 b	0.124 a	11.9 b
LSD (5%)	2.0	4.4	0.53	0.22	1.1	1.3	0.012	4.6
LSD (0.1%)	3.7	8.0	1.0	0.46	2.2	2.5	0.022	8.6
Significance	P<0.05	P<0.001	NS	P<0.05	P<0.05	P<0.05	NS	P<0.001

- [a] mean dry weight of plants after they were lateral pruned and wrenched in autumn and winter respectively.
- [b] mean dry weight of shoots after standard nursery topping at 30cm height in autumn. Above- and below- ground shoot weights were combined.
- Significant differences (P<0.05, P<0.001) in parameters are shown by different letters in each column (according to LSD test). NS = non-significant difference between treatments.

In this trial, the applied *Trichoderma* was found to colonise and persist in roots at relatively high levels of 30% and 46% in treatments FCC327 and PR6 respectively at six months after treatment, and 32 and 18% in treatments FCC327 and PR6, at 11 months after treatment. Untreated Control cuttings had 5% or less root colonisation, indicating low levels of environmental *Trichoderma* in the soil bed that therefore did not have an impact on the results.

The positive results of the 2017 trial led to the establishment of a larger validation cuttings trial in June 2018, with two hard-to-root clones and seven *Trichoderma* treatments (Figure 8). This trial was harvested in late July 2019 and results will be presented to the industry this year.

Figure 8: Setting of cuttings in June 2018 (left image) and overall view at harvest in July 2019 (right image) of the Te Ngae Nursery trial.

2.3.2 Forestry plantation validation trials for most-effective treatments 2018

Eight forest plantation trials of two to three hectare in size were established in four important forestry regions (Northland, Bay of Plenty/Waikato, Nelson and Gisborne) in winter 2018 (Table 3). Two locations, based on altitude, were chosen in each region to test the isolates under different temperature regimes. Treatments were the two most effective mixtures to date:

PR6, comprising:

- FCC55, *T. harzianum* - growth promotion (Hill, 2016)
- FCC318, *T. atrobrunneum* – disease suppression (Hill, 2016)
- FCC327, *T. harzianum* - growth promotion (Hill, 2016)
- FCC340, *T. harzianum* - disease suppression (Hill, 2016)

PR3a, comprising:

- FCC13, *T. asperellum*
- FCC14, *T. atroviride*
- FCC15, *T. atroviride*
- FCC180, *T. crassum* – growth promotion (Hill, 2016)

and an untreated Control.

The experimental design was robust with large plots of 9 x 9 seedlings (including two buffer rows of seedlings around the edge of each plot) and a high level of replication (seven and ten replicates per trial). An internal area of 5 x 5 seedlings will be marked and seedling survival, growth (height and diameter) and disease expression will be measured approximately 12 months after planting, and then on a less frequent basis.

Seedlings used in these trials were inoculated (5 x 10^5 spores/seed) with the two *Trichoderma* mixtures by a seed-coat method at PFOlsen Waiuku Nursery in September 2018. Seedlings were on-grown in containerised cell trays until winter 2018 and harvested a few days before being dispatched. On arrival, some forestry companies conditioned the seedlings by storing in a 2 to 4°C chiller for one week before planting. All trials were established with contractor crews under supervision of Dr. Helen Whelan, except Tauwhareparae, Gisborne, that was supervised by a contractor experienced in trial-work. Trial site preparation included spot-mounding, herringbone ripping or no soil disturbance and pre-planting herbicides for weed removal.

Endophytic *Trichoderma* root colonisation was confirmed in May 2018 (presence in 49%, 32% and 4% of root pieces in the PR6, PR3a and untreated Control treatments respectively; Whelan 2018) and deemed sufficient for trial work.

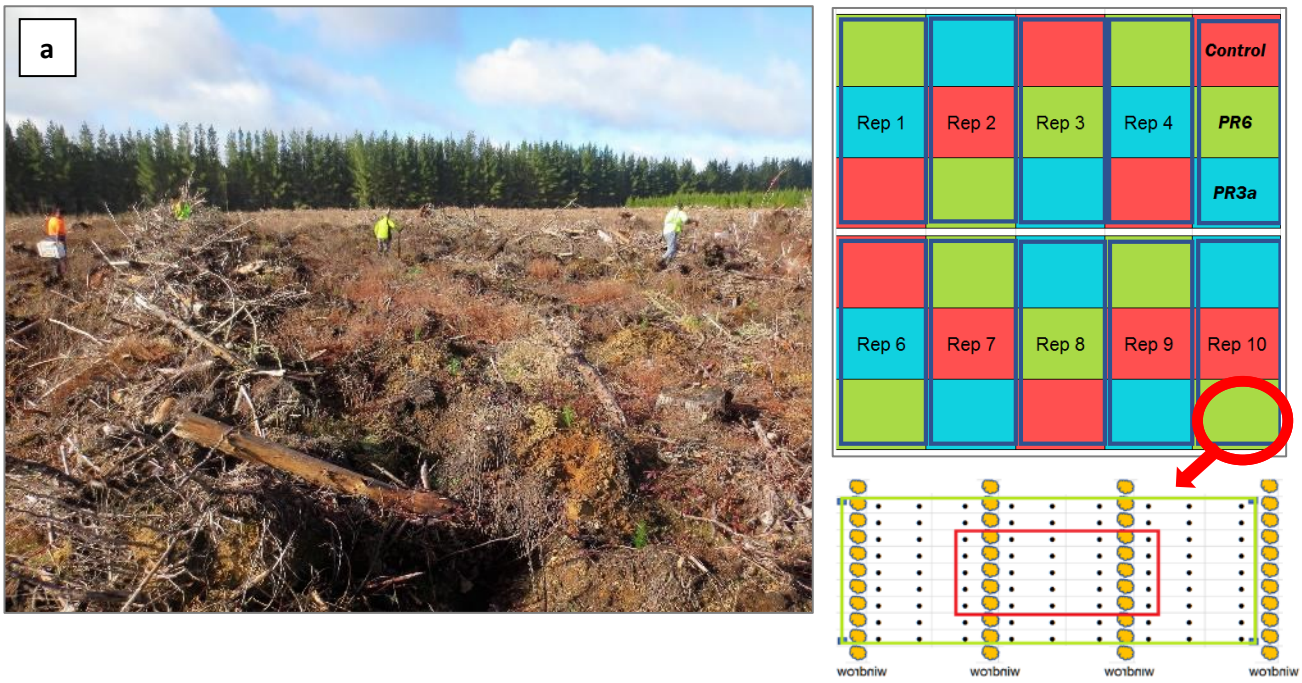

Figure 9: Large validation trials in a) Kaingaroa forest, Waikato/Bay of Plenty, b) Golden Downs forest, Nelson and c) Whatoro forest, Northland. The plot and replicate arrangement for the Kaingaroa trials is inserted.

Table 3: Establishment details for 2018 forestry plantation trials.

Region	Company	Forest / Trial Name	Location	Altitude (m)	Planting Date	No Replicates	Planting Density and spacing	Comments
Northland	Rayonier	Topuni	-36.225173 174.413915	40	23/08/18	8	1000 (2m x 5m)	Wet site with high clay soil type, West 5° slope, herringbone ripping
Northland	Hancock	Whatoro	-35.708955 173.676602	330-350	08/08/18	10	830 (4m x 3m)	10km from coast, flat, potential high pampas grass weed burden in future
Bay Plenty / Waikato	Timberlands	Kaingaroa xPKANG 209/4	-38.559711 176.445696	180	24/07/18	10	925 (4m x 2.7m)	Spot-mounded, flat, free-draining soils; fleabane
Bay Plenty / Waikato	Timberlands	Kaingaroa xPKANG 660/2	-38.868971, 176.280092	240	23/07/18	10	925 (4m x 2.7m)	Spot-mounded, flat free-draining soils; fleabane
Nelson	Nelson Forests	Golden Downs / Sherry	-41.448233 172.651675	310-380	03/09/18	8	800 (5m x 2.5m)	West slope (7-10° bottom half and 17-32° top half of trial); replicates placed across the slope. Cold, snow-prone site but more sheltered from winds compared to Berryman. Ex-Douglas-fir.
Nelson	Nelson Forests	Golden Downs / Berryman	-41.458333 172.90833	450	05/09/18	9	800 (5m x 2.5m)	South 8° slope with internal gullies; plots placed to avoid these. A range of slash levels but plots arranged to have similar levels in each replicate. Very cold, frosty, wind-exposed site. Lower growth potential compared to Sherry.
Gisborne	Juken	Patunamu	-38.90725 177.239278	200	18/07/18	9	1190 (2.9m x 2.9m)	East 15° slope, high amount of cut-over, potential high boxthorn/hawthorn burden in future
Gisborne	PFOlsen	Tauwhareparae	-38.198800, 178.099317	400-425	11/09/18	10	1000 (3m x 3.3m)	East 15° slope, small gullies through trial

2.3.3 Forestry plantation trials 2012 to 2015

Between 2011 and 2015, 24 forestry plantation trials were established in radiata pine growing areas at locations throughout New Zealand (Table 4). The most effective 11 isolates or isolate mixtures identified in nursery screening trials and disease assays were tested. In 2019, ten of these trials were selected for continued measurement (Table 4) based on:

1. quality of trial at establishment and durability over time
2. regional spread
3. plants responding to expectation of the general environment, and
4. a lower priority to the 2014 trials due to the different control seedlines (see section 5.6.3 of Hill, 2016 report).

Table 4: Establishment details for forestry plantation trials 2012 to 2015 and the trials selected for continuation after 2019.

Date established	Company	Locality	Forest plantation area	Trial continued
2012	Juken NZ Ltd	Gisborne	Wharerata	
2012	PF Olsen	Rotorua	Pinnacles	
2013	Timberlands	Kaingaroa	XP KANG	✓
2013	Juken NZ Ltd	Gisborne	Cricklewood	
2014	Timberlands	Kaingaroa	XP KANG	
2014	Hancock	Kinleith	Phoenix Horohoro	
2014	Hancock	Kinleith	Waituna_Kinleith	
2014	Hancock (now Tasman Pine)	Nelson	MTRN	✓
2014	Hancock (now Tasman Pine)	Nelson	Pearse	
2014	Hancock	Northland	Otaenga	✓
2014	Hancock	Northland	Pipiwai	
2014	Ernslaw One Ltd	Gisborne (Inland Tokomaru Bay)	Waiau	✓
2014	Ernslaw One Ltd	Gisborne (Inland Whatatutu)	Waipaoa	
2014	Ernslaw One Ltd	Ohakune	Karioi	
2014	Ernslaw One Ltd	Wanganui	Harakeke	✓
2014	Nelson Forests Ltd	Nelson	Ngaruru	
2014	Nelson Forests Ltd	Nelson	Kohatu	✓
2014	Nelson Forests Ltd	Nelson	Kings Ridge	
2014	Rayonier	Wanganui	Lismore	
2015	Timberlands	Kaingaroa	XP KANG	✓
2015	Rayonier	Northland	Glenbervie	✓
2015	Rayonier	Matariki	Maramarua Forest	
2015	Hancock	Kinleith	Phoenix Horohoro	✓
2015	Hancock	Kinleith	Giles Rd Kinleith	✓

Survival, height and health score measurements for many of the 2012 to 2014 trials, one year after establishment, were presented in Hill's (2016) report. Measurements taken in the trials between 2015 and 2017 were summarised in reports sent to the respective forestry companies (Hill and Whelan, 2016; Whelan, 2018i, j and k; Whelan, 2019h, i and j). Recent measurements (between 2018 and 2019) are summarised in the following section.

In Figures 10 to 21**,** significant differences (P<0.05) in the variable were shown by different letters (according to LSD test).

