

Enhancing *Pinus radiata* health and vigour using beneficial microbes and natural products

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1. Background

Fungal diseases cost New Zealand forestry over \$100M each year, mainly as a consequence of reduced growth but also from tree mortality. Economic losses are greatest for the three diseases *Dothistroma*, *Cyclaneusma* and *Armillaria*. In recent years *Nectria* flute canker has also increased in importance. There are also significant economic losses associated with bark beetles, and our forests are under constant threat from new pest and disease incursions.

The Bioprotection Research Centre and AgResearch have been investigating two beneficial fungi, *Trichoderma* spp. and *Beauveria* spp. as potentially useful in New Zealand forestry. *Trichoderma* contains strains and species active against fungi that cause plant and tree diseases. In addition *Trichoderma* isolates have been shown to induce the plants defense systems and enhance growth, vigour and rooting. Previous research on *Trichoderma* has led to products used to reduce plant disease impacts in New Zealand, including ArborGuard™, which is active against *Armillaria* disease in forest plantations. *Beauveria* is a genus of insect-killing fungi and previous research has shown that two species (*B. bassiana* and *B. caledonica*) are pathogenic to bark beetles in New Zealand forests (Reay et al. 2008).

A previous FBRC funded project and a related FRST project have demonstrated compatibility of the two fungi suggesting they could be applied as dual inocula in forestry nurseries. Methods for coating the fungi onto pines were also developed under a previous FBRC contract.

A prototype seed coating kit, suitable for coating *P. radiata* seed with selected beneficial microbes by forest nurseries, has been developed and tested. An effective seed coating procedure was established by mixing seeds with the microbe formulation and inexpensive and harmless PVA (interior) adhesive using either a concrete (10 kg seeds) or hand operated (1-5 kg seeds) mixer (please refer to last year's report for further detail).

In this new programme we will evaluate performance of dual inoculations of the fungi (in strain pairs) in the presence of both insect and plant diseases. The aim is to utilize developed technology which can be applied in nurseries (as was the goal of the previous project) and demonstrate the value in terms of plant growth of the use of these beneficial microbes.

2. Improved *Trichoderma* formulations for forest nurseries

2.1 PF Olsen Seedling Trial May 2011

In 2004, an application rate trial was carried out to assess the optimum inoculum concentration for ArborGuard™. Three different ArborGuard™ concentrations were tested with the results showing an inverse relationship between inoculum concentration and plant growth promotion. The lowest (best) concentration was then used as the basis for the recommended seed coat and spray application rates.

However, this inverse relationship has not been explored further. For this reason, another application rate trial was set up at the PF Olsen nursery in October 2010 to evaluate the effects of even lower inoculum concentrations of the ArborGuard™ formulation on *P. radiata* seedling growth. The trial comprised 21,000 seedlings.

Previous greenhouse trials showed enhanced growth and survival of *P. radiata* seedlings after inoculation with a mixture of *Trichoderma* isolates designated as WT3. In addition, there are two *Trichoderma* mixtures, PBI and IT3, that emerged from *P. radiata* cutting trials. All three mixtures, WT3, PBI and IT3, were therefore included in the PF Olsen seedling trial to verify their beneficial effects on *P. radiata* seedlings. Mortality assessments are shown in **Figure 2.1**. For trial design, layout and protocol, see **Appendix 1**.

Main outcomes:

- The PBI mixture reduced mortality by 50% (from 12% [Control] to 6%)
- WT3 and IT3 showed indications to reduce mortality by 27% and 29%, respectively (statistical differences could not be detected)
- Comparing three different ArborGuard (AG) concentrations, the lowest concentration (10% of standard application) gave the best result > reduction of 37% compared with the untreated control
- No treatment effects were observed on stem diameter

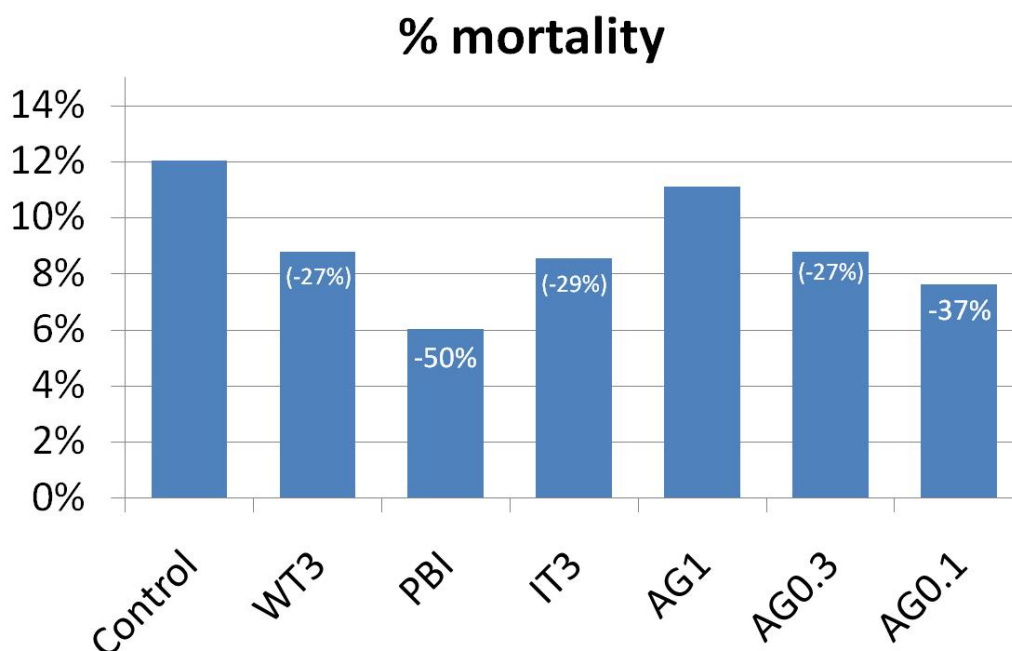


Figure 2.1 PF Olsen seedling trial - May 2011.

