Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Summary of *Trichoderma* Research Trials (Tasks 3.1, 3.4, 4.1 and 5.1) to December 2018

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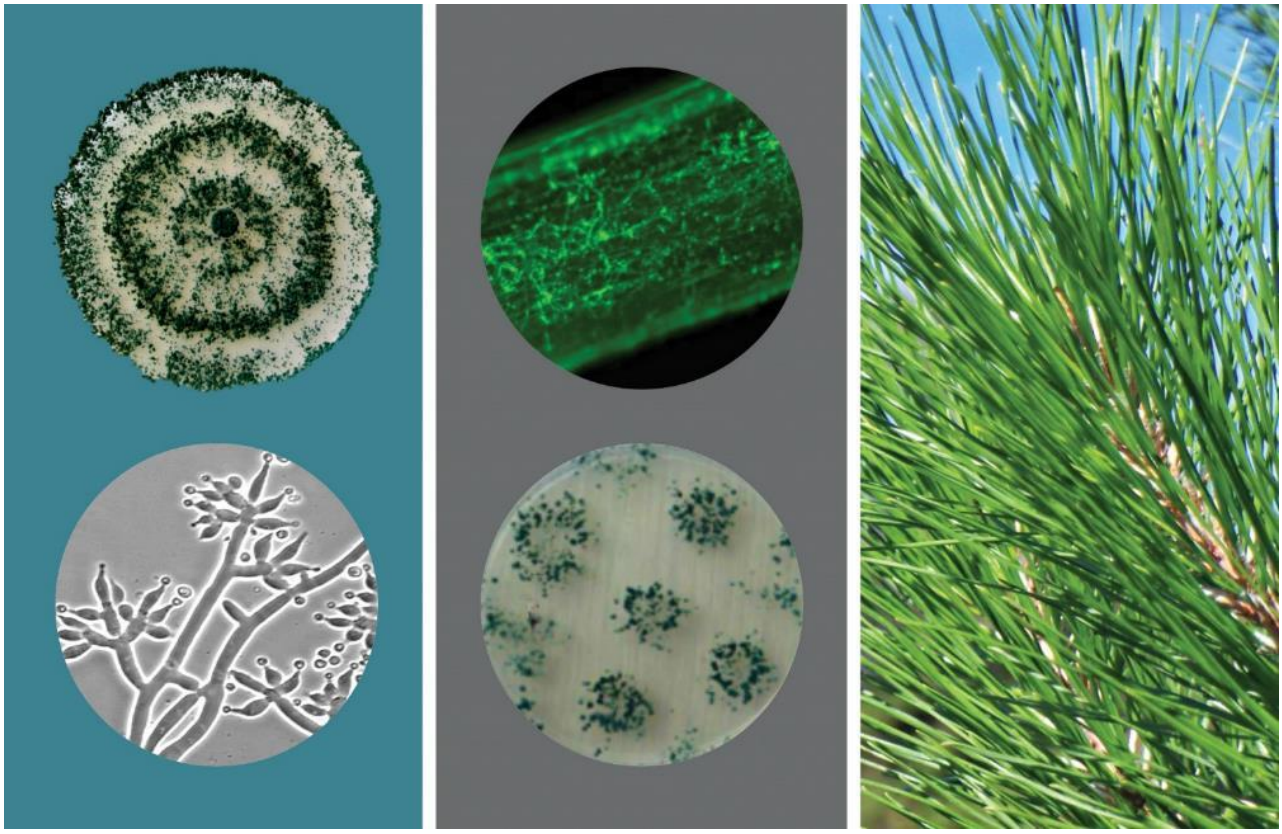

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SUMMARY:

Task 3.1: Effect of *Trichoderma* on a Hard-to-Root Clone of *Pinus radiata:*

- Two T*richoderma* treatments (isolate FCC327 and PR6 mixture) 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%), in *Pinus radiata* Clone 57 cuttings in Te Ngae Nursery in 2017.
- Approximately 8 to 10% more cuttings (average of 65.4%) survived to harvest in the *Trichoderma* treatments, compared to the untreated control (56.3%).
- The positive results in the 2017 trial led to the establishment of a larger cuttings trial in June 2018, with two hard-to-root clones and 7 *Trichoderma* treatments.

Task 3.5: Effect of *Trichoderma* Root Endophytes in Established *Pinus radiata* Trees:

- Eleven months after *Trichoderma* treatment of 21-year-old trees in Tawawera Forest, Kawerau, root colonisation levels were more than twice (average of 21% of root pieces tested) that of the untreated trees (average of 9% of root pieces tested).
- The *Trichoderma* impregnated root dowel treatment resulted in a doubling of the diameter at breast height increment (10.2mm), compared to the control (5.3mm).
- No disease assessments were made in 2018 due to low infection rates in the region.

Task 4.1: *Trichoderma* and Cypress Canker:

- Nursery *Trichoderma* inoculated seedlings were found to have sufficient *Trichoderma* in the roots to test the potential for *Trichoderma* root endophytes to control cypress canker in field trials. These seedlings are being distributed to the New Zealand Cypress Development Group members for trial establishment in multiple locations where canker is prevalent.
- The effect of six *Trichoderma* root endophyte mixtures on seedling growth will be assessed in a second containerised trial at Southern Cypresses Nursery, Ohoka, Canterbury that was established in 2018.

Task 5.1: *Trichoderma* and Swiss Needle Cast in Douglas-Fir:

- Roots of Oregon, California and Washington seedlot Douglas-fir nursery seedlings were readily colonised by beneficial *Trichoderma* isolates, with more than half the root pieces tested having *Trichoderma* present eleven months after inoculation.
- Survival of Douglas-fir seedlings was significantly higher (P<0.05; 81.3%) in the T1 (FCC320, FCC327 and LU633) treatment, compared to the untreated control (72.1%).
- The two *Trichoderma* treatments (T1 and T2 (PR3a mixture)) tested had large impacts on seedling 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.
- The effect of *Trichoderma* T1 and T2 treatments on Swiss needle cast will be determined in a field trial to be established in Kaingaroa Forest in winter 2019.
- The effect of eight *Trichoderma* root endophyte mixtures on seedling growth will be assessed in a second containerised seedling trial at Lincoln University Nursery, Canterbury that was established in 2019.

Task 3.1: Effect of *Trichoderma* Root Endophytes on Root Initiation in a Hard-to-Root Clone of *Pinus radiata*

Introduction: High quality clonal stock is advantageous in New Zealand forestry production because it enables improved crop quality and productivity compared 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 endophytes on cutting survival, root initiation and growth of an important *P. radiata* clone was assessed in a pilot trial at Timberland's Te Ngae Nursery, Rotorua in 2017. An additional trial was established in June 2018 to validate the results found in 2017.

Methods: Soil (Te Ngae soil series; sandy pumice with some Rotomahana Mud) was fumigated with Fumasol (ai. Metam as a sodium salt, 510g/litre) at 700 litres/ha, cultivated and treated with Agzyme (Appendix B) at 1 litre/ha, Ag Concepts Super Hume (Appendix B) at 5 litres/ha and Mynoke Vermicompost at 24 tonnes/ha, in winter 2017. Two beds were then formed and planting-holes mechanically formed four weeks before planting. A total of ten replicate blocks, with 3 plots each, were set out in the two beds. Plots contained 8 rows x 13 cuttings and were arranged in a completely randomised block design. Untreated buffer zones of 8 rows x 4 cuttings were marked out between the plots and black polythene plastic strips were placed (from ground-level to 200mm depth) in each buffer zone (Figure 1).