Isolate mixtures used in the trials are listed below:

2013 trials:

T1 = *Trichoderma* mixture 'A' = PBI (LU132, LU140, LU584, LU633)
T2 = *Trichoderma* mixture 'no. 11' = (FCC318, FCC319, FCC320, FCC322, FCC340)
T3 = *Trichoderma* mixture 'no. 6 + 180' = (FCC13, FCC14, FCC15, FCC16, FCC180)
T4 = *Trichoderma* mixture best 2012 trial = (FCC362, FCC368, FCC49, FCC55)
T5 = *Trichoderma* mixture 2nd best 2012 trial = (FCC333, FCC327, FCC410, FCC424)
T6 = Control, untreated

2014 trials:

T1 = *Trichoderma* mixture = PBI (LU132, LU140, LU584, LU633)
T2 = *Trichoderma* mixture = PR1 (FCC318, FCC319, FCC320, FCC322, FCC340)
T3 = *Trichoderma* mixture = PR2 (FCC362, FCC368, FCC49, FCC55)
T4 = *Trichoderma* mixture = PR3 (FCC180, FCC327, FCC275, FCC161)
T5 = Control, untreated

2015 trials:

T1 = *Trichoderma* FCC320
T2 = *Trichoderma* FCC327
T3 = *Trichoderma* mixture = PR5 (FCC161, FCC180, FCC275, FCC327)
T4 = *Trichoderma* mixture = PR6 (FCC55, FCC318, FCC327, FCC340)
T5 = *Trichoderma* mixture = PBI (LU132, LU140, LU584, LU633)
T6 = Control, untreated

Two mixtures were used in the large validation trials (Section 3.2):

- Treatment T3, from the 2013 trials, excluding FCC16, and
- Treatment T4, from the 2015 trials.

2.3.3.1 Timberlands Kaingaroa 2013 trial

Trial assessed at Year 5.5 (February 2019):

Height:

- Treatment T3 resulted in a 17% increase in tree height (significant at P<0.05) compared to the untreated Control (Figure 10).
- A similar pattern in treatment effects was found in the two-year assessment; where T3 had an 11% increased tree height (significant at P<0.05) compared to the untreated Control (Hill, 2016).

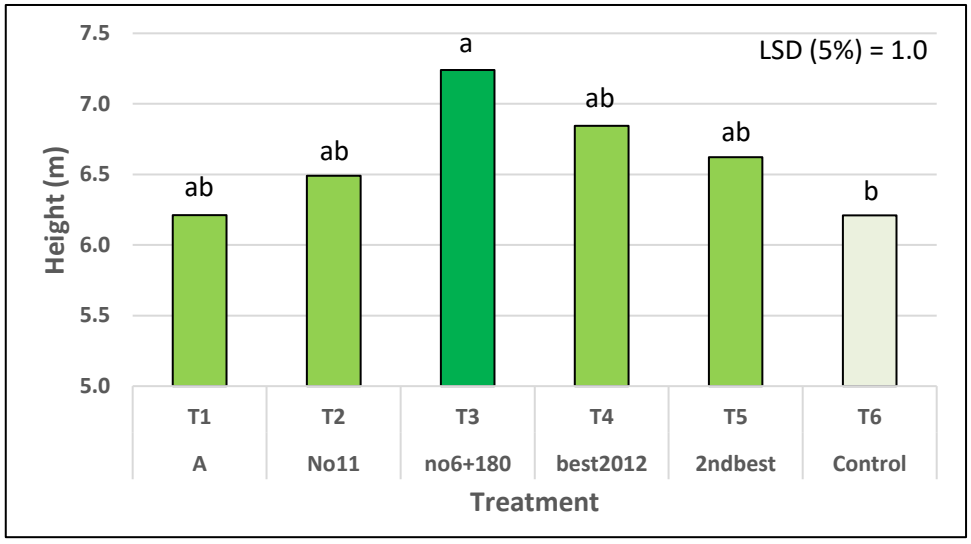

Figure 10: Effect of *Trichoderma* treatment on tree height 5.5 years after planting in the Kaingaroa plantation.

Trunk Diameter at Breast Height (DBH):

- There was no significant difference in DBH among treatments (Figure 11).
- A similar pattern in treatment effects was found in the four-year assessment (Whelan, 2018j); where Treatment T3 had a 13% increased DBH (not significant) compared to the untreated Control.

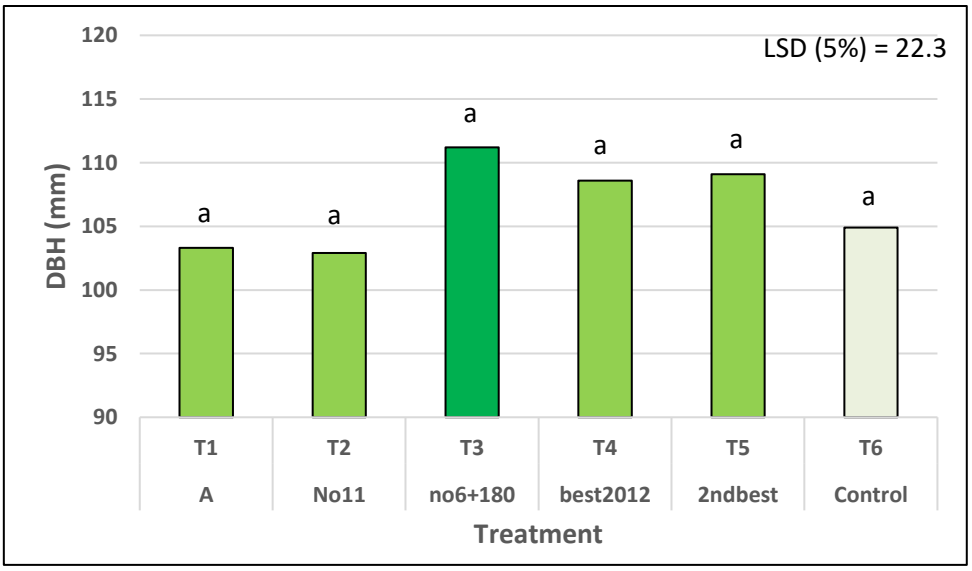

Figure 11: Effect of *Trichoderma* treatment on trunk DBH 5.5 years after planting in the Kaingaroa plantation.

Disease Severity (%) of Dothistroma Needle Blight Infection (February 2019):

- Treatments T4 and T5 resulted in a 33% and 49% reduction (significant at P<0.05) in disease severity (%) respectively, compared to the untreated Control (Figure 12).
- *Trichoderma* nursery seed-treatment reduced but did not eliminate the expression of disease in the field (Figure 13).

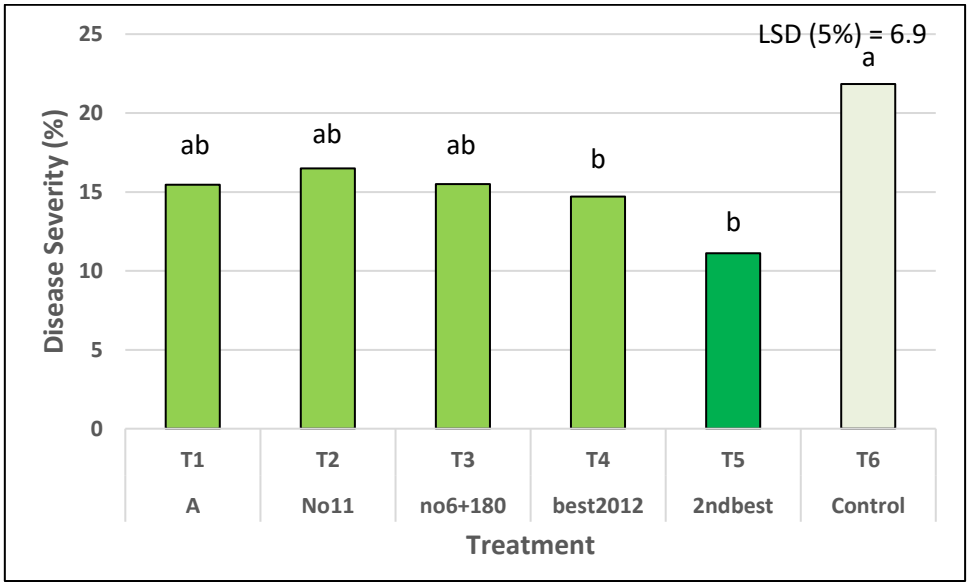

Figure 12: Effect of *Trichoderma* treatment on Dothistroma needle blight severity (%) 5.5 years after planting in the Kaingaroa plantation.

Figure 13: Dothistroma needle blight infection in a) Treatment T3 and b) untreated Control trees in Feb 2019.

2.3.3.2 Timberlands Kaingaroa 2015 trial

Trial assessed at Year 3.75 (March 2019):

Height:

- Treatment T1 resulted in a 7% increase in tree height, but this was not significantly different (P<0.05) compared to the untreated Control (Figure 14).
- A similar pattern in treatment effects was found in the 2.5-year assessment (Whelan, 2018j); where Treatment T1 and T4 had 10 to 11% increased tree height (not significant) compared to the untreated Control.

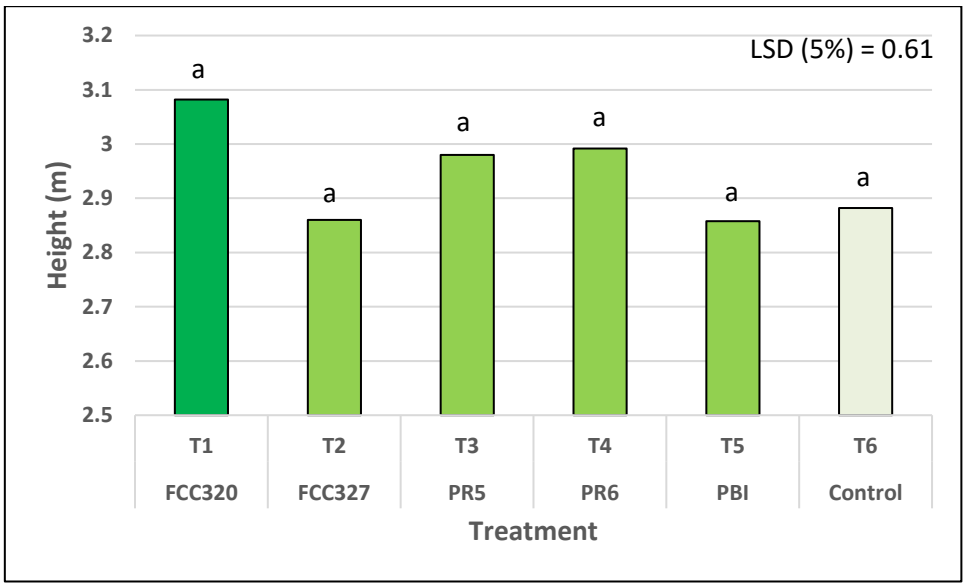

Figure 14: Effect of *Trichoderma* treatment on tree height (mm) 3.75 years after planting in the Kaingaroa plantation.

Trunk Diameter at Breast Height (DBH):

- Treatment T1 resulted in an 11% increased DBH, but this was not significantly different (P<0.05) to the untreated Control (Figure 15).