3. Enhancing health and vigour of *P. radiata* cuttings

3.1 Commercial validation trials using *P. radiata* cuttings

Last year's first indications of improved cutting survival in commercial pine nurseries were validated with increased replication at the PF Olsen, Te Ngae and Rangiora nurseries totalling ~140,000 cuttings. Treatments included the best *Trichoderma* isolates and the natural product, Fulvic acid. In addition, effective timing of application and combination treatments were under investigation. The main results of the PF Olsen and Te Ngae cutting trials are shown in **Figure 3.1 – 3.3**. The Rangiora cutting trial assessment was postponed to end of July. For trial design, layout and protocol, see **Appendix 2**.

Main outcomes - PF Olsen cutting trial – May 2011 (containerised)

- Fulvic acid (FA) reduced mortality by 52% (from 19% [Control] to 9.3%)
- WT3 and LU996 showed indications to reduce mortality by 38% and 43%, respectively (statistical differences could not be detected)
- Later applications of Trichoderma or fulvic acid treatments (2 and 4 months after setting) gave no improvements
- No treatment effects were observed on stem diameter

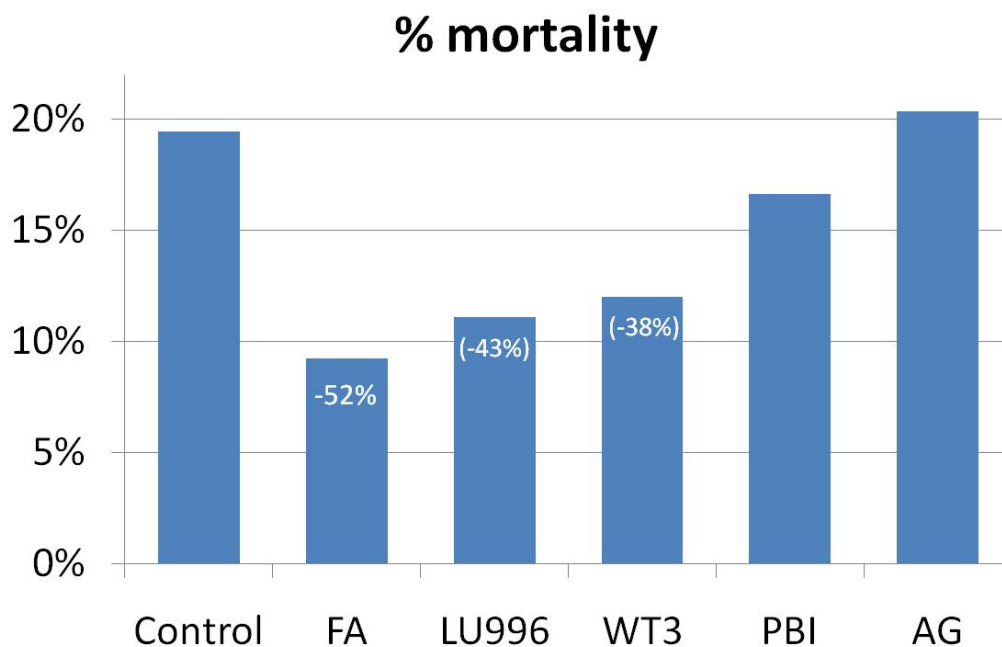


Figure 3.1 PF Olsen cutting trial - May 2011.

Main outcomes – Te Ngae cutting trial – May 2011 (soil bed)

- Fulvic acid reduced mortality by 75% (from 23% [Control] to 5.7%)
 - Fulvic acid increased stem diameter by 9.3%, but reduced root biomass by 15% (and showed indications to also reduce shoot biomass by 9% [not statistically significant])
 - Trichoderma treatments were applied 4 months after setting and gave no improvements on stem diameter and survival
 - However, PBI and LU996 increased root biomass both by 17%, and increased the root:shoot ratio by 14% and 15%, respectively
- It needs to be tested whether the root-promoting mixture PBI (or the isolate LU996) can compensate for the reduction in root biomass when treated in combination with fulvic acid
- Assessments at the Rangiora Nursery are in progress and will be reported when completed