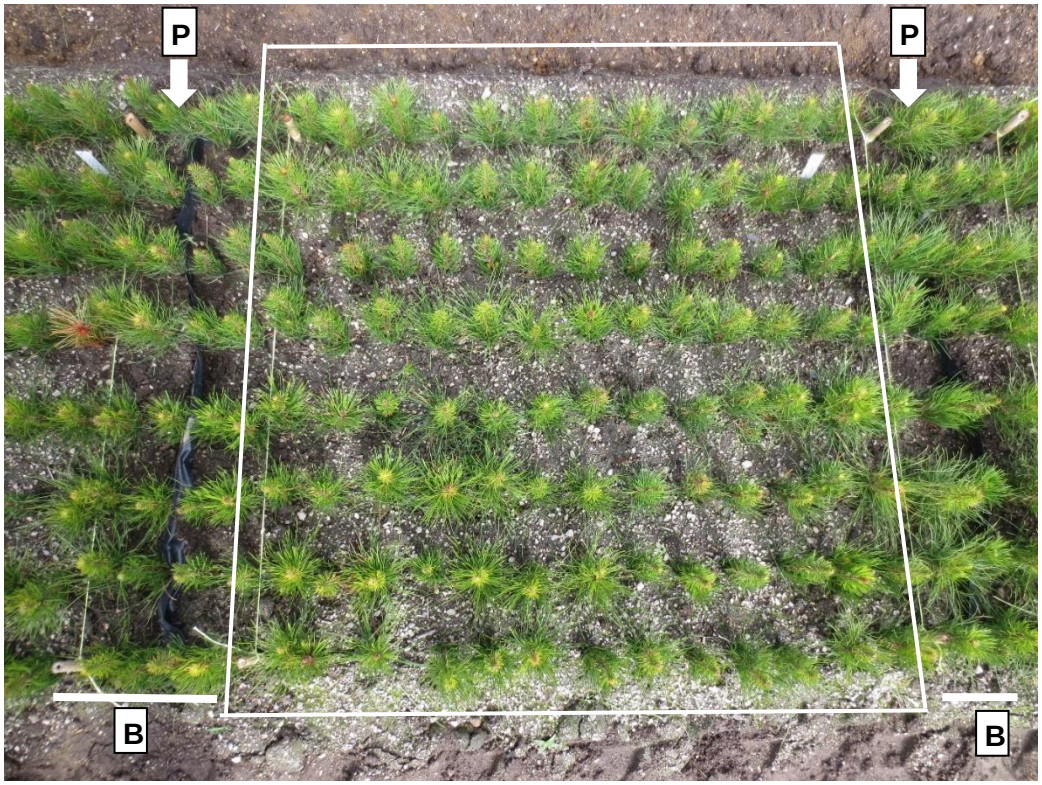

Figure 1: Plot with buffer zones (B) and plastic sheet inserts (P) on the left and right of the plot.

***Trichoderma* treatments included:**

1. *Trichoderma* PR6 mixture (containing isolates FCC55, FCC318, FCC327 and FCC340)
2. *Trichoderma* isolate FCC327
3. Untreated control

Trichoderma treatments (3000ml of 6.7 x 10^6 spores/ml per plot) were applied with a watering can to the plots (but not in the buffer zones) on 16 August 2017. The untreated control treatment received 3000ml of tap water. Two plastic sheets were secured over the soil bed to stop leaching of spores due to forecasted rain. Sheets were removed the following morning and bare-root cuttings (Clone 57, Forest Genetics Ltd, http://www.forest-genetics.com) were hand-planted into the soil bed rows.

Beds were covered with frost cloth for six weeks which was removed in mid-November 2017. One Blue Shield DF (ai. 500g/kg copper hydroxide) spray was applied at 2kg/ha in November 2017 as part of normal nursery practice. Cuttings were watered daily, if required. Plants were topped, and roots were cut by lateral disc in autumn and plants were wrenched in June 2018.

Trichoderma root colonisation (%) was measured by sampling 20 random cuttings in each treatment six and eleven months (25 July 2018) after setting and *Trichoderma* application. One hundred random root pieces per treatment were surface-sterilised and placed on *Trichoderma* isolation malt yeast extract agar with rose bengal (MRB; Appendix A) plates for growth of *Trichoderma* colonies, according to established protocols below:

- roots were thoroughly washed with tap water and sectioned into approximately 5 to 10mm lengths,
- root pieces were placed into a petri dish and soaked in Virkon (1% w/v) for 10 min for surface sterilisation, then rinsed in sterile distilled water,
- five pieces per treatment were aseptically transferred onto one of each of five replicate *Trichoderma* isolation MRB plates,
- agar plates with roots were incubated on a laboratory bench top with ambient light and temperature conditions for 14 days,
- following incubation, plates were visually assessed, the total number of *Trichoderma* colonies counted and mean root colonisation % calculated.

Thirty-two cuttings (central 4 rows x central 8 cuttings per plot) were harvested from each plot on 25 July 2018 and the following measurements or calculations were performed on a plant or plot basis:

1. roots were scored based on the presence of roots in each quadrant (refer to Figure 2)
2. survival (%), defined as any cuttings with roots present
3. root collar diameter was measured at ground level with a digital calliper
4. dry weight of shoot (including both above- and below- ground material); dried at 65°C for 72 hours
5. dry weight of root; dried at 65°C for 72 hours
6. root:shoot dry weight ratio
7. rooting depth of 4 cuttings per plot at harvest

The effect of *Trichoderma* treatments on plant dry weight was not measured accurately because the roots and shoots were trimmed during the season, as part of standard nursery practice. However, an estimate of the relative difference in dry weight between treatments, was determined.

Data were analysed for significance by analysis of variance (ANOVA) and least significant difference (LSD) tests (GenStat, v19).

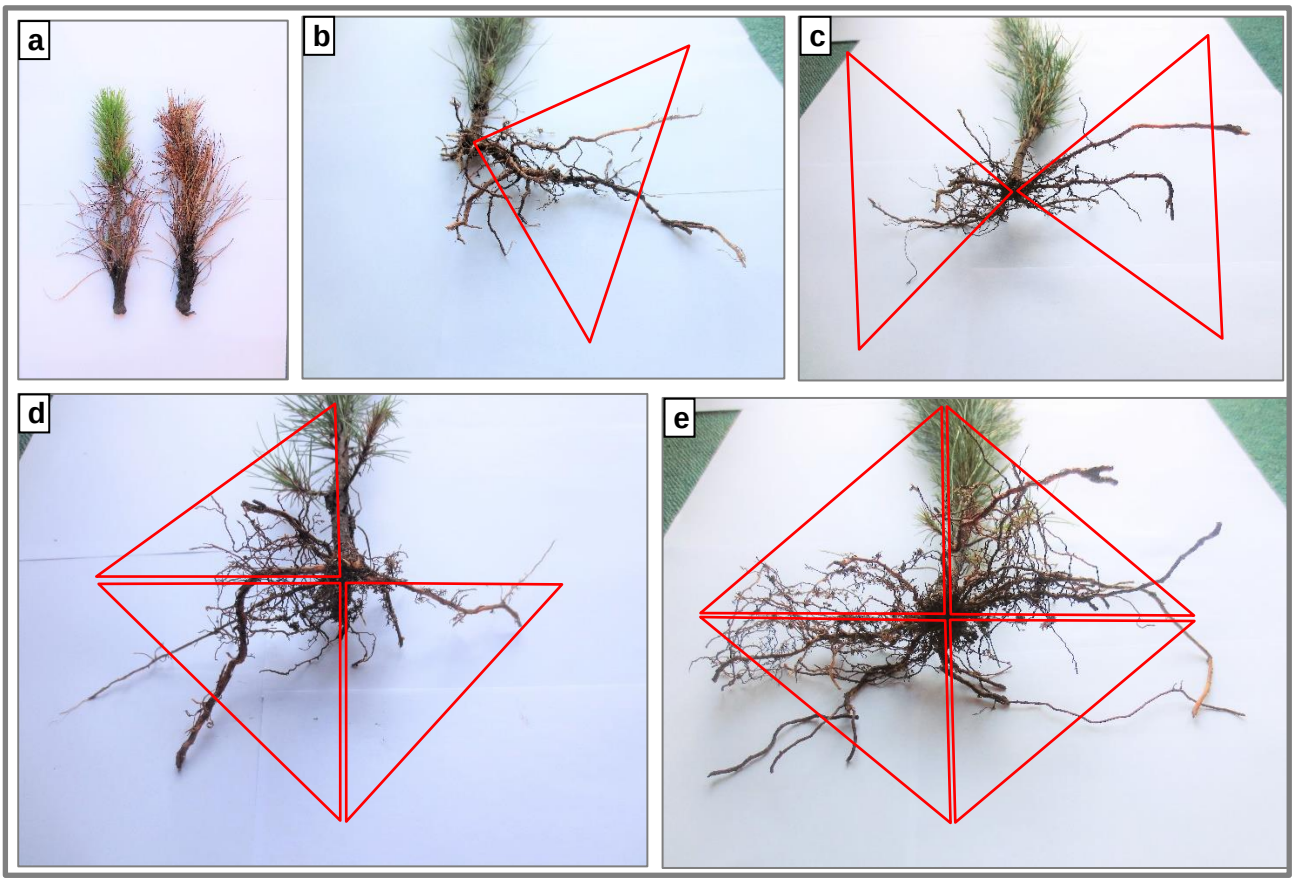

Figure 2: Root scores used at harvest; a) 0 or 0.5; green tipped or dead cuttings with no roots, b) 1; roots in one, c) 2; two, d) 3; three and e) 4; four quadrants.