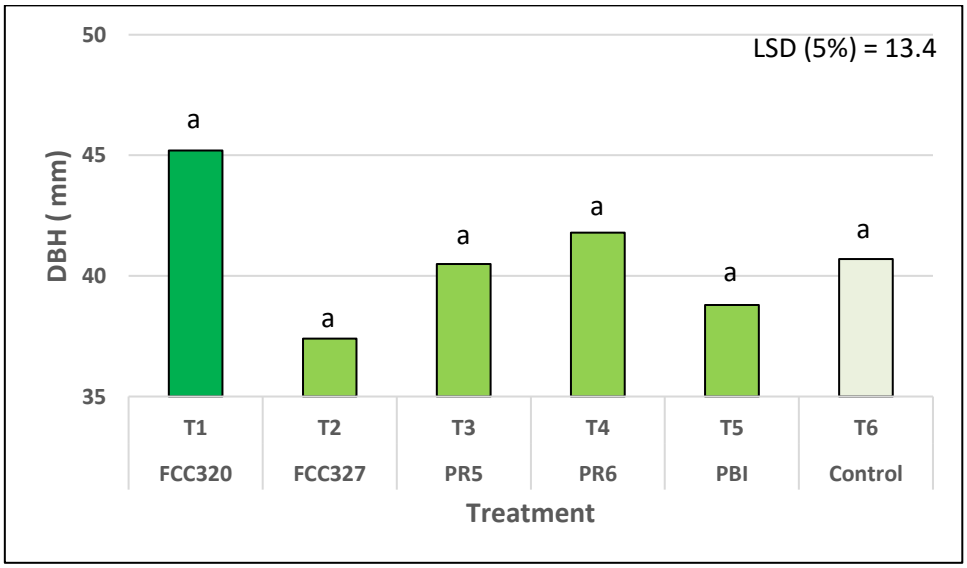

Figure 15: Effect of *Trichoderma* treatment on trunk DBH (mm) 5.5 years after planting in the Kaingaroa plantation.

Dothistroma needle blight was present at relatively low levels (approximately 9 to 13% disease severity) in March 2019, with little variation in levels among treatments (Figure 16).

Figure 16: Kaingaroa 2015 trial in May 2018

2.3.3.3 Ernslaw One Ltd Harakeke 2014 trial

Trial assessed in Year 3 (September 2017) and Year 4 (October 2018):

Height (Year 3):
Compared with the Control:

- Treatments T1, T2, T3 and T4 resulted in a 12.1%, 11.5%, 10.6% and 8.5% increase in tree height, respectively (significant at P<0.05; Figure 17).

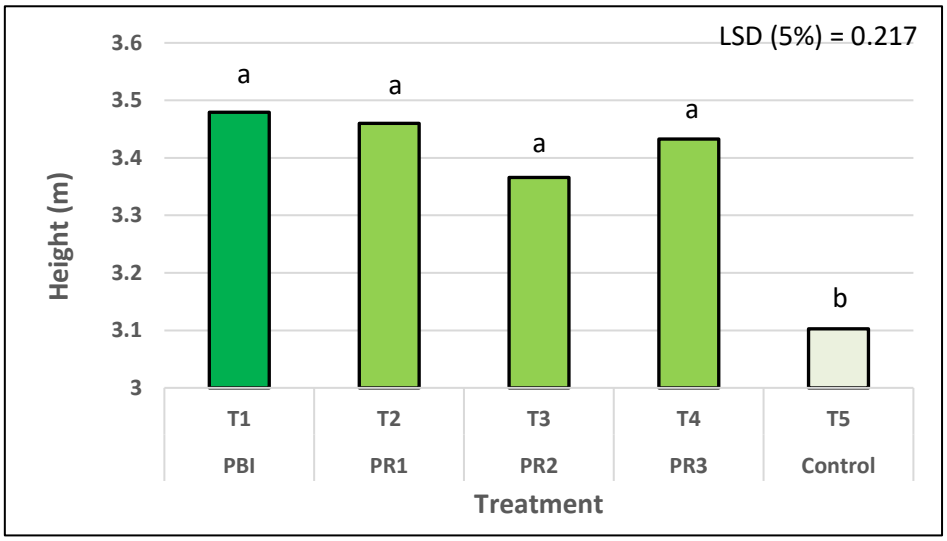

Figure 17: Effect of *Trichoderma* treatment on tree height (m) three years after planting in the Harakeke plantation.

Trunk Diameter at Breast Height (DBH; Year 3):

Compared with the Control:

- Treatments T1 resulted in a 17.2% increase in trunk diameter (significant at P<0.05; Figure 18 and 19).
- Treatments T2, T3 and T4 resulted in a 12.3%, 11.8% and 9.3% increase in trunk diameter, respectively (not significant at P<0.05) (Figure 18).

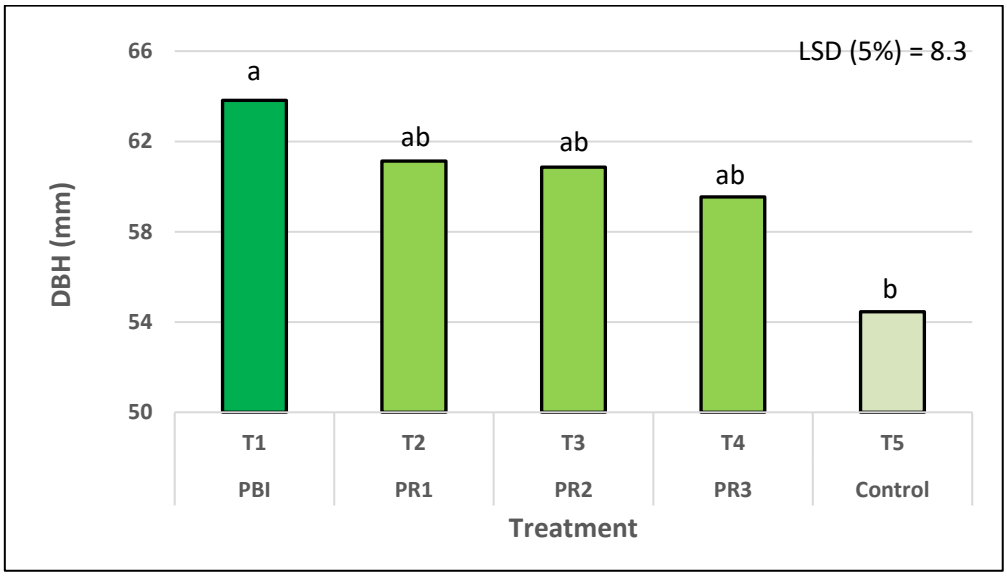

Figure 18: Effect of *Trichoderma* treatment on DBH (mm) three years after planting in the Harakeke plantation.

Figure 19: Trees treated with T1 (PBI *Trichoderma* mixture), left row, and untreated, right row, in Harakeke 2014 trial, October 2018.

Vigour Score (Year 4):

- No disease was present at the assessment date. However, all *Trichoderma* treatments had an impact on vigour score, with a significant increase (P<0.05) of 8 to 12%, compared to the untreated Control (Figure 20).
- A similar pattern in treatment effects was found in the Year 3 assessment (Whelan, 2018i); where Treatment T1 had a 17% increased vigour score (significant at P<0.05) compared to the untreated Control.

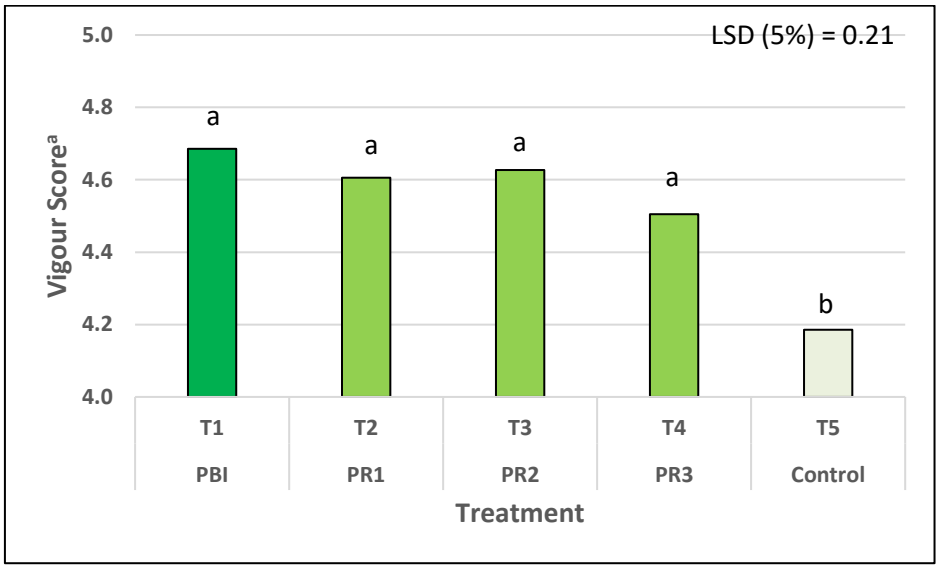

Figure 20: Effect of *Trichoderma* treatment on vigour score four years after planting in the Harakeke plantation.

[a] Vigour Score ranged from 5 (excellent) to 1 (very poor)

Survival (Year 4):

- Survival (%) was similar (between 89 and 100%) in all treatments (not significantly different at P<0.05) (Figure 21).

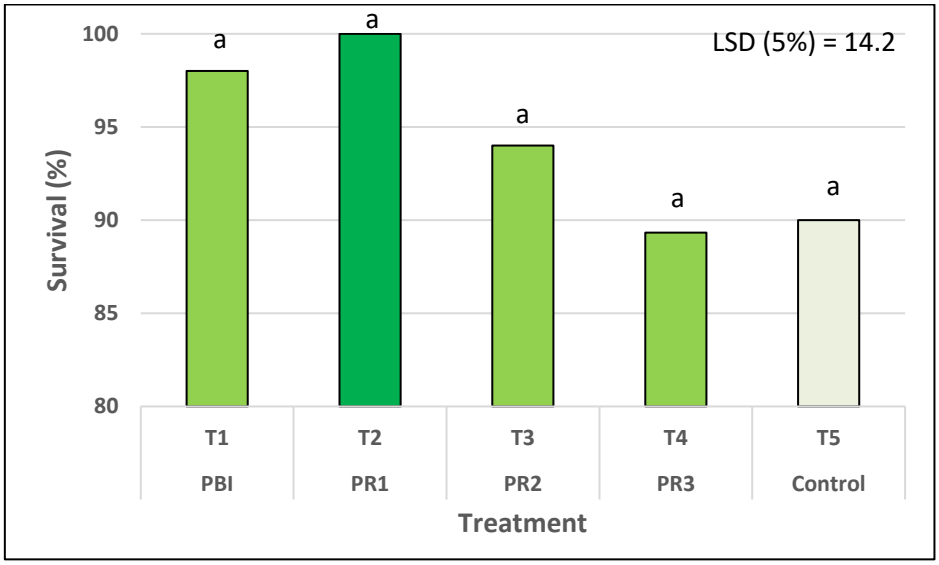

Figure 21: Effect of *Trichoderma* treatment on survival (%) four years after planting in the Harakeke plantation.

2.3.3.4 Ernslaw One Ltd Karioi 2014 trial

Trial assessed in Year 3 (September 2017) and Year 4 (October 2018):

Height (Year 3):

- Treatment T3 resulted in an 8% increase in tree height, but was not significantly different compared to the untreated Control (Figure 22).