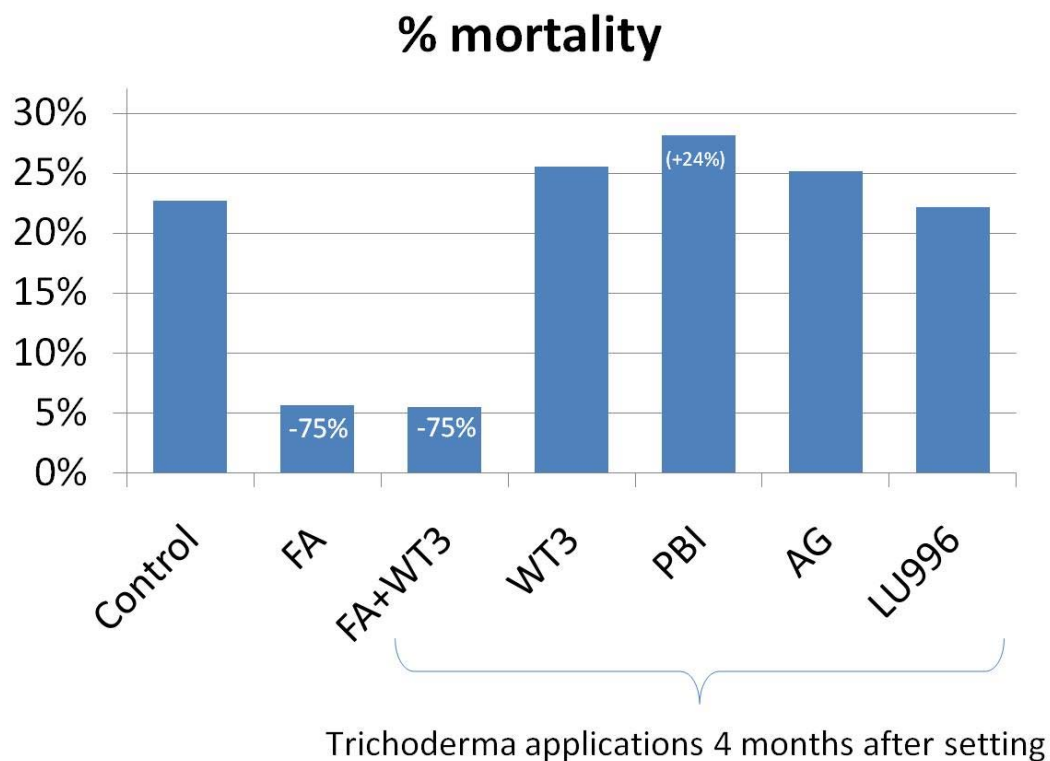


Figure 3.2 Te Ngae cutting trial - May 2011

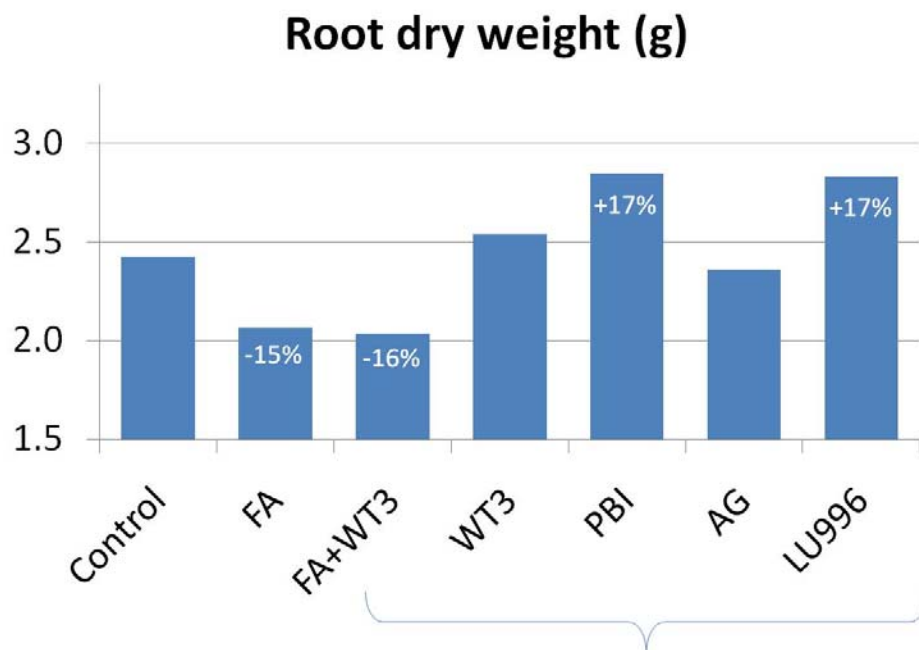
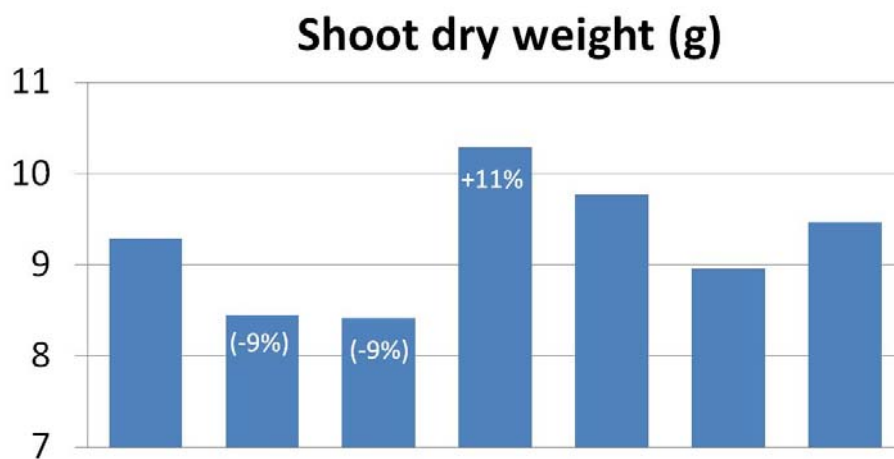
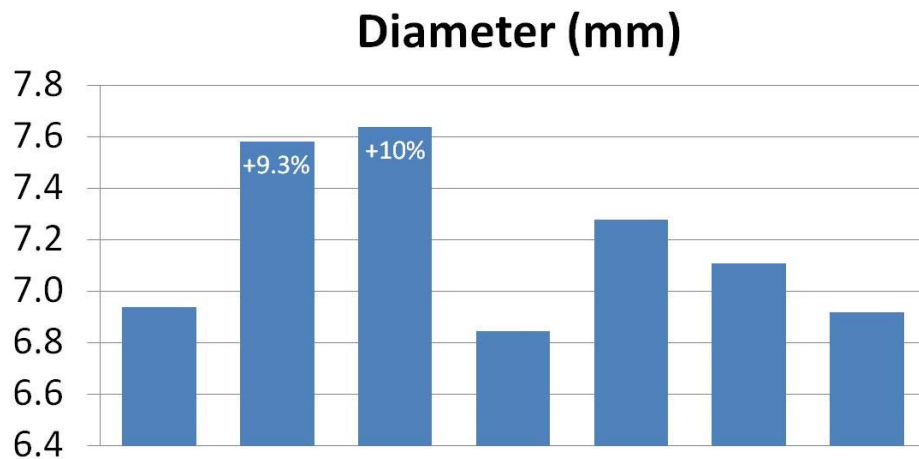