Results:

High levels of root colonisation (approximately one third of root pieces tested) were found in the FCC327 treatment six months after setting and *Trichoderma* application; this level was maintained until harvest, eleven months later (Table 1). *Trichoderma* PR6 treatment also had high levels (45.5%) of root colonisation at six months but these levels were reduced to 18.4% at harvest. Untreated control cuttings had 5% or less root colonisation at the two sampling dates, indicating low levels of environmental *Trichoderma* in the soil bed which therefore did not have an impact on the results.

Table 1: Colonisation (%) of *P. radiata* roots with *Trichoderma* treatments, sampled six and eleven months after setting of cuttings and *Trichoderma* application, based on MRB plating data.

Treatment	Root Colonisation (%)	
	22 February 2018	25 July 2018
Trichoderma PR6	45.5	18.4
Trichoderma FCC327	30.0	31.7
Untreated Control	1.1	5.0

Cutting survival (%) was significantly lower in the untreated control treatment, three ($P<0.05$) and eleven ($P<0.001$) months after setting and *Trichoderma* application, compared to the *Trichoderma* treatments (Table 2; Figure 3). At harvest, approximately 8 to 10% more cuttings were present in the *Trichoderma* treatments, compared to the untreated control.

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.

Both FCC327 and PR6 *Trichoderma* treatments significantly (P<0.001) increased the number of cuttings with root scores of 3 or 4 by 136 and 152% respectively (ie a 2.5-fold increase), compared to the untreated control (Table 2). The number of plants with commercially acceptable root scores of 3 or 4 was low, at 30% or less, (Table 2) because this clone is very sensitive to late setting (the trial was established late due to the principal researcher being unavailable) and two flooding events occurred during the growing season. This clone may also be sensitive to setting depth. Cuttings appeared to initiate more roots if the bottom of the cutting was deeper in the soil bed (Table 3).

Table 3: Mean depth of *P. radiata* cuttings measured at harvest for *P. radiata* plants with different root scores.

Root Score	Mean Depth of cutting measured at harvest (mm)
0, 0.5, 1	56
3, 4	65
LSD (5%)	6.0
Significance	P<0.001

Trichoderma had no effect on root collar diameter or root/shoot ratio (Table 2). However, treatment with FCC327 resulted in root, shoot and plant dry weights being significantly (P<0.05) greater, compared to the untreated control (Table 2), even though roots and shoots were trimmed during the year.

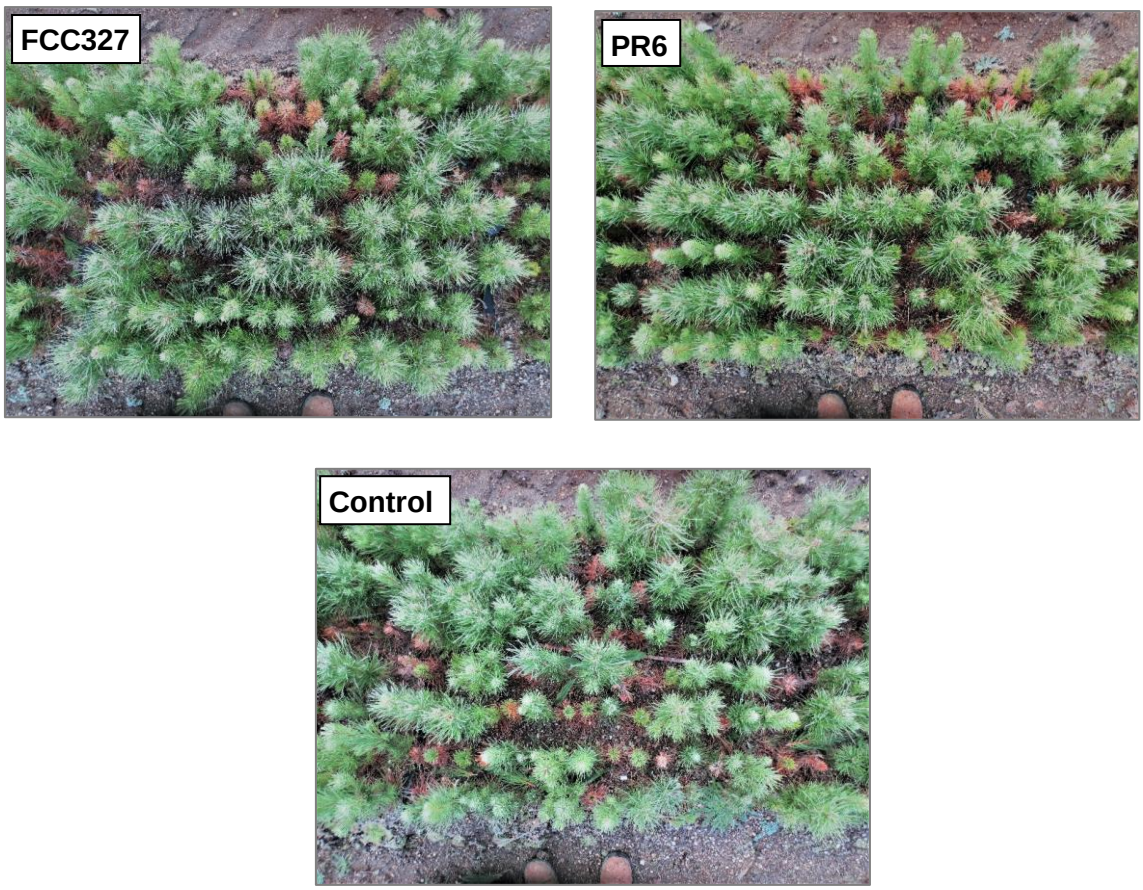

Figure 3: Treatment plots eleven months after setting and *Trichoderma* application.

New 2018 Trial:

The positive results in the 2017 trial led to the establishment of a larger trial in June 2018 (Figure 4). The trial comprised of 2 hard-to-root clones (Clone 48 and 57; Forest Genetics Ltd, http://www.forest-genetics.com), seven *Trichoderma* treatments and nine replicates, arranged in a completely randomised block design. Soil preparation in 2018 was similar to that of the 2017 trial and planting-holes were made three weeks before planting. Bare-root cuttings were hand-planted into three soil bed rows in plots of 1m wide and 3m length on 19 June 2018. *Trichoderma* treatments were applied as a soil drench (2.1 litres at 2.8 x 10^6 spores/ml per plot) the day after setting with a watering can. Cuttings were covered with frost cloth until cloth was removed in October 2018.

***Trichoderma* treatments included:**

1. *Trichoderma* PBI (LU132, LU140, LU584 and LU633), no fungicide
2. *Trichoderma* modified ArborGuard mixture (LU655, LU659, LU660, LU661, LU663), no fungicide
3. *Trichoderma* general Mixture (FCC320, FCC327, FCC633), no fungicide
4. *Trichoderma* mixture A (LU297, LU668, LU753, LU996, LU1328), no fungicide
5. *Trichoderma* PR6 (FCC55, FCC318, FCC327, FCC340), no fungicide
6. Control untreated no fungicide
7. Control untreated + fungicide

Figure 4: Setting of cuttings in June 2018 (left image) and overall view of the Te Ngae Nursery trial in October 2018 (right image).

Future Measurements in 2018 trial:

1. Root *Trichoderma* colonisation in February 2019 and harvest (June 2019)
2. Survival, root score, root and shoot dry weight at harvest.