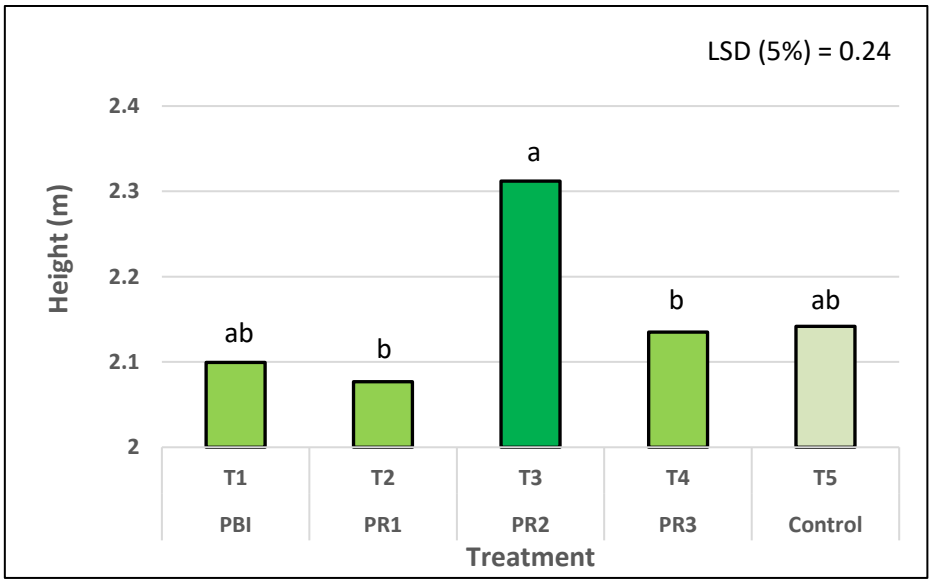

Figure 22: Effect of *Trichoderma* treatment on height (m) three years after planting in the Karioi plantation.

Trunk Diameter at Breast Height (DBH; Year 3):

Compared with the Control:

- Treatment T1 resulted in a 19.0% increase in trunk diameter (significant at P<0.05; Figure 23).
- Treatments T2 resulted in a 9.9% increase in trunk diameter, but was not significantly different.

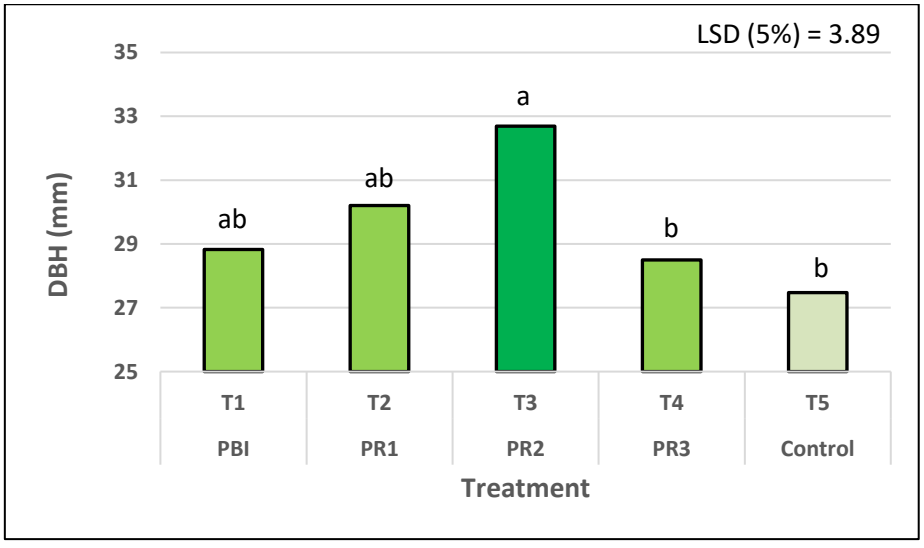

Figure 23: Effect of *Trichoderma* treatment on DBH (mm) three years after planting in the Karioi plantation.

Disease Score of Dothistroma Needle Blight Infection (October 2018):

- Treatment T3 resulted in a 60% reduction (significant at P<0.05) in disease severity, compared to the untreated Control (Figure 24).

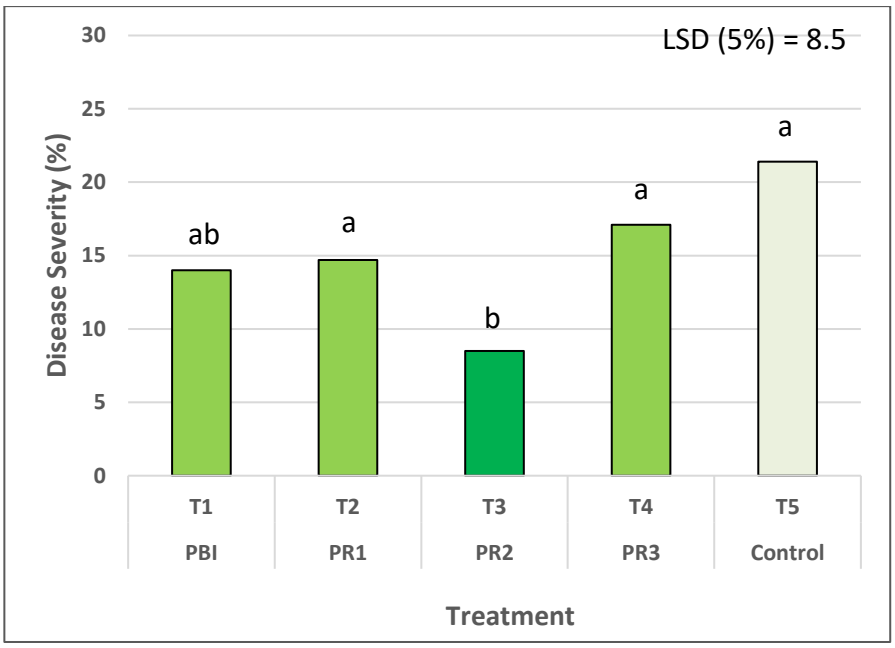

Figure 24: Effect of *Trichoderma* treatment on Dothistroma needle blight score four years after planting in the Karioi plantation.

Figure 25: Karioi 2014 trial in October 2018.

2.3.3.5 Tasman Pine North Moutere 2014 trial

Trial assessed in Year 4.5 (November 2018):

Height:

- Treatments T1 and T3 resulted in an 8% increase in tree height, but this was not significantly different compared to the untreated Control (Figure 26).
- A similar pattern in treatment effects was found in the one-year assessment; except Treatments T1, T3 and T4 resulted in a significant increase (P<0.05) of 14% to 19% in tree height, compared to the untreated Control (Hill *et al.,* 2015).

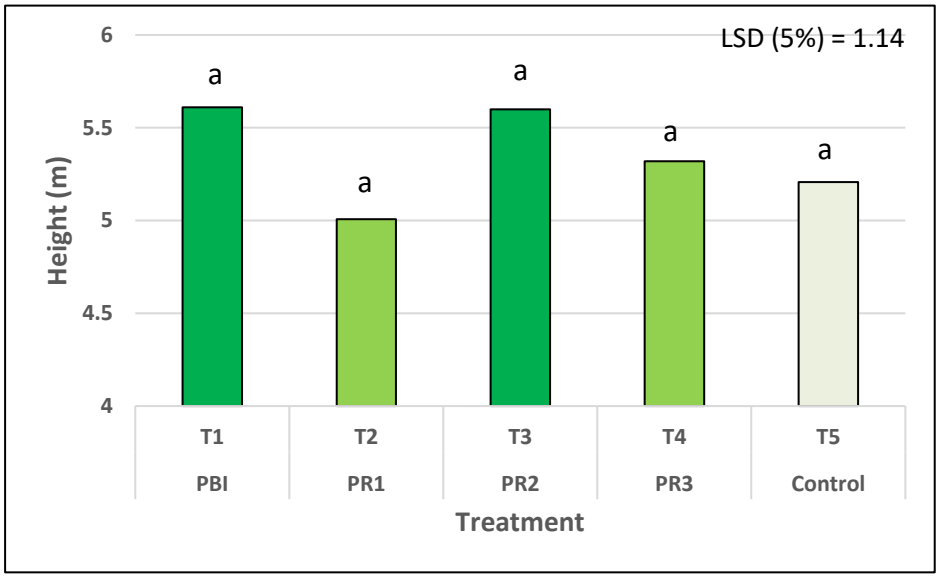

Figure 26: Effect of *Trichoderma* treatment on height (m) 4.5 years after planting in the North Moutere plantation.

Diameter at Breast Height (DBH):

- Treatments T1 and T3 resulted in a 13% and 14% increase in DBH respectively, but this was not significantly different compared to the untreated Control (Figure 27).

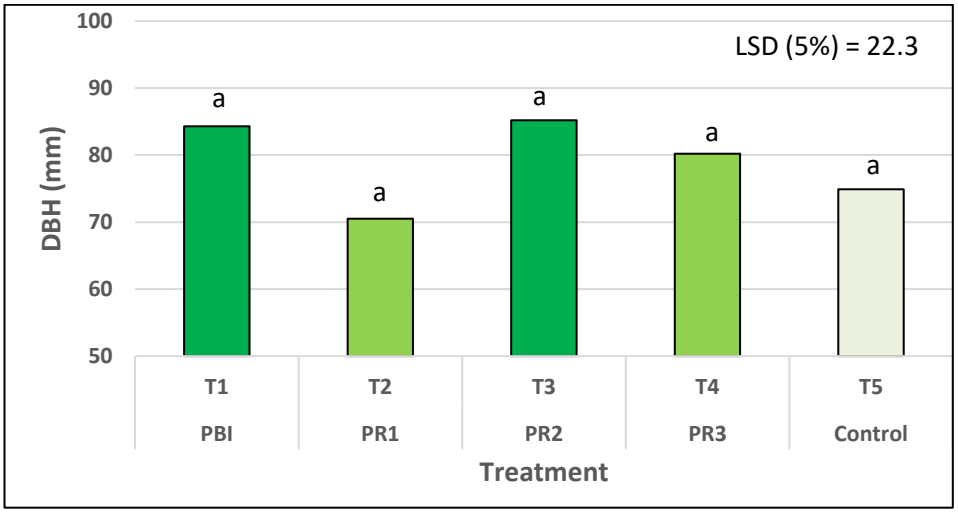

Figure 27: Effect of *Trichoderma* treatment on DBH (m) 4.5 years after planting in the North Moutere plantation.

2.3.3.6 Hancock Phoenix Horohoro 2014 trial

Trial assessed in Year 3.5 (December 2017):

Height:

- *Trichoderma* treatments resulted in a 10% to 13% increase in tree height, but these were not significantly different (P<0.05) from the Control (Figure 28).

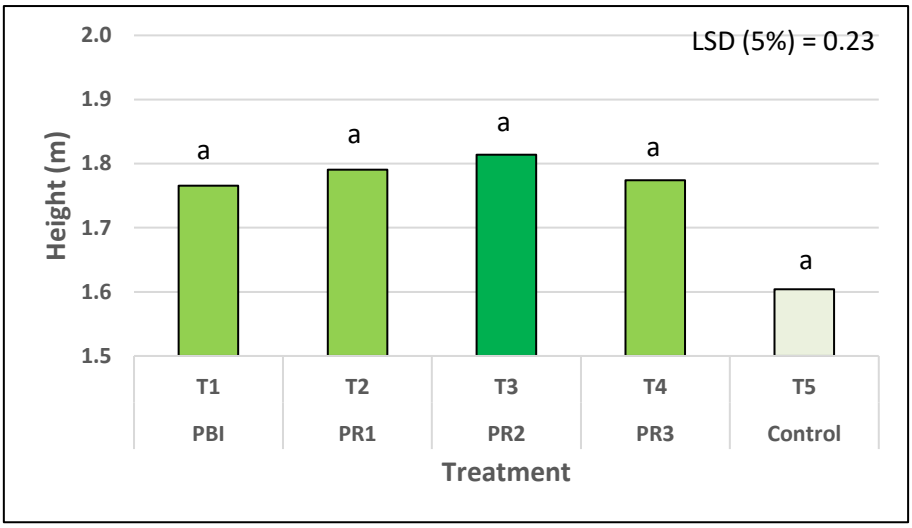

Figure 28: Effect of *Trichoderma* treatment on height (m) 3.5 years after planting in the Horohoro plantation.