Figure 3.3 Te Ngae cutting trial - May 2011

4. Beneficial entomopathogenic fungi

4.1 Summary

To effectively utilize entomopathogenic fungi as agents in bioprotection strategies for bark beetle populations, cost-effective delivery systems are needed.

In addition to active directly against insect species, *Beauveria bassiana* is a recognised endophyte that has been successfully introduced into a diverse range of plant species, and in several instances colonisation of plant tissues by the fungus has provided protection against insect damage, or has inhibited insect establishment and development.

Survival of *B. bassiana* in seed coating adhesives has been previously assessed and two superior adhesives have been identified. Delivery of this biocontrol agent to the seedling root zone of radiata pine via seed coatings would be simple to implement by the forestry industry.

This project had the following objectives:

- to isolate and assess indigenous isolates of endophytic *B. bassiana* by screening for insecticidal activity
- to use molecular techniques to characterise these endophytic isolates
- to use seed coating and root dipping techniques to deliver the fungus to the soil rhizosphere
- to determine whether seed coating and root dipping techniques could produce endophytic establishment of *B. bassiana* in the pine seedlings

Indigenous isolates of endophytic *B. bassiana* were recovered from NZ pines in early 2009. Insect activity was confirmed by bioassay against *Hylastes ater* and *Hylurgus ligniperda*, and phylogenetic relationships with other *Beauveria* spp. was determined. A paper detailing the recovery, insecticidal activity and molecular characterisation has been published in Biological Control (Reay et al, 2010).

A pot trial was set up May/June 2009 that inoculated pine seedlings with two promising isolates of *B. bassiana* recovered from the pine endophyte survey, using seed coating and root dipping techniques. Destructive sampling took place at 2, 4 and 9 months, using needle and root assays to confirm endophytic colonisation of the seedlings, and soil plating to assess establishment in the potting mix.

Results to date show that using methylcellulose as the adhesive either via root dipping or seed coating has produced some endophytic activity in treatments using isolate F647, with root colonisation observed at all three sampling dates. Soil colonisation for both isolates and all treatments in the root zone was evident at 2 months but tapered off at 4 months, and was barely detectable at 9 months.

This project had the following milestone objectives:

1. Isolation, insecticidal activity and molecular characterization of indigenous endophytic *Beauveria*
2. Colonisation of radiata pine by indigenous endophytic *Beauveria*.



Page 6 **Figure 4.1** Root from *Pinus radiata* colonised by *Beauveria bassiana* on agar medium

4.2 Methods

4.2.1 Isolation, insecticidal activity and molecular characterisation of indigenous endophytic *Beauveria*

Isolation

Asymptomatic foliage, seeds and roots were sampled from a range of trees and stand types over a year from February 2008 to January 2009. A total of 132 trees from the genus *Pinus* were sampled, of which the majority (125) were *Pinus radiata*. Branches were sampled from the lower canopy of trees, and wherever possible these were second or third year needles as these have been found to contain a greater proportion of fungal endophytes (Johnson and Whitney, 1992). Four root samples and one seed sample were also collected.

Needle, seed and root samples were surface sterilised by soaking in ethanol (96%) for 1 minute, followed by 10% sodium hypochlorite (NaOCl) for 5 minutes, before two 1 min rinses in sterile distilled water. Following sterilisation each needle or root was cut into three sections, while each seed was cut into thin (1mm) sections. Samples were placed onto antibiotic-mended PDA and incubated at 20°C for 10-14 days. Developing colonies were then sub-cultured onto new PDA plates and incubated at 20°C until sporulation occurred. Morphological characterisation under a dissecting microscope was used to confirm the identity of *Beauveria* spp.