Task 3.5: Effect of *Trichoderma* Root Endophytes in Established *Pinus radiata* Trees

Introduction:

In New Zealand, very promising results have been obtained in *Trichoderma* endophyte biocontrol trials, with the best *Trichoderma* treatments increasing tree height in three- to six- year-old stands by up to 20% (Hill and Whelan, 2017). In these trials, application of *Trichoderma* inoculum has been by seed-coat in the nursery; a practical, effective, low-cost and socially acceptable method to apply biocontrol agents. However, the majority of the approximately 1.5 million hectares (Ministry for Primary Industries, 2017) of New Zealand's *P. radiata* plantations have not been seed-coated with *Trichoderma* endophytes. This pilot study will investigate whether it is feasible to treat established plantation trees with *Trichoderma* endophytes in order to induce disease resistance and growth benefits.

Trial Location and Tree Selection (29/30 November 2017)

A pilot trial was established in a clonal *P. radiata* stand, located in the Tarawera Forest (owned by Hancock Natural Resource Group) approximately 8km south-south-east of Kawerau (Figure 5). The stand, planted in 1996, had received standard industry management and was pruned and thinned to 300 stems per hectare. The stand now receives no fungicides or herbicides due to the age of the trees.

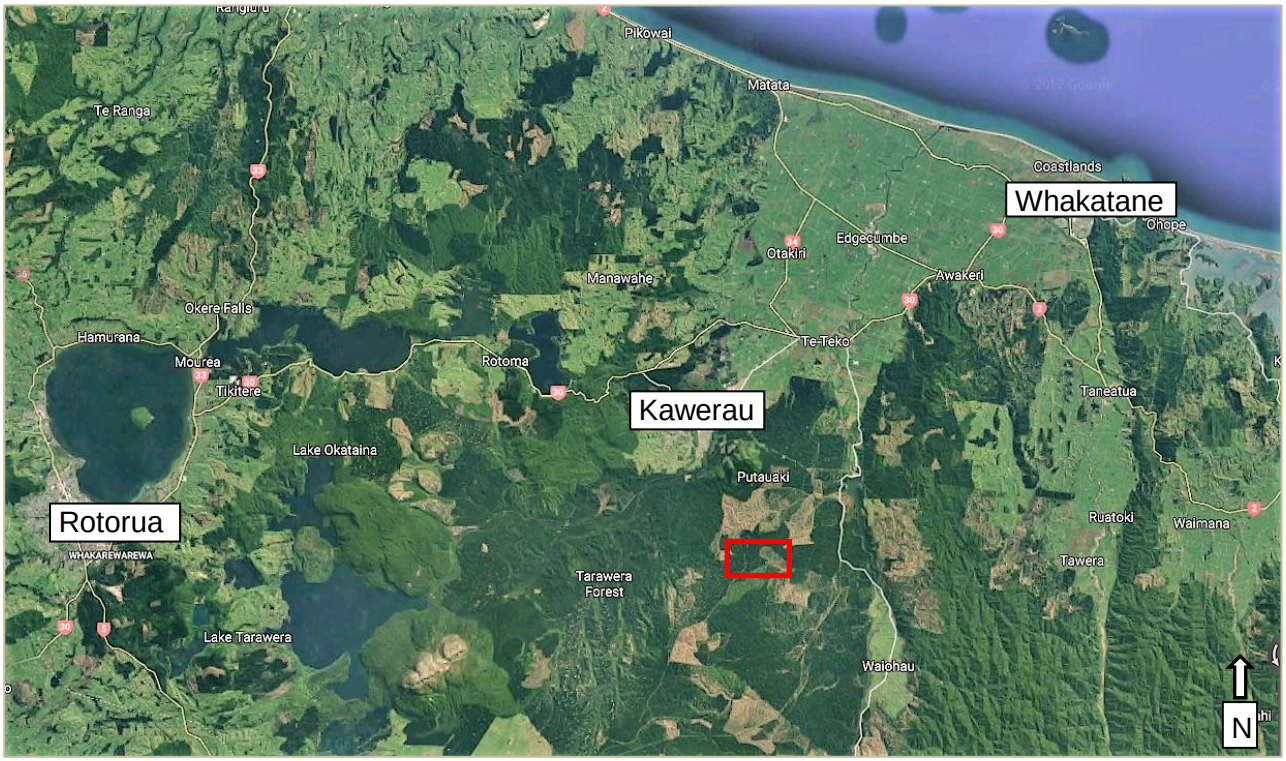

Figure 5: Location of trial (red rectangle) near Kawerau.

Trees selected for the trial were on a south-facing outer row beside a forestry access road (Station Rd; Figure 6)). Trees were of a relatively similar height (approximately 35m) and had trunk diameters at breast height (DBH) of between 462 and 700mm. The bottom canopy mostly contained branches with sparse red/brown necrotic needles (Figure 7) caused by high infection of red needle cast disease (*Phytophthora pluvialis*) in spring of 2017 (M. Baker *pers. comm.)*. Approximately 15 to 20% of the top canopy contained relatively green needles.

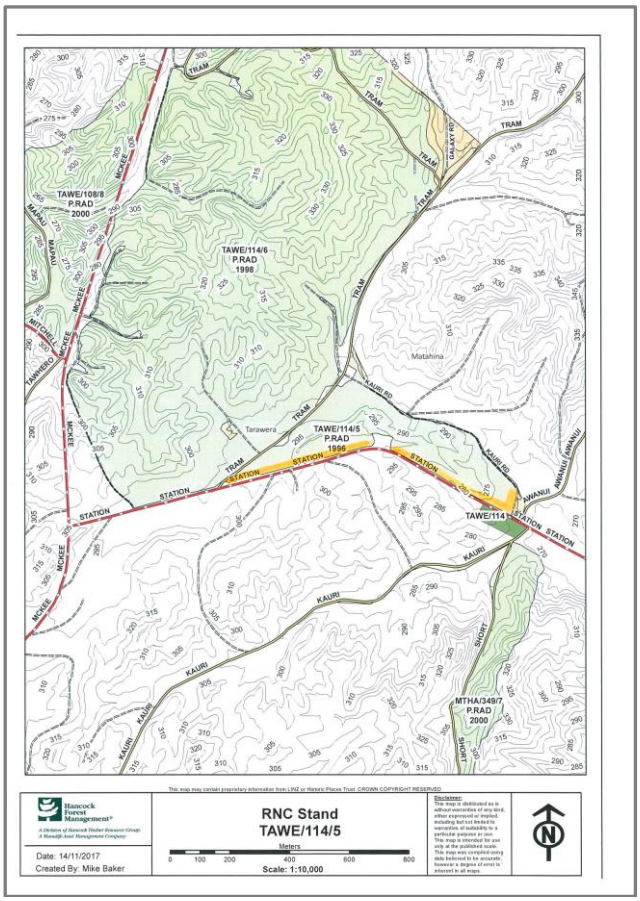

Figure 6: Plan of the Kawerau Established Tree trial (the yellow line marks the trees in the trial).