Dothistroma needle blight disease assessment:

- *Trichoderma* treatments resulted in a 20 to 24% reduction in disease severity (%), but this was not significantly different (P<0.05), compared to the Control (Figure 29, 30).

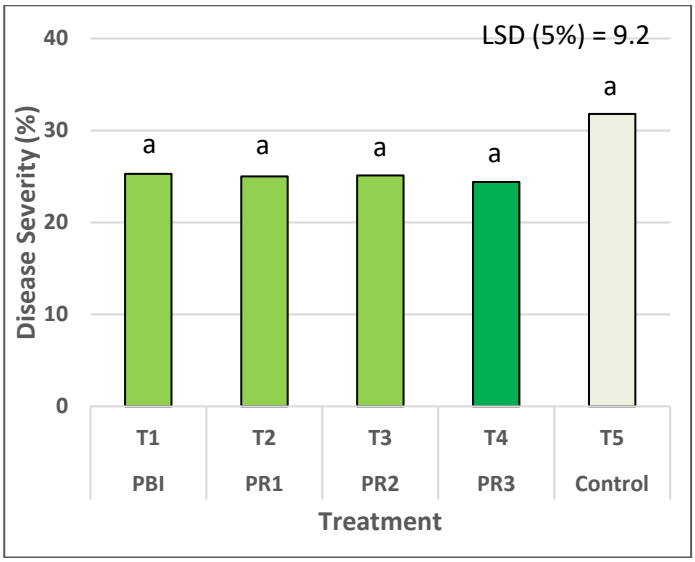

Figure 29: Effect of *Trichoderma* treatment on Dothistroma Needle Blight severity (%) 3.5 years after planting in the Phoenix Horohoro plantation.

Figure 30: Phoenix Horohoro 2014 trial in December 2017.

2.3.3.7 Hancock Phoenix Horohoro 2015 trial

Trial assessed in Year 2.5 (December 2017):

Height:

- Tree height was similar (between 1.5 and 1.6m) in tree height in all treatments (not significantly different at (P<0.05) (Figure 31).

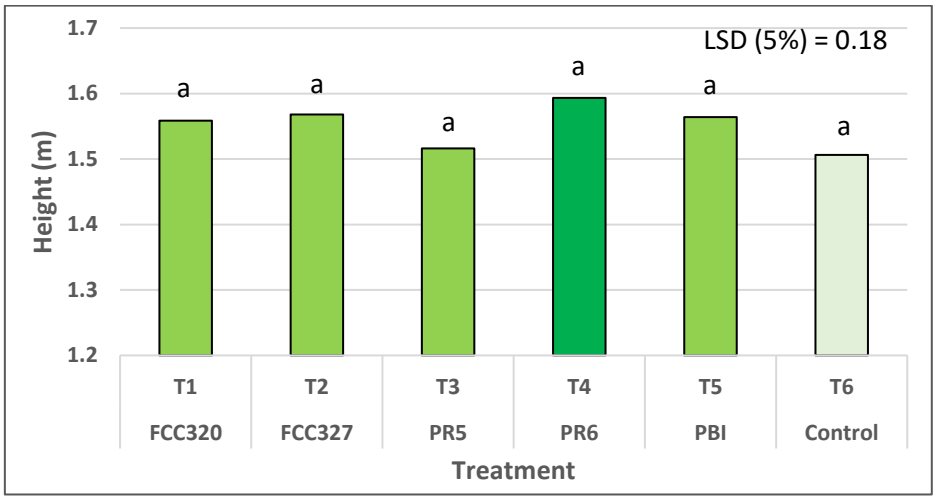

Figure 31: Effect of *Trichoderma* treatment on height (m) 2.5 years after planting in the Horohoro plantation.

Dothistroma needle blight disease assessment:

Compared to the Control:

- Treatment T3 resulted in a 31% significant (P<0.05) reduction in disease severity (%) (Figure 32, 33).
- Treatments T4 and T5 resulted in a 16% and 21% reduction in disease severity (%), but this was not significantly different (P<0.05).

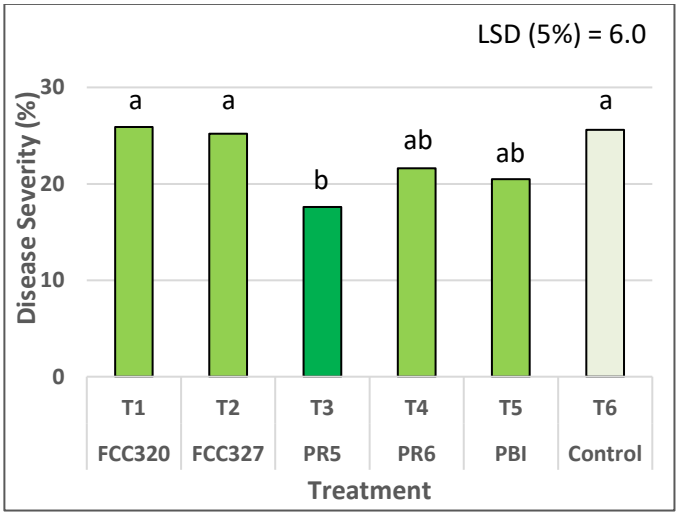

Figure 32: Effect of *Trichoderma* treatment on Dothistroma Needle Blight severity (%) 3.5 years after planting in the Horohoro plantation.

Figure 33: Phoenix Horohoro 2015 trial in December 2017.

Further measurements will be completed in the other trials (Nelson Forests Kohatu, Hancock Otaenga, Ernslaw One Waiau and Rayonier Glenbervie) by the end of 2019.

2.3.4 Feasibility of treating older trees with *Trichoderma* root bioprotectants to mitigate disease problems

In New Zealand, promising results have been obtained in *Trichoderma* bioprotection trials, with the most effective *Trichoderma* treatments increasing tree height in one- to 5½- year-old stands by up to 20.4% (Hill and Whelan, 2016, Hill *et al.,* 2015, Whelan, 2019j; see Section 2.3.3). In these trials, application of *Trichoderma* inoculum has been by seed-coat in the nursery; this being a practical, effective, low-cost and socially acceptable method to apply bioprotection agents. However, the majority of the approximately 1.5 million hectares of New Zealand's radiata pine plantations have not had *Trichoderma* applied. A pilot study (Whelan and Hill, 2017, Whelan, 2018k) was established in a 22-year-old clonal radiata pine stand in Tarawera Forest, Kawerau to investigate whether it is feasible to treat established plantation trees with *Trichoderma* bioprotectants in order to induce disease resistance and growth benefits.

Five *Trichoderma* treatments, using a single mixture (PR6 comprising isolates FCC55, FCC318, FCC327 and FCC340), were applied to individual trees in five replicate blocks, by:

1. *Trichoderma* infused-root dowels (Figure 34)
2. trunk injection with spore suspension (Figure 34)
3. trunk spray with spore suspension
4. soil drench around the perimeter of the trunk, and
5. an untreated Control.

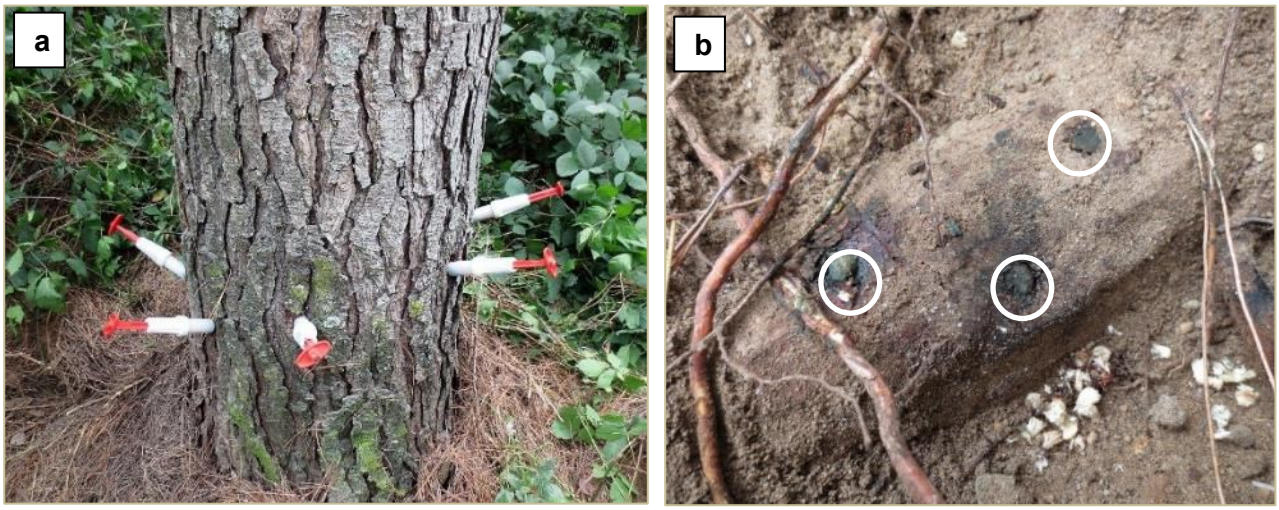

Figure 34: Inserted a) injectors containing *Trichoderma* spore suspension in trunks, and b) *Trichoderma* spore-infused wooden dowels in roots of radiata pine trees.

Trees had previously had a high infection of red needle cast disease (*Phytophthora pluvialis*) in spring of 2017 (Figure 35).

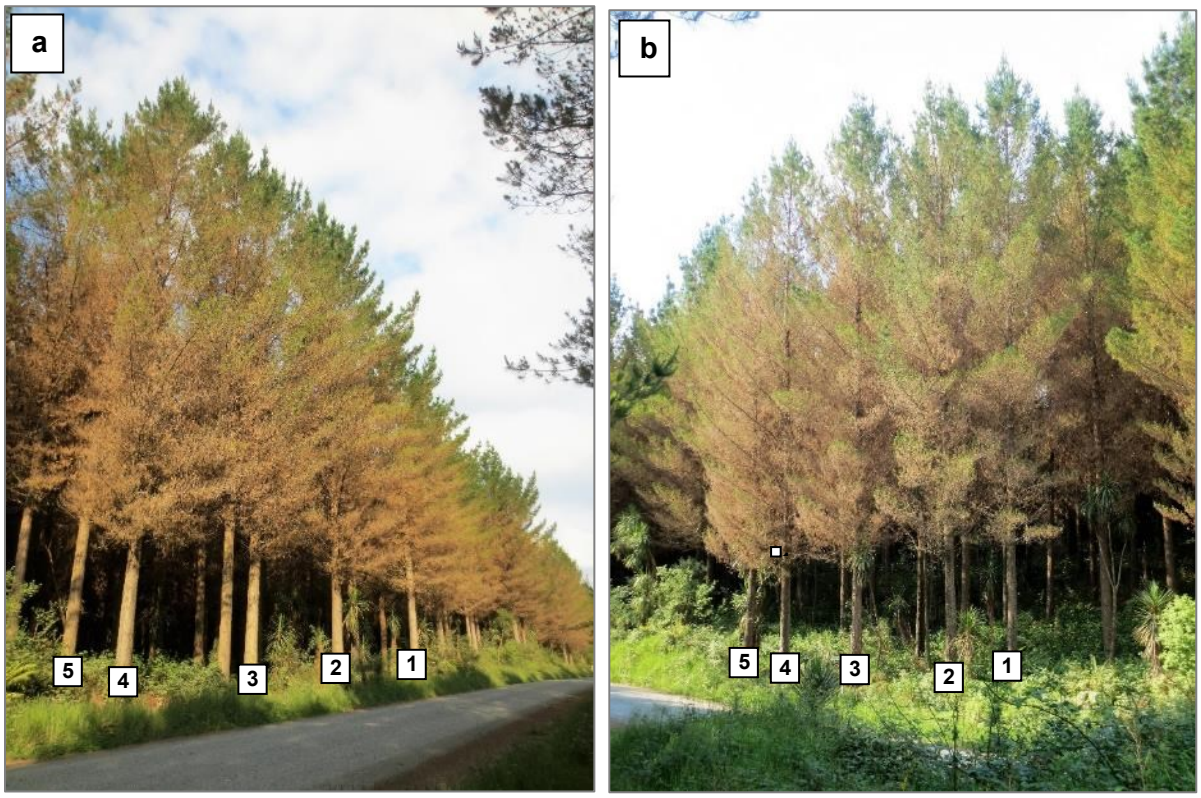

Figure 35: Individual trees treated with *Trichoderma* treatments in replicates (a) 2 and (b) 3.