Insecticidal activity

To confirm entomopathogenicity of the isolated *Beauveria* spp, laboratory bioassays were carried out using field-collected *Hylurgus ligniperda* adults and commercially supplied *Tenebrio molitor* (Coleoptera) larvae. A total of 21 *Beauveria* isolates were selected and tested against insects, using representative isolates from a range of geographic locations from where needle, seed and root samples were collected. Conidial suspensions were prepared by harvesting conidia into 0.01% Triton X-100, and conidial viability was confirmed by using a 20 hour germination test based on germ tube formation. For the *H. ligniperda* assays, batches of 12 beetles were inoculated by placement in 5mL of a 5×10^7 conidia/mL suspension and agitating for 10 seconds. The beetles were then transferred to individual wells in a 24-well cell culture plate (BD Falcon™) lined with 1cm x 1cm squares of filter paper. Control beetles were immersed in 0.01% Triton X-100 only. For each *Beauveria* isolate, 3 replicates of 12 beetles were treated but the experiment was only replicated once due to limited beetle availability. For *T. molitor*, bioassays were performed by applying 0.5mL of a 5×10^6 conidia/mL suspension to 55mm diameter filter paper in plastic dishes (55mm) with snap-top lids. Control dishes were treated with 0.5mL of 0.01% Triton X-100. Batches of 5 larvae were tested using 5 replicate containers per *Beauveria* isolate. The bioassays were replicated three times.

All bioassays were incubated at 20°C in enclosed plastic bags containing moistened paper towels to maintain high humidity. Data (live/dead) was collected at 2 day intervals for 14 days and at each observation any *T. molitor* cadavers were removed from 6 communal dishes to prevent cross-contamination of survivors and placed in petri dishes on damp filter paper. *H. ligniperda* cadavers were left in their individual wells. Cadavers were monitored for infection, which was considered to have occurred when fungal outgrowth was observed from joints in the exoskeleton, indicating internal origin. Cadavers were held until conidiation occurred, to confirm infection by *B. bassiana*.

Molecular characterisation

DNA was extracted from fungal hyphae and amplification of a terminal region of the elongation factor 1- α (EF1- α) locus was performed. PCR products were sequenced (Canterbury

Sequencing and Genotyping Facility, University of Canterbury, New Zealand) and all sequences have been deposited in GenBank.

4.2.2 Endophytic establishment of *Beauveria bassiana* in *Pinus radiata*

A pot trial was set up May/June 2009 inoculating pine seedlings with two promising isolates of *B.bassiana* (F647 and F668) recovered from the pine survey, using seed coating and root dipping techniques (Posada and Vega, 2005). Two coating adhesives, methylcellulose and Xanthum gum, were selected based on results from previous seed-coating trials (Brownbridge et al, 2009).

Fungi

Beauveria bassiana isolates F647 and F668 were used throughout. Both are pathogenic to bark beetles and were selected as representative isolates of indigenous endophytic *Beauveria*. To prepare the coatings, conidia were harvested from 12-day old cultures grown on PDA overlain with clear cellophane; this system allows easy removal of conidia by peeling the cellophane off the surface of the medium. Suspensions containing ca. 10^8 conidia/mL 0.01% Triton X-100 were prepared for incorporation into the coating matrix.

Seed coating

P. radiata seeds were treated by soaking in 99% ethanol for 1min before being sterilised in 10% NaOH for 5mins. Following this they were rinsed twice in sterile distilled water for 1min. Seeds were then soaked in sterile distilled water for 24 hours at 4°C. Sets of 10 seeds were placed on sterile filter paper in a 55mm Petri dish, and allowed to air dry prior to treatment. Seeds were coated with conidia suspensions mixed with either xanthum (0.2%) or methylcellulose (2%) applied via a spray gun. After coating, seeds were dried and spore loadings on the seeds were determined after preparation by cfu counts. For each coating and fungal isolate, ten seeds were placed into 10 mL sterile 0.01% Triton X-100 in a sterile 20 mL plastic tube. Two replicate tubes were prepared for each seed coating. Seeds were allowed to soak for 30 minutes to allow the coatings to re-hydrate before shaking the tubes on a wrist shaker set at maximum for ten minutes. Serial dilutions were prepared and 100µL aliquots plated on to antibiotic-amended quarter-strength PDA medium. Two duplicate plates were prepared for each dilution. Plates were incubated at 20°C and cfus counted after 10 days.

Root coating

Seeds were surface sterilised as per the seed coating, but then placed on to damp filter paper held in petri dishes and incubated at room temperature in the dark by placing in a black plastic bag for 2-3 weeks until they germinated. When the radicle was 2-3cm long seed was dipped in the coating (Fig 4.2) prepared by adding 5 mL of spore suspension at 2.9×10^8 conidia/mL to 20ml of either xanthum (0.2%) or methylcellulose (2%) coating to obtain a final suspension of 5×10^7 conidia/mL.



Planting

Coated seeds were planted in a bark-based potting mix in multi-cell plant trays and held in a greenhouse at ~15°C in the dark until seeds began to germinate. Once germinated, seedlings were allowed to grow for 2-4 weeks before being transplanted into 6-cell plant trays for remainder of the trial. Coated seed radicles were planted directly into 6-cell plant trays. All seedlings were left to grow in the greenhouse for the duration of the trial, temperatures ranged from 15°C in July when sowing took place through to 25°C in summer when the 9 month sampling took place.