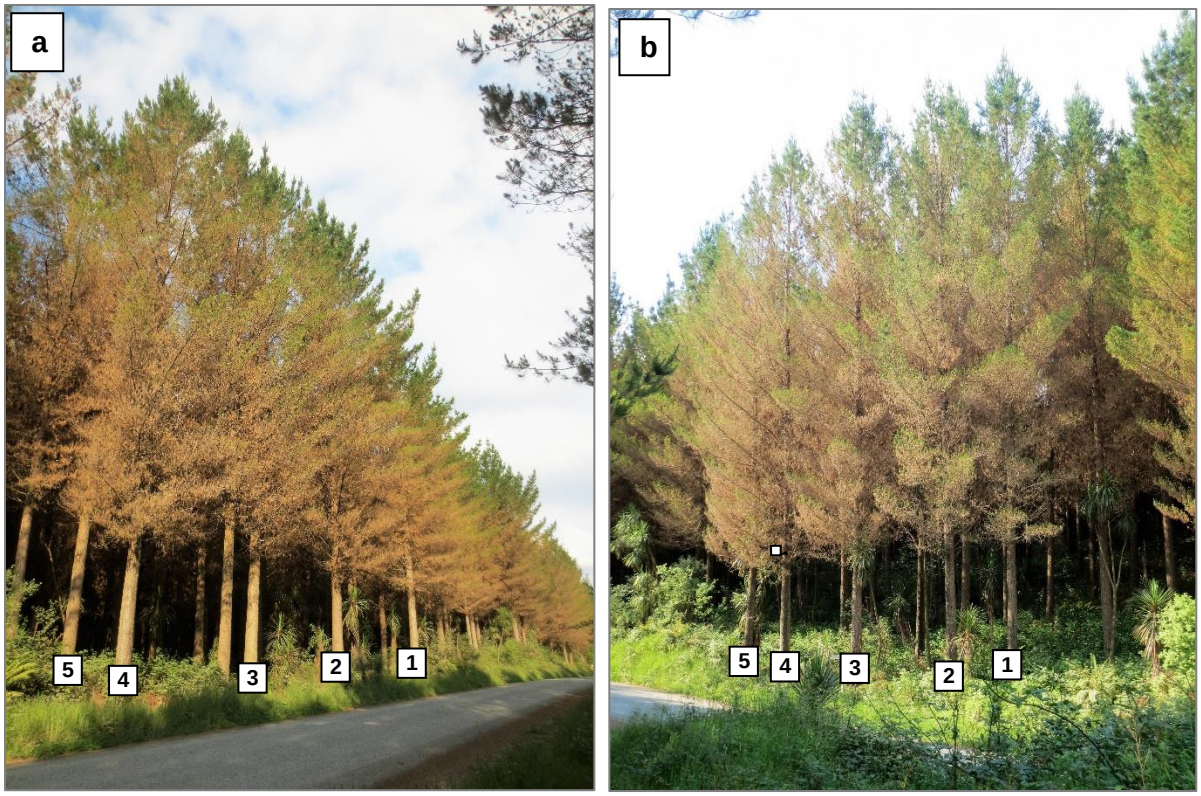

Figure 7: Individual trees treated with *Trichoderma* treatments in replicates (a) 2 and (b) 3.

Treatments:

Trees were randomly allocated treatments, apart from trees receiving the soil drench treatment which were separated from neighbouring trees with an untreated (buffer) tree. Four *Trichoderma* treatments (using one isolate mixture of FCC55, FCC318, FCC327 and FCC340; PR6 mixture) and one untreated control, were applied to five replicate blocks (Table 4). Weeds were not disturbed at the base of the trunks, apart from blackberry canes which were cut at above ground level to allow access to the trunk.

Table 4: Number, volume and spore concentration of *Trichoderma* applications and total spore number applied per tree in each treatment

Treatment Name	Number and volume of *Trichoderma* applications per tree	Spore concentration per application (spores/ml)	Total *Trichoderma* spore number applied per tree
Soil Drench	1 application of 20000ml	5.0×10^6	1.0×10^{11}
Trunk Injection	7 injections x 5 to 10ml per injection	3.0×10^8	1.1 to 2.1 $\times 10^{10}$
Root Dowel	15 dowels x 0.08ml per dowel	2.2×10^8	3.3×10^9
Trunk Spray	1 application of 2000ml	5.0×10^6	1.0×10^{10}
Control	0	0	0

Soil Drench:

- mulch material removed from the base of the tree
- spore suspension applied with a watering can within a 1.5m radius from the edge of the tree trunk
- mulch material replaced

Trunk Injection:

- 7 holes (6mm diameter and 45mm depth) were drilled into bark fissures in the trunk at a 30° downward angle approximately 300 to 500mm from base of tree with an Ozito 18V Li-Ion Drill Driver
- spore suspension loaded into Chemjet Tree Injector (www.chemjet.com.au) and screwed into trunk (Figure 8)
- injector handle released, and spore suspension injected under pressure
- injectors removed between two hours and one or two days after placement of injectors due to slow infiltration rate of suspension (low tree water evaporation rates)

Figure 8: Inserted injectors containing *Trichoderma* spore suspension in *P. radiata* trunks.

Root Dowel:

- dowels (balsa wood, 6mm diameter x 5mm length) were added to a plastic bag containing 1.32g dry *Trichoderma* spores (at 7.6 x 10^{10} spores/g) mixed with 6ml of sterile 0.01% Tween 80 and potato dextrose broth (24g/litre).
- bag left overnight in fridge to allow absorption of spore suspension into dowels
- patches of top soil removed from within 1m of trunk base and 15 holes (6mm diameter and 5mm depth) drilled into large feeder and small support roots (maximum diameter of 40mm)
- dowels placed into single holes and gently hammered until sitting flush with the outside of the root surface (Figure 9)
- top soil replaced

Figure 9: Inserting (left image) and inserted (right image; white circles) dowels containing *Trichoderma* spore suspension in *P. radiata* roots.

Trunk Spray:

- spore suspension mixed with Penatra (an organosilicone surfactant, http://www.sstnewzealand.co.nz; 0.2%) was sprayed with a 4-litre sprayer, from base of trunk to 2m above ground level, particularly spraying into bark fissures.
- *Trichoderma* spore germination was not affected by Penatra in laboratory germination tests.

Measurements at Trial Establishment:

Trunk diameter:

DBH (mm) was measured at 1.4m above ground level with a diameter tape and the position marked with paint. Data were analysed for significance by analysis of covariance (ANCOVA) and least significant difference (LSD) tests (GenStat, v19).

Natural *Trichoderma* Fungal Presence:
Presence of *Trichoderma* fungi, before treatment application, was estimated by sampling feeder roots from the five trees allocated treatments in replicate 5 and another three trees within or near replicate 5 and processing according to established protocols (refer to methods in Task 3.1). Forty root pieces per tree were tested. *Trichoderma* root colonisation, before treatment application, was measured at a mean of 6% (Table 5).

Disease and Green Canopy Levels:
Disease and green canopy levels were estimated by visually assessing needle symptoms and the amount of green canopy, with the aid of photographs, taken from ground level, of each tree.

First Assessment, eleven months after treatment application on 30 October 2018:

Root Colonisation:

Eleven months after *Trichoderma* 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%.

Table 5: Colonisation (%) of *P. radiata* roots in trees sampled in and around replicate 5. Measurements taken prior, and eleven months after, treatment applications, based on MRB 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**

Disease Assessment:

No foliar disease was present in the trial in October 2018 (Figure 10). The trees had a spring flush of green needles with an open canopy due to the intense disease presence in spring 2017.

Trunk Diameter:
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 control. 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 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).

Figure 10: Tree canopy (left image) and trunk diameter measurements (right image) in the Kawerau Established Tree trial on 30 October 2018.

Future Measurements in Kawerau 2017 trial:

1. Root colonisation (%) in spring 2019 and beyond
2. Disease and green canopy level assessments when new infections occur
3. DBH in spring 2019 and beyond

New Timberlands Kaingaroa 2019 Trial:

The positive result in the 2017 trial has led to an agreed establishment of a trial in Timberland's Kaingaroa forest in February 2019. A similar trial design is proposed, except an additional replicate, insertion of buffer trees between treated trees, measurement of tree height and root colonisation levels in all trees will be attempted.

Task 4.1: *Trichoderma* and Cypress Canker

Introduction: Endophytic *Trichoderma* fungi show great potential for improving growth and health of forest trees. In New Zealand, numerous beneficial *Trichoderma* isolates have been screened for their ability to promote growth and suppress foliar diseases in laboratory and nursery *P. radiata* trials, with the most effective isolates applied as nursery seed-coat treatments and placed into plantation trials. Very promising results have been obtained with best treatments increasing tree height in three- to six- year-old stands by up to 20% (Hill and Whelan, 2017).

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 *Trichoderma* root endophytes to control cypress canker was assessed in a pilot trial at Southern Cypresses Nursery, Ohoka, Canterbury in 2017.

Methods:

Treatments included:

Three seedlots:

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

Two *Trichoderma* treatments:

1. Beneficial *Trichoderma* isolates FCC320, FCC327 and LU633 (+ *Trichoderma*)
2. Untreated control (- *Trichoderma*)

C. macrocarpa and *C. lusitanica* seeds were mechanically planted into 6x8 cell plastic trays containing nursery potting mix (4 and 2 seeds per cell for *C. macrocarpa and C. lusitanica* respectively) on 31 March 2017. Seeds were covered with a thin layer of vermiculite. Multiple seeds were planted in each cell because of low germination rates of the seedlots and extra plants were thinned and placed into additional trays 4 weeks after initial planting. *C. x leylandii* seeds were planted in plastic trays in September 2016.