The presence of natural *Trichoderma* fungi, before treatment application, was estimated at 6%, by sampling feeder roots from the five trees allocated treatments in replicate 5 and another three trees within or near replicate 5. Eleven months after treatment, mean root colonisation levels had increased by 136% in the treated trees, compared to the untreated trees (Table 5). Root colonisation levels in the untreated trees remained relatively low at 9%. Root colonisation will be measured in all replicates in the future.

Table 5: Colonisation (%) of radiata pine roots in trees sampled in and around replicate five. Measurements taken prior to and eleven months after, treatment applications, based on malt yeast extract / rose bengal agar plating data.

Treatment	**Measurement Date**	
	30 Nov 2017 (before treatments applied)	30 Oct 2018 (11 months after treatment)
Treated Trees:		
Trunk Injection	0	18
Root Dowel	0	25
Trunk Spray	4	15
Soil Drench	8	25
Mean of treated trees	**4**	**21**
Untreated Trees:		
Control	4	5
neighbouring tree	8	5
neighbouring tree	20	13
neighbouring tree	0	13
Mean of untreated trees	**6**	**9**

The effect of *Trichoderma* on tree growth and disease expression was measured eleven months after application by trunk diameter at breast height (DBH) and disease severity (%). Change in DBH was found to be influenced by the initial DBH (ie: larger trees grew more than smaller trees irrespective of the treatment applied), therefore initial DBH was used as a covariate to calculate the adjusted change in DBH in ANCOVA analysis. DBH increased in all five treatments eleven months after treatment application (Table 6). The root dowel treatment resulted in approximately twice the increase ($P<0.05$) in DBH increment, compared to the untreated Control. No disease was present at the assessment; therefore, the growth response in the root dowel treatment may have been due to increased disease resistance and quicker recovery of canopy green tissue compared to the Control trees.

Table 6: Adjusted change in DBH (mm) increment eleven months after treatment application in the Kawerau Established Tree trial.

Treatment	**Adjusted Change in DBH (mm) Increment [a]**
Root Dowel	10.2 a
Trunk Injection	7.8 ab
Trunk Spray	6.4 ab
Soil Drench	6.1 b
Control	5.3 b
LSD (5%)	4.1

[a] Significant difference ($P<0.05$) are shown by different letters (according to LSD test).

The positive result in the 2017 trial led to the establishment of a trial in Timberland's Kaingaroa forest in February 2019. The design was similar to that of the 2017 trial, apart from one additional replicate and insertion of buffer trees between treated trees. Measurement of tree height and root colonisation levels in all trees was undertaken.

2.4 Milestone 4 – Nursery and forest plantation trials in Douglas-fir and Cypress

2.4.1 Potential of *Trichoderma* to control cypress canker in cypress

Cypress is an important timber and shelter species for small-scale foresters, lifestylers and farmers. It can be strongly affected by cypress canker disease, caused by *Seiridium cardinale* and *Lepteutypa cupressi,* and trees may not reach maturity. The potential for BPRC's *Trichoderma* root endophytes to control cypress canker was assessed in a pilot trial at Southern Cypresses Nursery, Ohoka, Canterbury in 2017 (Whelan 2018).

One *Trichoderma* mixture of isolates FCC320, FCC327 and LU633 (spore suspension of 1000ml of 5.0×10^6 spores/ml per tray), and water (untreated Control) were applied to containerised seeds of:

1. *Cupressus macrocarpa* (susceptible)
2. *C. lusitanica* var. lusitanica (low susceptibility), and
3. *Cupressus x leylandii* var. Ferndown (not susceptible).

Cypress seedlings were colonised with *Trichoderma* at high levels six months after seeding (37, 38 and 73% of *C. macrocarpa, lusitanica* and *leylandii* root pieces respectively, Figure 36a). At 15 and 16 months, root colonisation levels had reduced to 25, 28 and 40% in *C. macrocarpa, lusitanica* and *leylandii* respectively (Figure 36b). A relatively small number of *Trichoderma* colonies (mean of 7%) were found in the untreated Control seedlings, likely from environmental *Trichoderma* isolates (e.g. from the potting mix and/or airborne spores in the nursery). They therefore did not have an impact on the results.

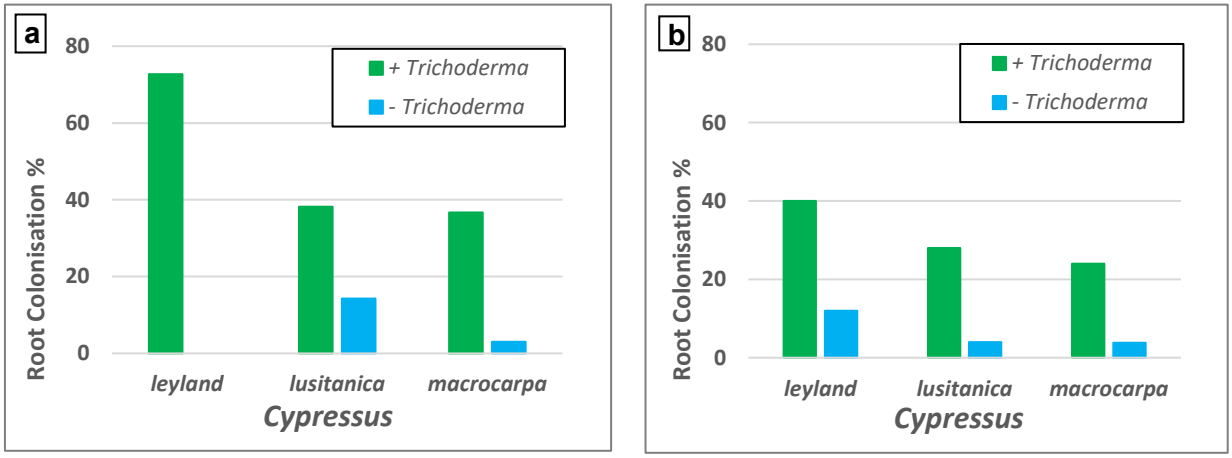

Figure 36: Root colonisation (%) six months (a), and mean of 15 and 16 months (b) after *Trichoderma* application, based on MRB plating data**.**

Colonisation was deemed sufficient for field trial work and these seedlings are being distributed to members of the New Zealand Cypress Development Group (an interest group of the New Zealand Farm Forest Association comprising foresters, landowners and lifestylers) in multiple locations where canker is prevalent.

In the 2017 trial, the incidence and severity of an opportunistic nursery pathogen *Pestalotiopsis guepinii* was observed to be less in the *Trichoderma* inoculated *C. x leylandii* seedlings, compared to the untreated trays (Figure 37).

Figure 37: Untreated (-*Trichoderma*) and treated (+ *Trichoderma*) *C. x leylandii* seedlings six months after treatment. The brown tissue in the untreated seedlings was caused by *Pestalotiopsis guepinii*.

A second containerised trial at Southern Cypresses Nursery was established in November 2018 to determine the effect of six *Trichoderma* root endophyte mixtures on seedling growth.

Treatments included two seedlots (*C. macrocarpa* and *C. lusitanica* var. lusitanica), six *Trichoderma* treatments (various isolate mixtures) and two untreated Controls. Two of the treatments were *Trichoderma* isolates collected from a susceptible *C. macrocarpa* clonal (Kukupa) stand in Murchison (a region prone to canker infection) with a history of low canker levels and high levels of natural *Trichoderma* (Whelan 2018). Seedling root colonisation, survival and growth will be measured in December 2019.

2.4.2 Potential of *Trichoderma* to control Swiss needle cast in Douglas-fir

Douglas-fir (*Pseudotsuga menziesii)* is the second most widely planted forestry plantation crop in New Zealand and can be affected by nursery and plantation foliar diseases, including Swiss needle cast (*Phaeocryptopus gaeumannii).* The effect of *Trichoderma* root isolates on seedling growth and disease resistance to Swiss needle cast in Douglas-fir was assessed in two trials at the Lincoln University Nursery, Canterbury.

A containerised nursery trial with three seedlot treatments:

1. 12/663 with California provenance (generally low tolerance)
2. 12/706 with Washington provenance (generally medium tolerance)
3. 12/662 with Oregon provenance (generally high tolerance), and

three *Trichoderma* treatments (5ml of 5.0 x 10^6 spores/ml spore suspensions into each tray cell):

1. T1: *Trichoderma* general mixture of isolates FCC320, FCC327 and LU633
2. T2: *Trichoderma* PR3a mixture (isolates FCC13, FCC14, FCC15 and FCC180)
3. Untreated Control

was established in October 2017.

Five replicates of inoculated and untreated seedlings (approximately 3500 plants) were selected and lined out at ArborGen Edendale Nursery, Invercargill in March 2018 (Figure 38b) for use in future plantation trials in Timberlands Kaingaroa forest. Unfortunately, a large number of the seedlings were damage during undercutting, and, after discussion with relevant parties, the plants were abandoned. New seedlings will be inoculated in spring 2019 at Lincoln University Nursery. The remaining six replicates were harvested for growth parameters in September 2018 (Figure 38a).

Figure 38: Inoculated Douglas-fir seedlings in a) Lincoln University Nursery in July 2018 and b) ArborGen Edendale Nursery (the centre row, December 2018).

Root colonisation (%) was very high (between 56 to 84% of root pieces tested) eleven months after seeding and *Trichoderma* inoculation, in the nursery grown plants (Figure 39). The untreated Control seedlings had relatively low (0 to 12%) root colonisation from environmental sources (e.g. from the potting mix and/or airborne spores in the nursery) and therefore did not have an impact on the results. Survival of root *Trichoderma* in the field plants was less than or similar to that of the nursery plants (Figure 39), and did not decline below 24%.