Establishment of *Beauveria* in soil root zone

At each sampling time a sample of soil was taken from immediately around the root zone of each seedling. A 1g sub-sample of this soil was placed into 9 mL of 0.01% Triton X-100 and 100µL aliquots were plated on to antibiotic-amended quarter-strength PDA medium. Plates were incubated at 20°C and *Beauveria* cfus counted after 10 days.

4.3 Results and Discussion

4.3.1 Isolation of endophytic *Beauveria*

Pure cultures from a total of 21 fungal colonies morphologically resembling *Beauveria* spp. were isolated from source material. Colonies were considered to have originated from source material only if they grew (Table 1) from the cut end of needles or roots, or the cut surface of seeds.

Table 4.1 *Beauveria* isolates, host/substrate and collection site

Isolate	Origin of isolate
F641	Needles, Single large <i>P. radiata</i> Mt Thomas, Canterbury, NZ.
F643	Needles, 15yr approx. small block, Mt Thomas, Canterbury, NZ.
F646	Needles, <i>Larix</i> sp. Mt Thomas, Canterbury, NZ.
F647	Needles, single <i>P. radiata</i> , 25 yr approx, Staverly, South Canterbury, NZ.
F648	Needles, single <i>P. radiata</i> , 15 yr approx, Geraldine, South Canterbury, NZ.
F668	Needles, single mature <i>P. radiata</i> , Te Hana, Auckland, NZ.
E83	Needles, unpruned mature <i>P. radiata</i> , Pinedale Forest, Central North Island, NZ.
E84	Needles, unpruned <i>P. radiata</i> sapling, Pinedale Forest, Central North Island, NZ.
E85	Needles, unpruned mature <i>P. radiata</i> , Pinedale Forest, Central North Island, NZ.
E87	Needles, unpruned <i>P. radiata</i> , Pinedale Forest, Central North Island, NZ.
E89	Needles, unpruned mature <i>P. radiata</i> , Pinedale Forest, Central North Island, NZ.
E94	Needles, unpruned mature <i>P. radiata</i> , Pinedale Forest, Central North Island, NZ.
E97	Needles, unpruned mature <i>P. radiata</i> , Pindale Forest, Central North Island, NZ.
E106	Needles, <i>P. radiata</i> seedling, Pinedale Forest, Central North Island, NZ.
E107	Needles, <i>P. radiata</i> seedling, Pindale Forest, Central North Island, NZ.
E110	Needles, <i>P. radiata</i> seedling, Pindale Forest, Central North Island, NZ.
E112	Needles, <i>P. radiata</i> seedling, Pindale Forest, Central North Island, NZ.
E115	Needles, <i>P. radiata</i> seedling, Pindale Forest, Central North Island, NZ.
E119A	Roots, <i>P. radiata</i> seedling, Pindale Forest, Central North Island, NZ.
E134A	Seeds, mature single <i>P. radiata</i> , Putaruru, Central North Island, NZ.

Table 4.2 Mean percent mortality of *Tenebrio molitor* larvae and *Hylurgus ligniperda* adults 10 days after treatment with selected *Beauveria bassiana* isolates.

Treatment ¹	<i>T. molitor</i> mortality (%)	<i>H. ligniperda</i> mortality (%)
Control	38	8
E106	100	92
E107	100	100
E110	100	100
E112	100	100
E115	100	100
E119A	100	100
E134A	100	100
F647	100	100
E83	100	92
E84	100	100
E87	100	100
E89	100	100
E94	100	67
E97	100	100
LSD	8	n/a

1 Insects treated with *B. bassiana* conidia suspended in 0.01% Triton X-100 at 5×10^6 conidia/mL

4.3.2 Insecticidal activity

Pathogenicity of strains was confirmed in bioassays against both *H. ligniperda* adults and *T. molitor* larvae. All 15 isolates recovered from pines were pathogenic to both test organisms (Table 2).

4.3.3 Molecular characterisation

Molecular characterisation of all the *Beauveria* fungi isolated by PCR amplification and sequence analysis of the EF1- α gene classified them as *B. bassiana* Clade A in the scheme proposed by Rehner and Buckley (2005). The majority of isolates were recovered from *P. radiata* needles, with just one seed-isolate (E134A) and one root-isolate (E119A) found. *Beauveria bassiana* Clade A was the only species to be detected as an endophyte. Isolate F624, recovered by Ganley and Newcombe (2006) from *Pinus monticola* in North America, was included as a comparison with PCR analysis confirming that this isolate also classified as *B. bassiana* Clade A.(Reay et al, 2010).

4.3.4 Endophytic establishment of *Beauveria bassiana* in *Pinus radiata*

Methylcellulose used as a root coating proved to be the most effective means of introducing *B. bassiana* as an endophyte into pine seedlings. *Beauveria* was recovered from both needles and roots of seedlings treated with isolate F647 nine months post-inoculation (Fig 4.3). Xanthum gum gave a comparable result to methylcellulose for *Beauveria* recovery from needles in root-dipped seedlings, but there was no recovery from roots after 9 months. When used as a seed coating, again methylcellulose was more effective than xanthum when using isolate F647 (Fig 4.4). Establishment of the fungus in roots was found in three of the five sampled seedlings after two months growth, although this dropped away to just one in five after nine months. However, needle establishment was only observed in one plant, and that was at the four month sampling period.