Treatments were applied as a spore suspension (1000ml of 5.0 x 10^6 spores/ml per tray) with a 10-litre watering can on 31 March 2017. Tap water (1000ml per tray) was applied to the control trays. Trays were placed in a covered area for six months then put outside. Trays were watered daily when required. An application of Goal (ai. 480 g/l oxyfluoren) was made at 1 l/ha in September 2017 and foliar fertiliser were applied every six weeks. Seedlings were topped at 28cm height in Nov 2017.

Trichoderma root colonisation (%) was measured by sampling five random seedlings in each treatment at six (26 August 2017), 15 (11 June 2018) and 16 months (11 July 2018) after seeding and *Trichoderma* application, and processing according to established protocols (refer to methods in Task 3.1). Between twenty-five and forty random root pieces per treatment were tested at each date.

Results:

It was observed that the *Trichoderma* treatment reduced the incidence and severity of a nursery pathogen *Pestalotiopsis guepinii* during *C. x leylandii* seedling growth (Figure 11).

Figure 11: Untreated (-*Trichoderma*) and treated (+ *Trichoderma*) *C. x leylandii* seedlings six months after treatment. The brown tissue in the untreated seedlings was caused by an opportunistic nursery pathogen *Pestalotiopsis guepinii*.

High levels of root colonisation (%) were found in samples six months after seeding (37, 38 and 73% of *C. macrocarpa, lusitanica* and *leylandii* root pieces were colonised respectively (Figure 12a and c). At 15 and 16 months, root colonisation levels were reduced to 25, 28 and 40% in *C. macrocarpa, lusitanica* and *leylandii* respectively (Figure 12b). A relatively small number of *Trichoderma* colonies (mean of 7%) were found in the untreated control seedlings in this period, probably from environmental *Trichoderma* strains (e.g. from the potting mix and/or airborne spores in the nursery).

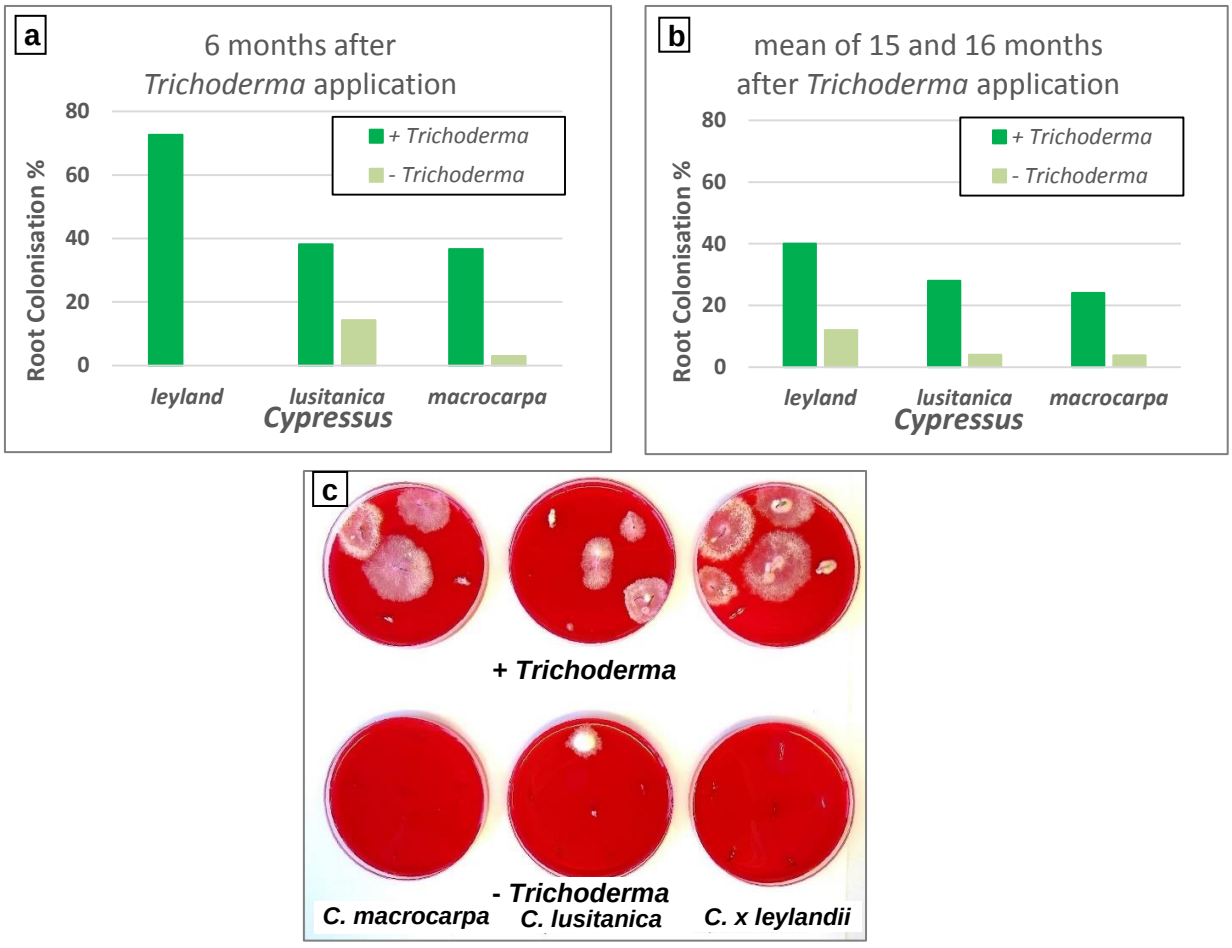

Figure 12: Root colonisation (%) six months (a), and 15 and 16 months (b) after *Trichoderma* application, based on MRB plating data**.** Examples of *Trichoderma* isolation MRB plates for each treatment and seedlot 8 days after plating in the roots sampled 6 months after seeding (c).

The inoculated seedlings had sufficient *Trichoderma* in the roots to test the potential for *Trichoderma* root endophytes to control cypress canker. These seedlings are being distributed to the New Zealand Cypress Development Group[a] members for trial establishment in multiple locations where canker is prevalent.

New 2018 Containerised Trial:

The effect of *Trichoderma* root endophytes on seedling growth will be assessed in a second containerised trial at Southern Cypresses Nursery, Ohoka, Canterbury that was established in 2018.

Treatments included:

Two seedlots:

1. *Cupressus macrocarpa* (susceptible)
2. *Cupressus lusitanica* var. lusitanica (low susceptibility)

Eight *Trichoderma* treatments:

1. Control, no fungicide
2. Control, + fungicide
3. *Trichoderma* isolates FCC320, FCC327 and LU633, no fungicide
4. PR6 (FCC55, FCC318, FCC327, FCC340), no fungicide
5. Modified ArborGuard mixture (LU655, LU659, LU600, LU661 and LU663), no fungicide
6. PBI (LU132, LU140, LU584 and LU633), no fungicide
7. Cypress Murchison Selection A (*T. harzianum*), no fungicide
8. Cypress Murchison Selection B (*Trichoderma* species to be determined), no fungicide

C. macrocarpa and *C. lusitanica* seeds were mechanically planted (6 to 8 seeds per cell) into 48 of 6x8 cell plastic trays containing nursery potting mix on 30 November 2018. Seeds were covered with a thin layer of vermiculite. Multiple seeds were planted in each cell because of low germination rates of the seedlots. Each cell will be thinned to one seedling and surplus seedlings will be removed from the trial.

Trichoderma treatments (5ml of 5.0×10^6 spores/ml spore suspensions) were pipetted into each cell of six replicate trays the day after seeding (Figure 13) and arranged in a completely randomised block design. Tap water (240ml per tray) was applied to the control trays and trays were placed in a covered area.

[a] New Zealand Cypress Development Group is an interest group of the New Zealand Farm Forest Association and comprises foresters, landowners and lifestylers.

Figure 13: Seeded trays inoculated with *Trichoderma* spore suspensions on 30 November 2018; ready to be arranged in the shade house.