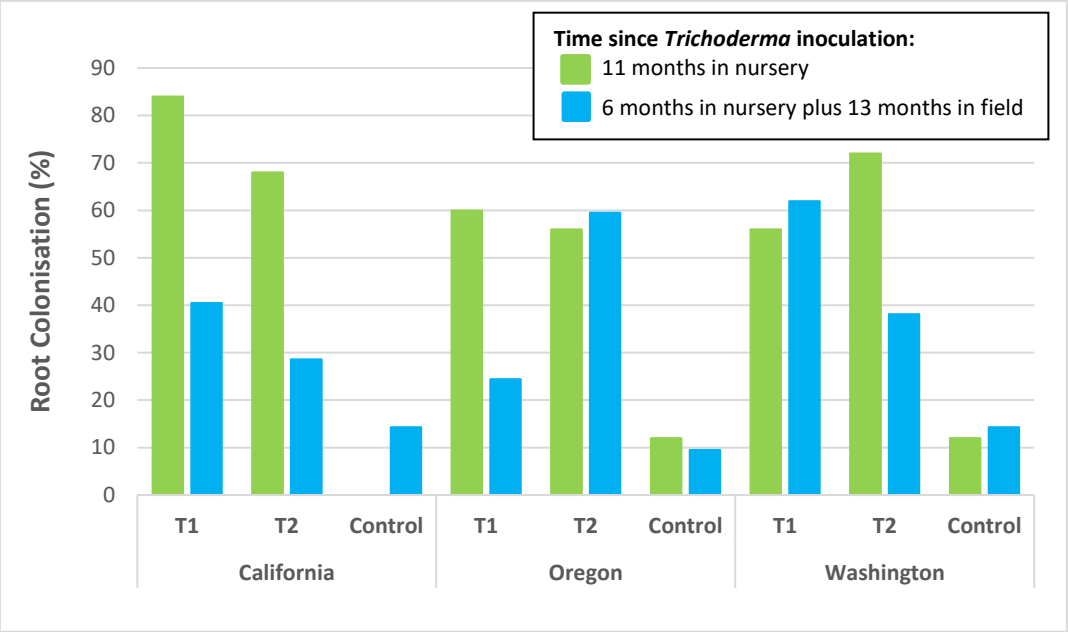

Figure 39: Root colonisation (%) eleven months after seeding and *Trichoderma* inoculation for Treatment T1 (FCC320, FCC327 and LU633 mixture), T2 (PR3a mixture) and untreated Control treatments, based on MRB plating data**.**

Seedling emergence and survival rates were at expected levels for the seedlots, with Washington being greater than Oregon and California (Whelan 2018). The seedlot type did not significantly affect other growth parameters.

Trichoderma treatments did not affect seedling emergence, but at harvest, survival was 13% higher ($P<0.05$) in the T1 treatment, compared to the untreated Control (Table 7). Both *Trichoderma* treatments had large impacts on growth parameters, with highly significant ($P<0.001$) increases in root collar diameter, height, shoot, root and plant dry weights by approximately 13, 18, 32, 29 and 30% respectively, compared to the untreated Control (Figure 40).

Table 7: Emergence, survival and growth parameters in the 2017 Douglas-fir Lincoln University Nursery trial harvested on 19 September 2018.

Treatment	Emergence (4 weeks after seeding) (%)	Survival (%)	Root Collar Diameter (mm)	Height (cm)	Mean Dry Weight (g)		
					Shoot [a]	Root	Plant
Trichoderma:							
T1 (FCC320, FCC327, LU633)	79.1 a	81.3 a	2.45 a	18.4 a	1.25 a	1.16 a	2.41 a
T2 (PR3a)	77.1 a	74.1 ab	2.50 a	18.1 a	1.26 a	1.16 a	2.42 a
Control	78.9 a	72.1 b	2.19 b	15.5 b	0.95 b	0.90 b	1.86 b
LSD (5%)	2.7 (NS)	7.2 (*)	0.13 (*)	1.5 (*)	0.17 (*)	0.14 (*)	0.27 (*)
LSD (0.1%)			0.23 (***)	2.6 (***)	0.30 (***)	0.24 (***)	0.47 (***)

Significant differences (P<0.05 and P<0.001) in parameters are shown by different letters in each column (according to LSD test). NS = non-significant difference among *Trichoderma* treatments.

[a] Shoot dry weight from potting mix level to the seedling tip.

Figure 40: Oregon Douglas-fir seedlings at harvest for Treatment T1 (FCC320, FCC327 and LU633 mixture), T2 (PR3a mixture) and untreated Control treatments.

A second containerised trial at Lincoln University Nursery was established in September 2018 to determine the effect of eight *Trichoderma* root isolate mixtures on seedling growth of two Douglas-fir seedlots (California and Oregon). Two of the treatments were *Trichoderma* root isolates collected from healthy, actively growing Douglas-fir trees sampled in Rayonier Matariki Forests Glendhu Forest, South Otago. Seedling root colonisation, survival and growth will be measured in December 2019.

2.5 Milestone 5 - Isolation and characterisation of fungal partners in the radiata pine root microbiome

The research programme has selected a range of beneficial *Trichoderma* isolates for use in New Zealand plantation forestry. However, a wide range of fungal (and other organism) species from various genera may also internally colonise radiata pine roots and have an inhibitory or promontory effect on the applied *Trichoderma* isolates. Therefore, an important question about the deployment of these *Trichoderma* isolates is how persistent and effective they are in the presence of other fungi resident in the plant roots or surrounding soil.

There is limited information on the fungal root endophytes naturally present in plantation radiata pine trees in New Zealand. In Milestone 5.0, roots were collected from twelve plantations in the North, and South Island and endophytic fungi were isolated and characterised using DNA analysis of partial *tef1* and ITS gene sequences to species level for *Trichoderma* and other fungal genera, respectively (Hill *et al.,* 2017). Additional information for mature seedlings grown in greenhouse and nursery conditions was included to increase the knowledge of fungal endophytes in younger radiata pine trees. A novel culturing method of agar-filled tissue culture plates was developed as an alternative to the traditional agar-filled petri dish method (Figure 41). Use of tissue culture plates was convenient, efficient and less time demanding and was recommended for microbiome studies involving large-scale sampling of plant tissues.

Key results:

- Eighty-nine endophytic fungal isolates, representing 26 genera and 57 species, were identified. In addition, 44 endophytic fungal isolates, representing 16 genera and 21 species, were identified from roots for 8-month and 2-year-old radiata pine seedlings grown in greenhouse and nursery conditions. A total of 34 genera and 69 species were identified in the three studies combined (Appendix 3).

- The genera with the highest species diversity were *Aspergillus, Fusarium, Ilyonectria, Penicillium* and *Trichoderma,* while the most common genera were *Absidia, Cladophialophora, Fusarium, Mucor, Penicillium* and *Phialocephala* species.

- Ten *Trichoderma* species (*T. asperellum, T. atroviride, T. crassum, T. gamsii, T. hamatum, T. harzianum, T. koningii, T. koningiopsis, T. spirale* and *T. tomentosum*) were isolated from plantation sites in this study and this has contributed to the characterisation of the background population of *Trichoderma* in New Zealand radiata pine plantation systems.

A few of the endophytic species found were recognised as weak or latent plant pathogens and may cause disease under certain conditions. This study provided information on the natural endophytes in New Zealand radiata pine systems that may become emergent fungal pathogens. This is particularly important with climate change which may induce warm-tolerant latent endophytes to become pathogenic (e.g. *Erythricium salmonicolor,* a species of the family *Corticiaceae,* that causes wood cankers in tropical citrus and rubber crops). *Resinicium bicolor,* a pathogen of Douglas fir (*Pseudotsuga menziesii*) may need to be monitored for any change in its impact in radiata pine plantations.

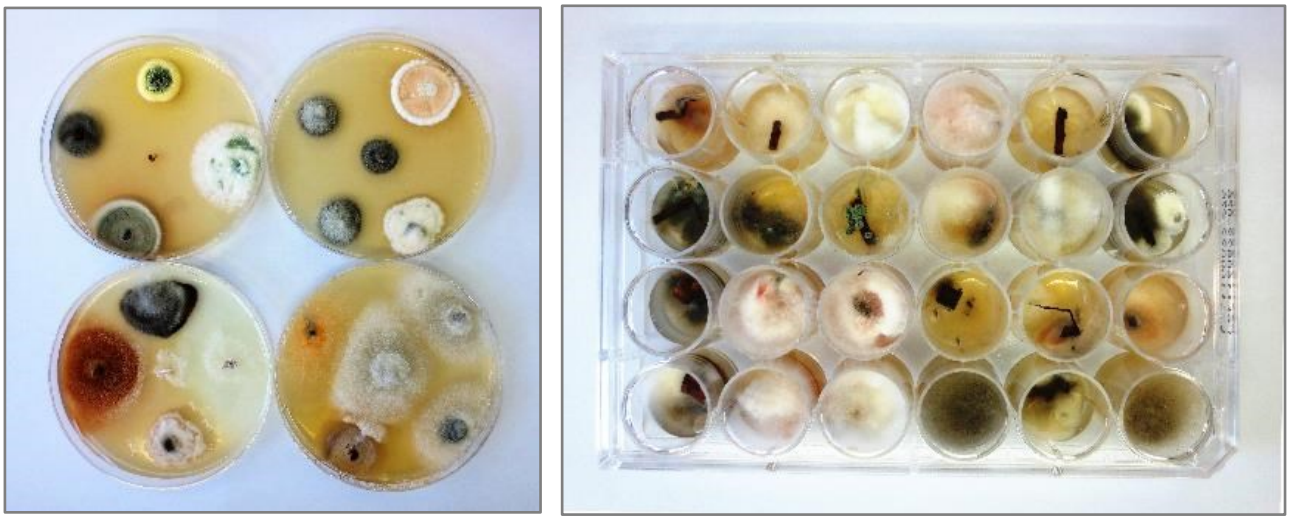

Figure 41: Seven-day old fungal colonies grown from single pieces of surface-sterilised plantation radiata pine root plated on malt yeast extract agar, amended with antibiotics, in a) petri dishes and b) tissue culture plates.

Aspects of the isolation methods, including culturing temperature and type of isolation media, were likely to select for faster-growing or otherwise more competitive species (e.g. *Mucor, Rhizopus, Neurospora* sp.) which may obscure the recovery of slower-growing or less competitive species. These isolation conditions may indicate fungal microbes that flourish in warm, nutrient-rich environments but may not represent the microbes that can grow in more extreme conditions.

This study characterised some of the fungal endophyte species in plantation radiata pine roots and provided important prerequisite information for future examination of the effectiveness of the applied isolates. In addition, other species that could improve radiata pine health were potentially identified.

3. COMMERCIALISATION OF *TRICHODERMA* ISOLATES

Many effective endophytic *Trichoderma* isolates for foliar disease control and enhanced growth in radiata pines were selected in the research programme between 2012 and 2017. These isolates were applied as part of a seed-coat treatment, and the inoculated seedlings were established in large validation trials in winter 2018. As part of the current research programme, the viability of these *Trichoderma* treatments for commercial production is currently being determined by Agrimm Technologies Ltd, Lincoln, Canterbury.

4. PROJECT OUTPUTS

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5. CONCLUSIONS

This bioprotection research project in forestry has used innovative approaches to enhance plant health and growth and improve sustainability in radiata pine nurseries and plantations. In order to alleviate losses caused by existing diseases and reduce potential impacts of biosecurity threats, research is being conducted to establish a long-term symbiotic relationship between radiata pine and beneficial endophytic *Trichoderma* species. These fungi can induce a broad-spectrum activity response from their host plants, enhancing the speed and strength of the plant's response to diseases. *Trichoderma* species have also been shown to significantly improve growth rates of radiata pine that will lead to considerable economic benefits for forest owners through increased productivity in plantations. In addition, the project will reduce chemical use in nurseries, contribute to healthier forests with faster growth and may protect against biosecurity incursions.

6. PROPOSED RESEARCH AND DRAFT WORK PLAN

Priorities for future research most likely to lead to beneficial outcomes for the forestry industry will be submitted to FGR in August 2019.