Fig 4.3 *Beauveria* recovered from needles and roots after root dipping using F647

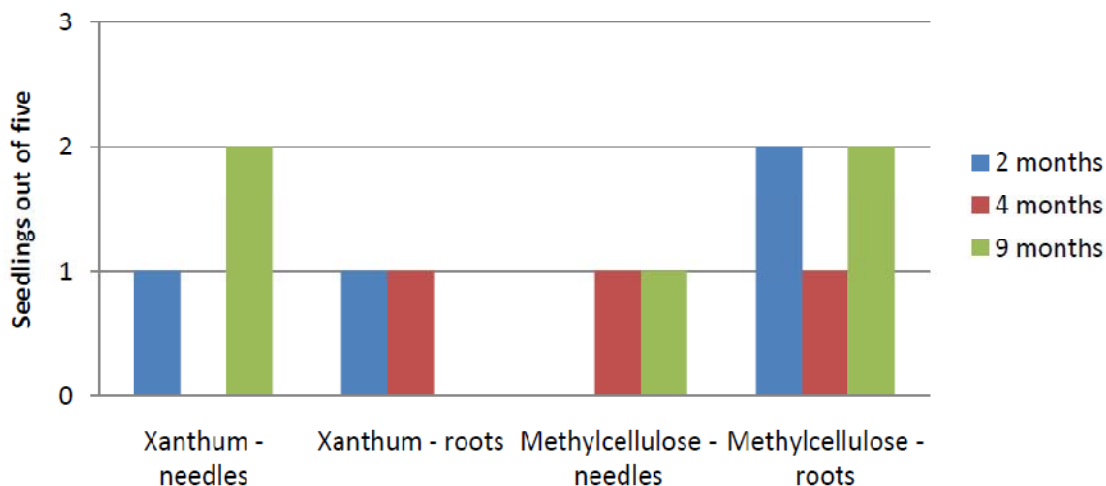
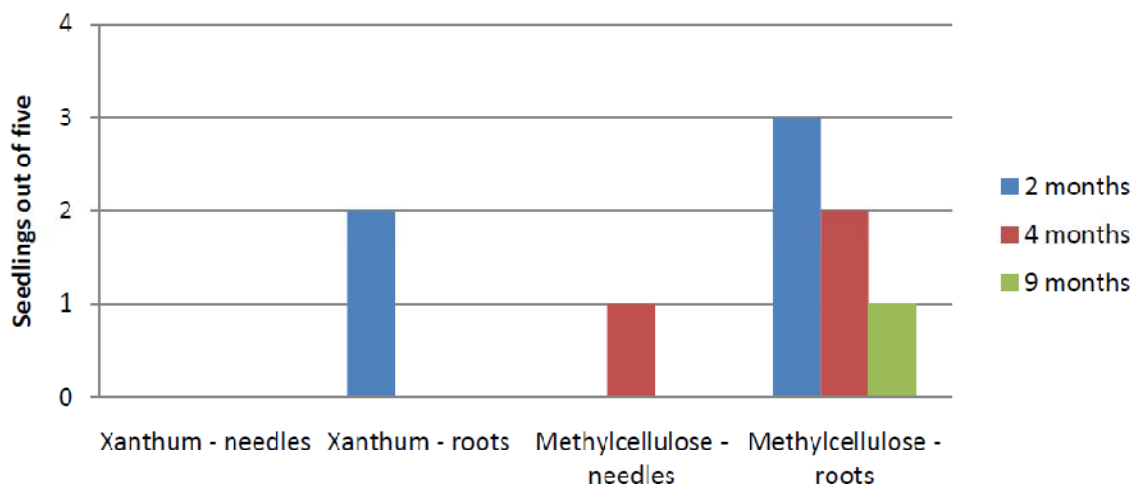
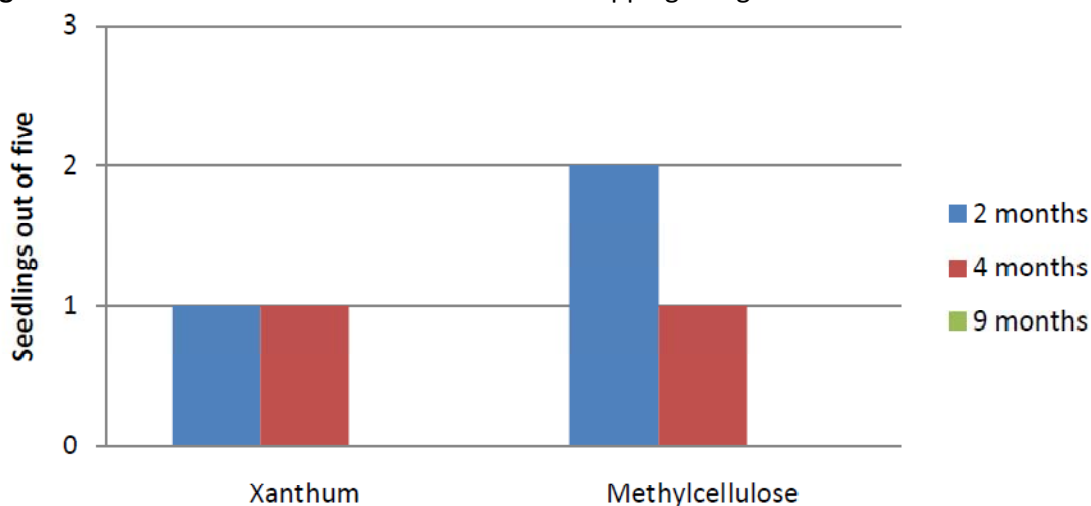