Murchison Isolates:

Two *Trichoderma* isolates collected from a 23-year-old *Cupressus macrocarpa* Kukupa clone stand in Murchison (-41.69960, 172.49701) were included in the trial (Figure 14). Kukupa was one of the first commercial Forest Research clones and has gained a reputation for being canker-prone. The Murchison area is prone to canker infection, however, the Murchison stand has low canker disease expression (<5%; http://www.nzffa.org.nz/farm-forestry-model/people-and-places/case-studies/gavins/) and high natural levels of *Trichoderma* (Table 7). Natural *Trichoderma* root colonisation in other *Cupressus macrocarpa* stands will be determined in 2019 for comparison to the levels in the Murchison stand.

Figure 14: 23-year-old *Cupressus macrocarpa* Kukupa clone stand in Murchison

Tree roots were sampled from six healthy, strongly growing trees and *Trichoderm*a isolates were isolated and purified according to established protocols (Hill, *et al.,* 2017). Three isolates with rapid mycelial growth and profuse sporulation on MYE agar were selected and the isolates identified to species level with DNA analyses of partial *tef1*α gene sequences and BLAST analysis ((https://blast.ncbi.nlm.nih.gov/Blast.cgi; Hill *et al.,* 2017; Table 7). Two of these isolates were tested in the trial.

Table 7: Root colonisation (%) of selected trees in a Kukupa clone stand in Murchison, and the species identified using DNA analyses of partial *tef1*α gene sequences for two of the isolates.

Tree Identification	% of roots colonised	Identification by DNA sequencing	Treatment Identification
8	36	*T. harzianum* strain DAOM242937 KX463434.1	Cypress Murchison Selection A
9	4	-	-
15	24	-	-
16	4	-	-
UN1	36	To be determined	Cypress Murchison Selection B
UN2	52	*T. harzianum* strain DAOM242937 KX463434.1	-
Mean	26		

Future Measurements:

- Root *Trichoderma* colonisation in June 2019
- Survival, root collar diameter, height and root and shoot dry weight at harvest
- Potted disease assay trial when the seedlings are older.

Task 5.1: *Trichoderma* and Swiss Needle Cast in Douglas-Fir

Introduction: Endophytic *Trichoderma* fungi show great potential for improving growth and health of forest trees. In New Zealand, numerous beneficial *Trichoderma* isolates have been screened for their ability to promote growth and suppress foliar diseases in laboratory and nursery *P. radiata* trials, with the most effective isolates applied as nursery seed-coat treatments and placed into plantation trials. Very promising results have been obtained with best treatments increasing tree height in three- to six- year-old stands by up to 20% (Hill and Whelan, 2017).

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 endophytes on seedling growth and disease resistance to Swiss needle cast in Douglas-fir was assessed in trials at the Lincoln University Nursery, Canterbury.

2017 Lincoln University Nursery Trial:

Treatments included:

Three seedlots:

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)

Three *Trichoderma* treatments:

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 (- *Trichoderma*)

Douglas-fir seeds (sourced from Proseed, Amberley, Canterbury) were stratified by soaking overnight, placing on slightly moist paper towels, and storing at 4°C in the dark for 35 days. After 35 days stratification, seed germination was measured by placing approximately 43 seeds of each seedlot on moist seed germination paper, rolling up and sealing in a plastic bag, then placing in a 25ºC room, in natural light, for 11 days. Seed germination was measured at 83, 88 and 93% for the California, Oregon and Washington seedlots respectively.

Seeds were sown on 9 October 2017 in plastic trays (BCC Sweden) with each tray containing either 63 or 81 cells with a volume of 100mL per cell (39mm diameter and 85mm depth). Seed was sown at a depth of 10mm into unsterilised potting mix (400L composted bark, 200L peat, 200L perlite medium grade, 4000g Osmocote Exact (16-9-11, 12-14 month), 3200g gypsum, 1200g dolomite and 800g Hydroflo wetting agent) and topped with a 50% vermiculite/potting mixture.

Trichoderma treatments (5ml of 5.0 x 10^6 spores/ml spore suspensions) were pipetted into each cell of 12 replicate trays the day after seeding and arranged in a completely randomised block design. Control trays received 5ml of sterile distilled water per cell. The trays were covered with a plastic sheet for the first five days to maintain moisture levels, then lightly hand-watered once a day. Seedling emergence (%) was measured on 6 November 2017, by counting the number of seedlings in each tray. Trays were transferred from the glasshouse to a shaded nursery area three weeks after emergence (at 30mm seedling height) and were lightly hand-watered every second or third day to avoid risk of damping-off disease. Trays were covered with bird-netting cloth to stop bird and rabbit damage. At 4 months of age, the trays were moved outside (Figure 15).

Five replicates (replicates 8 to 12) of inoculated and untreated seedlings (approximately 3500 plants) were lined out at ArborGen Edendale Nursery, Invercargill in March 2018 (Figure 15) and will be

planted in one or two plantation trials in Timberlands Kaingaroa forest (an area conducive to Swiss needle cast) in winter 2019.

The remaining six replicates (replicates 2 to 7) were harvested for growth parameters in September 2018. Replicate one was excluded due to poor growth (positioned on the exposed north side of the trial). All seedlings were harvested from each tray, apart from the seedlings in the outer row of each side of the trays. Data were analysed for significance by analysis of variance (ANOVA) and least significant difference (LSD) tests (GenStat, v19).

Figure 15: Inoculated Douglas-fir seedlings in Lincoln University Nursery (12 July 2018) and ArborGen Edendale Nursery (middle row, 14 December 2018). The Lincoln University Nursery seedlings had growth parameters measured on 19 September 2018 and the Edendale Nursery seedlings will be established in plantation trials in winter 2019.

Results:

Root colonisation (%) was very high (over half the root pieces tested) in the three seedlots eleven months after seeding and *Trichoderma* inoculation in the 2017 Lincoln University Nursery trial (Figure 16). The untreated control seedlings had relatively low (0 to 12%) colonisation from environmental sources (e.g. from the potting mix and/or airborne spores in the nursery).

Seedling emergence and survival rates were at expected levels for the seedlots, with Washington being greater than Oregon and California (Table 8). Other growth parameters were not significantly affected by the seedlot.

Trichoderma treatments had no effect on seedling emergence, but at harvest, survival was 10 and 13% higher ($P<0.05$) in the T1 treatment, compared to the T2 treatment and untreated control respectively (Table 8). Both *Trichoderma* treatments had large impacts on growth parameters, with highly significantly ($P<0.001$) 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 17).