7. REFERENCES

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8. ACKNOWLEDGEMENTS

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- Marika Fritzsche, John Sydney, Satoru Kuwabara, Rachel Smith and Wayne Cameron, Timberlands
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- Keith Wood, Pat McCarthy, Paul Silcock, Stephen Wycherley and Dave Grogan, Ernslaw One Ltd
- Mike Baker, Hancock Forest Management (NZ) Ltd
- Kate Muir and John Whitehead, Juken
- Craig Brown, Nelson Forests Ltd
- Simon van Haandel and Kevin Haine, PF Olsen NZ
- Mitchell Haberkorn, Tasman Pines Ltd
- Terry O'Neill, Ashburton District Council
- Tony Walls, Simons Hill Forest
- Andrew Simpson, Balmoral Station
- Claire and Terry Gavin, Kawatiri-Murchison Highway, Owen Junction

9. APPENDICES

Appendix 1. Milestones and tasks for the period 1 July 2014 to 30 June 2016

Task no.	Activity
	Milestone 1 – Controlled environment trials
1.1	To identify and propagate at least three research clones, which have been tested for resistance to *P. pluvialis*, for milestone 1.
1.2	Production of *Trichoderma* inoculum for treatment of plant material and supplied to research partners.
1.3	Identify endophyte/elicitor combinations that enhance seedling resistance to diplodia dieback in at least two pine seedlots.
1.4	Evaluate the most effective treatments for potential to control foliar pathogens (*Dothistroma septosporum* and *Phytophthora pluvialis*) in at least two pine seedlots.
1.5	Production of *Phytophthora pluvialis* for challenging endophyte-inoculated plant material to research partners. Challenge and assess endophyte-inoculated tissue culture plants (seedlings and clones) with *P. pluvialis* and D. septosporum.
1.6	Propagate research clones, which have been tested for resistance to *P. pluvialis*, for milestone 1.
1.7	Production of *Trichoderma* inoculum for treatment of plant material and supplied to research partners.
1.8	Verification of disease screening by comparing the most effective endophyte/elicitor treatment on clonal radiata pine selections.
1.9	Provide tissue samples from parallel inoculation trials to Lincoln and Massey for testing *Trichoderma* persistence (sample set 1 for milestone 2) and biochemical and molecular studies of induced resistance (sample set A for milestone 4).
1.10	Using the best-performing endophytes identified in year 1, continue to challenge and assess endophyte-inoculated tissue culture plants (seedlings and clones) with *P. pluvialis* and/or *D. septosporum*.
	Milestone 2 – Persistence of *Trichoderma*
2.1	Colonisation and persistence of *Trichoderma* confirmed in seedlings and cuttings in forest nurseries.
2.2	Persistence of *Trichoderma* in existing forest trials continue to be monitored by outgrowth of cultures onto plates.
2.3	Plate cultures of *Trichoderma* identified to species level by EF-1 amplification and strain-specific primers if available.
2.4	Strain-specific primers for LU633 and LU584 validated.
2.5	Quantitative PCR methods developed for LU633/584.
2.6	Provide tissue culture plant samples with inoculated endophytes to Lincoln/Massey for endophyte persistence testing during the year.
2.7	LU633/584 primers tested on forest and controlled environment samples already provided by Lincoln, PFR and Massey.
2.8	Confirm taxonomic identifications of the most control *Trichoderma* isolates based on sequence data from the EF-1 gene.
2.9	Evaluate the use of UP-PCR to distinguish between *Trichoderma* strains used in seed treatments.
2.10	Specific primers developed for at least two new *Trichoderma* strains using UP-PCR or candidate gene sequences such as EF-1 if suitable sequence differences are identified.
2.11	Further development of *Trichoderma* assays.
2.12	Provide sterile tissue culture plant samples (sample set 3) to Lincoln/Massey during the year.
2.13	PFR/Scion plants (sample set 1 from Milestone 1.9) tested for endophyte persistence.

2.14	Nursery and forest plants (sample set 2 from Milestone 3.14) tested for endophyte persistence.
2.15	The Tree Lab tissue culture plants (sample set 3 from Milestone 2.12) tested for endophyte persistence.
2.16	Obtain and store tissue culture plants (with all combinations of untreated/treated and pathogen challenged/unchallenged; sample set B) for Massey.
	Milestone 3 – Nursery and forest plantation trials
3.1	Production of *Trichoderma* inoculum for treatment of plant material.
3.2	Inoculation of *P. radiata* seedlings and/or cuttings with selected *Trichoderma* treatments.
3.3	Produce endophyte-inoculated tissue culture plants for nursery trials.
3.4	Establishment and monitoring of trials at Lincoln, commercial nurseries and forestry plantations.
3.5	Design an appropriate statistical methodology for determining treatment effects on disease expression (Cyclaneusma needle cast, Dothistroma needle blight, physiological needle blight and red needle cast) in established field trials.
3.6	Assess disease in at least four established field trials, if levels of disease are sufficient.
3.7	Assess plant growth (tree height, stem diameter) in nursery and plantation trials.
3.8	Production of *Trichoderma* inoculum for treatment of plant material.
3.9	Establishment and monitoring of trials at Lincoln, commercial forest nurseries.
3.10	Inoculation of *P. radiata* seedlings and/or cuttings with selected *Trichoderma* treatments.
3.11	Assess plant growth (tree height, stem diameter) in forestry plantation trials.
3.12	Assess disease in established field trials to determine the effect of *Trichoderma* inoculation on foliar disease expression.
3.13	Compare findings with results from controlled environment trials using the same *Trichoderma* isolates.
3.14	Provide nursery and forest samples to Lincoln and Massey for persistence studies (Milestone 2 sample set 2).
	Milestone 4 (MBIE funded) – Biochemical and molecular responses to endophytes and elicitors
4.1	Inoculum of selected *Trichoderma* treatments produced and supplied by Lincoln.
4.2	Monitor temporal changes in monoterpene content and enzyme activity in seedlings exhibiting induced resistance to pathogen inoculation.
4.3	Sequences of candidate induced resistance genes (e.g. peroxidases; beta pinene) obtained from *Pinus radiata* genome.
4.4	Quantitative PCR assays developed for pine genes identified in year 1. M
4.6	Biochemical responses quantified and compared to expression of candidate genes from Milestone 4.7.
4.7	Expression of candidate genes quantified in potted clonal plants (sample set A, Milestone 1.9) and possibly tissue culture plant samples (sample set B).

Appendix 2: Radial growth rate (mm/day) and sporulation levels of selected cultures of *Trichoderma* isolated from radiata pine roots in cold and warm regions and beneficial BPRC isolates, after five months incubation at 2°C**.**

Isolate	**Mean Radial Growth Rate (mm/day)**	
	Five months incubation	**Sporulation Level [a]**
Cold Region:		
T. sp. 273	0.03	x
T. sp. 787 (1)	0.20	✓ ✓
T. sp. 787 (2)	0.21	✓✓
T. atroviride	0.08	✓
T. composticola	0.15	✓
T. fertile	1.30	✓✓✓
T, polysporum	1.30	✓✓
T. spirale	0.00	✓✓✓
T. viridescens	0.13	✓
Warm Region*:*		
T. crassum	0.01	✓✓
T. koningii	0.02	✓✓
T. viride	0.01	✓
T. viridescens	0.11	✓
T. caerulescens	0.01	✓
T. spirale	0.00	✓✓✓
T. atroviride	0.00	✓✓
T. harzianum	0.00	✓✓
T. hamatum	0.00	✓
Beneficial Isolates:		
FCC55 *(T. harzianum)*	0.00	✓✓✓
FCC318 *(T. atrobrunneum)*	0.00	✓✓✓
FCC327 *(T. harzianum)*	0.00	✓✓✓
FCC340 *(T. harzianum)*	0.00	✓✓
FCC13 *(T. asperellum)*	0.00	✓✓✓
FCC14 *(T. atroviride)*	0.00	✓✓
FCC15 *(T. atroviride)*	0.00	✓✓
FCC180 *(T. crassum)*	0.00	✓✓✓

[a] Plates were removed from the 2°C growth cabinet and placed on a laboratory bench for three weeks. Resultant isolate sporulation was described as: x (nil, mycelial growth only), ✓(minimal), ✓✓ (medium) and ✓✓✓ (high) levels.

Appendix 3: Root fungi identification from trees sampled from seven New Zealand forestry regions.

Isolate Code	Collection Locality Code [a]	Identification [b]	Comments
110 and 111, 44	Ka, Ko	*Absidia glauca*	
28	P	*Ab. psychrophilia*	
45	KR	*Aspergillus cervinus*	
11	P	*As. niger*	
42	KR	*As. parvulu*	
11sub	P	*As. welwitschiae*	
63, 64	W	*Cadophora orchidicola*	Synonym = *Leptodontidium orchidicolav*
96	M	*Chaetomium funicola*	
16sub	P	*Cladophialophora chaetospira*	Saprophytic endophyte in plant litter
40R	Ko	*Coniochaeta mutabilis*	Genus includes tree pathogens
122	O	*Corticiaceae* sp.	
7sub	P	*Cunninghamella elegans*	
54 and 67, 58	W, Wp	*Epicoccum nigrum*	
93	M	*Eurotiales* sp	
69R	W	*Fusarium acuminatum*	
52, 59	W	*F. avenaceum*	
61R	W	*F. lateritium* or *avenaceum*	
5	O	*F. oxysporum*	
18a, 26sub	P	*Gongronella butleri*	
14sub, 17sub	P	*Hyaloscyphaceae* sp.	Saprophytic fungi of dead wood and other plant matter
55 and 57, 60	W, Wp	*Ilyonectria cyclaminicola*	Genus often pathogenic
104	Ka	*I. destructans*	
6	O	*I. radicicola*	
46	KR	*I. rufa*	
43	Ko	*Mortierella* sp.	
53	W	*M. alpine*	
19a, 109	O	*Mucor fuscus*	
68, 70R	W	*Mu. hiemalis*	
98	H	*Mu. moelleri*	
9, 10sub	O	*Penicillium amaliae*	
1aR	O	*P. bilaiae*	
23sub	O	*P. canescens*	
12	P	*P. citreonigrum*	
2	O	*P. glabrum*	
1, 9a	O	*P. lilacinoechinulatum* or *bilaiae*	
4d	P	*P. miczynski*	
66	W	*P. montanese*	
106	R	*P. murcianum*	
25sub	P	*P. pancosmium* or *ubiquetum*	
4a	P	*P pasqualense* or *restrictum*	
3sub	O	*P. sanguifluum*	

8a	O	*P. spinulosum*	
10	O	*P. thomaii*	
27	O	*Pestalotiopsis disseminata*	Opportunistic fungi; also a pathogen in banana
97	T	*Pezizales* sp.	
65	W	*Phacidiopycnis washingtonensis*	Described as a post-harvest fruit pathogen
62, 100	W, T	*Phialocephala fortinii*	Described as beneficial to tree growth
51	Wp	*Resinicium bicolor*	Pathogen of Douglas fir
50	Wp	*Rhizoscyphus* sp.	Some species are endophytic
20	P	*Talaromyces acaricola*	Some species are endophytic and described as potential biocontrol agents
13sub	P	*Ta. proteolyticus*	
123	O	*Trichoderma asperellum*	
34TB	O	*T. atroviride*	
80	T	*T. crassum*	
37TC	P	*T. gamsii*	
22, 22R, 32Asub, 36Asub	O	*T. hamatum*	
81, 125, 126, 127	T	*T. harzianum*	
30T, 33AR	O, P	*T. koningii*	
31TA, 32sub	O	*T. koningiopsis*	
74, 95	Ko, H	*T. spirale*	
38Asub	O	*T. tomentosum*	
41	Ko	*Umbelopsis changbaiensis*	Described as potential biocontrol agent
56	Wp	*U. ramanniana*	
91	R	*U. vinace*	
15sub	O	*Verticillium* sp.	

a Ka = Ohakune, Manawatu-Whanganui; Ko = Kohatu, Nelson; P = Whangarei, Northland; KR = Kikiwa, Nelson; W = Inland Tokomaru Bay, Gisborne; M = Banks Peninsula, Canterbury; Wp = Inland Whatatutu, Gisborne; O = Kaikohe, Northland; R = Rakaia, Canterbury; T = Kawerau, Bay of Plenty.

b Species identification based on analyses of *tef1* and ITS sequences for *Trichoderma* and other fungi genera, respectively.