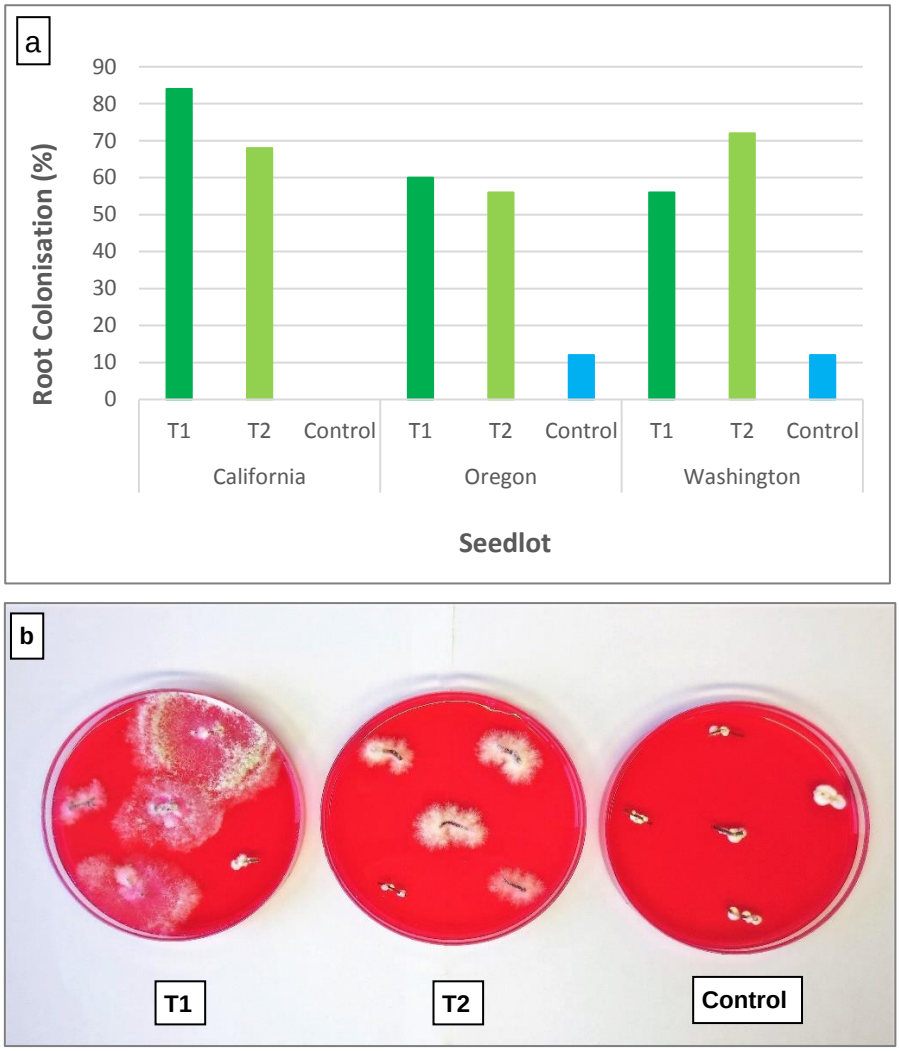

Figure 16: Root colonisation (%) eleven months after seeding and *Trichoderma* application for T1 (FCC320, FCC327 and LU633 mixture), T2 (PR3a mixture) and untreated Control treatments, based on MRB plating data**.** Examples of *Trichoderma* isolation MRB plates for the California seedlot treatment (b). Non-*Trichoderma* colony growths were generally *Penicillium* species.

Figure 17: Oregon Douglas-fir seedlings at harvest for T1 (FCC320, FCC327 and LU633 mixture), T2 (PR3a mixture) and untreated Control treatments.

Table 8: 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 (***)
Seedlot:							
Washington	87.0 a	85.2 a	2.37 a	17.4 a	1.15 a	1.02 a	2.27 a
Oregon	82.3 b	80.5 a	2.36 a	17.1 a	1.14 a	1.10 a	2.13 a
California	65.8 c	61.7 b	2.41 a	17.1 a	1.17 a	1.10 a	2.24 a
LSD (5%)	2.7 (*)	7.2 (*)	0.13 (NS)	1.5 (NS)	0.17 (NS)	0.14 (NS)	0.27 (NS)
LSD (0.1%)	4.8 (***)	12.6 (***)					

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 between treatments or seedlot.

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

New 2018 Containerised Nursery Trial:

A second containerised nursery trial investigating the effect of a larger number of beneficial *Trichoderma* root endophytes on seedling growth in Douglas-fir was assessed at the Lincoln University Nursery, Canterbury.

Treatments included:

Two seedlots:

1. 12/663 with California provenance (generally low tolerance)
2. 12/662 with Oregon provenance (generally high tolerance)

Eight *Trichoderma* treatments:

1. *Trichoderma* general mixture FCC320, FCC327 and LU633
2. *Trichoderma* PR3a (isolates FCC13, FCC14, FCC15 and FCC180)
3. *Trichoderma* PR6 (isolates FCC55, FCC318, FCC327, FCC340)
4. *Trichoderma* modified ArborGuard (isolates LU655, LU659, LU660, LU661, LU663)
5. *Trichoderma* FCC327
6. *Trichoderma* PBI (isolates LU132, LU140, LU584 and LU633),
7. *Trichoderma* Douglas-fir A (*T. atroviride* strain CIB T93, assession number EU279997.1)
8. *Trichoderma* Douglas-fir B (*T. polysporum* strain BMCC: LU1359, assession number KJ871225.1)
9. Untreated Control (- *Trichoderma*)

Seed source, stratification, sowing and establishment methods were similar to those described for the 2017 Douglas-fir Lincoln University Nursery trial (page 21). Seed germination was measured at 65 and

89% for the California and Oregon seedlots respectively. Seed was sown on 27 September 2018 in plastic trays with no 50% vermiculite/potting mix topping (Figure 18). Eight replicate trays were used in the trial. Emergence levels were low in the California trays and this treatment may be removed from the analysis.

Figure 18: Inoculated Douglas-fir seedlings in Lincoln University Nursery on 10 October 2018.

Glendhu Forest Isolates: Two *Trichoderma* isolates cultured from strongly growing Douglas-fir trees were included in the 2018 trial. Roots from nine, healthy, strongly growing trees were sampled in 4-, 8- and 34-year-old stands in Rayonier Matariki Forests Glendhu Forest (-45.867803, 169.700868), South Otago (Figure 19a). *Trichoderm*a isolates were isolated and purified according to established protocols (Hill, *et al.,* 2017). Two isolates with rapid mycelial growth and profuse sporulation on MYE agar were selected and the species identified with DNA analyses of partial *tef1*α gene sequences and BLAST analysis for species affiliation (Figure 19 b and c; Hill *et al*., 2017).

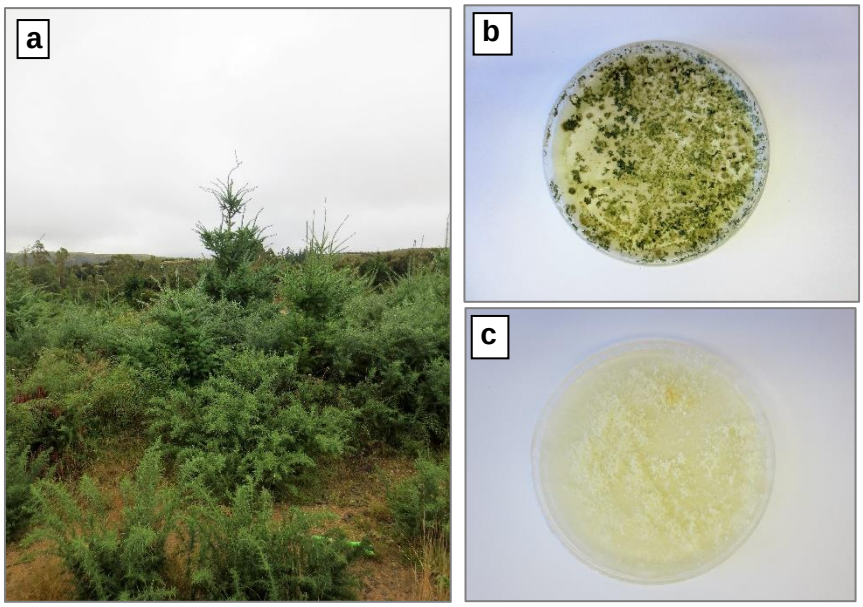

Figure 19: A strongly growing Douglas-fir trees sampled in Rayonier Matariki Forests Glendhu Forest, South Otago (a) and *Trichoderma* isolates *T. atroviride* strain CIB T93 (b) and *T. polysporum* BMCC: LU1359 (c) growing on MYE plates.

Future Measurements:

1. Root *Trichoderma* colonisation in June 2019
2. Survival, root collar diameter, height and root and shoot dry weight at harvest.

ACKNOWLEDGEMENTS

The following people are warmly thanked for their assistance in establishing trials, obtaining samples or providing source material:

- Claire and Terry Gavin
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- Nigel Heron, Timberlands Te Ngae Nursery
- Mark Ryan, ArborGen
- Shaf van Ballekom, Proseed New Zealand Ltd.

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APPENDICES:

APPENDIX A:

Malt Yeast Extract Agar with Rose Bengal (MRB) Recipe:

Malt extract	10 g
Yeast extract	1 g
Rose Bengal (50 mg/mL)	3 ml
Terrachlor 75WP	0.2 g
Agar	20 g
Chloramphenicol stock solution (100 mg/ml)	1 ml

Make up to 1 L with distilled water.

APPENDIX B:

AgZyme:

0.02%	Boron
0.0005%	Cobalt
0.10%	Iron
0.0005%	Manganese
0.05%	Zinc
<1.00%	Thiamine
<1.00%	Riboflavin
<1.00%	Carrageenan
<1.00%	Kelp Extract
<1.00%	Bromelain
<1.00%	Papain
<1.00%	Sucrose

The chelating agent is EDTA (ethyleneduaminetetraaceic acd) and HEDTA (hyroxyethylenediaminetetraacetic acid).

Ag Concepts Super Hume:

6%	Humic acids, derived from Leonardite
0.6%	Kelp Extract and organic acids
93.4%	Inert Ingredients