Fig 4.4 *Beauveria* recovered from needles and roots after seed coating using F647



Isolate F647 had greater endophytic activity than isolate F668. There was recovery from needles and roots using methylcellulose as either a root dip or seed coating, and xanthum as a root dip. F668 was only recovered from roots of seedlings treated by root dipping at the two and four month sampling periods (Fig 4.5), using both xanthum and methylcellulose. There was no recovery from needles or roots in seedlings treated by seed coating with F668.

Fig 4.5 *Beauveria* recovered from roots after root dipping using F668



4.3.5 Establishment of *Beauveria* in soil

Beauveria was recovered from soil at 10^3 cfus per gram for all treatments at the two and four month samplings, except for the xanthum root dipped treatment using F668. But by nine months there was no recovery from soil for any of the seed coating treatments, and just trace amounts in three planting bags for root-dipped F647 treatments (Figs 4.6 and 4.7).

Fig 4.6 Conidia cfus per gram of soil in root zone for root dip treatments

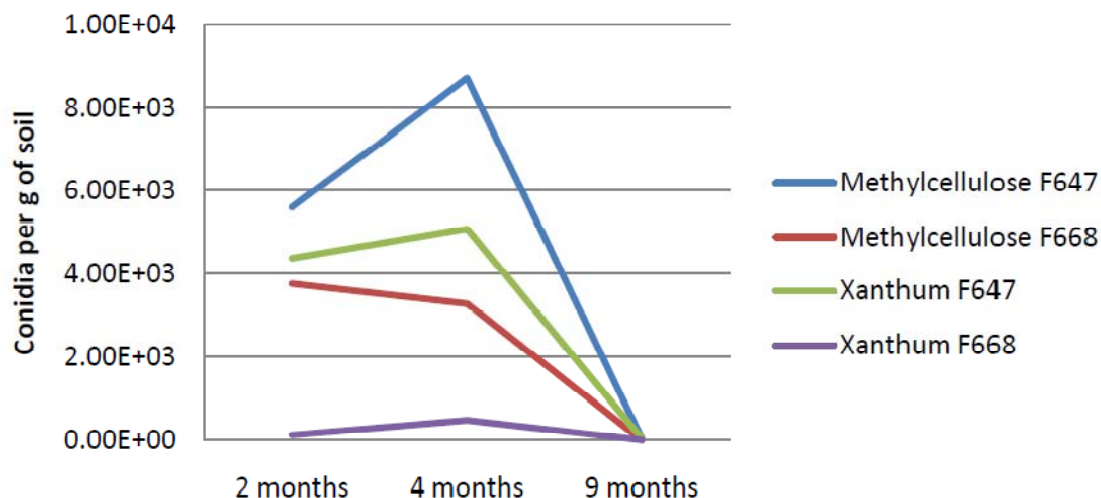
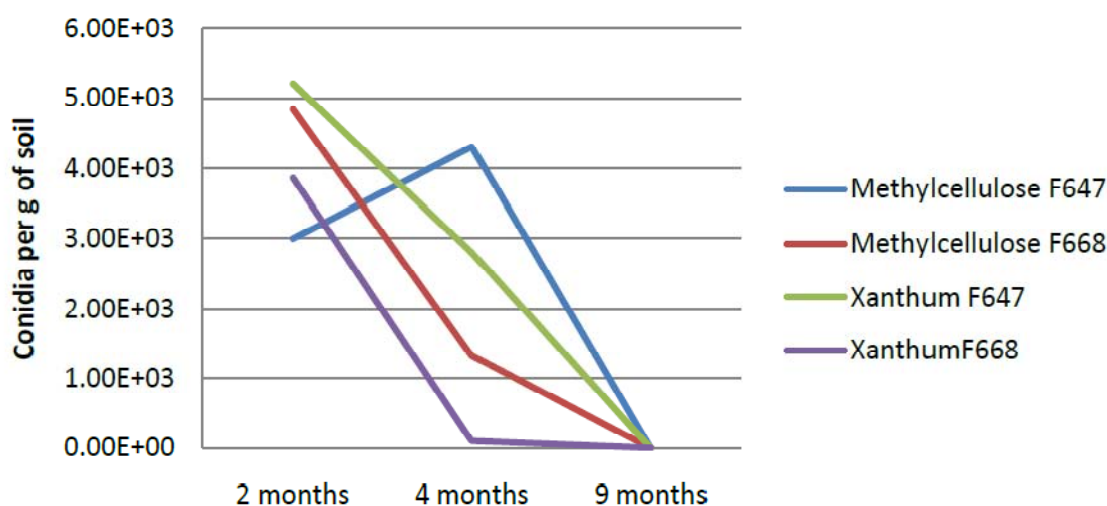


Fig 4.7 Conidia cfus per gram of soil in root zone for seed coated treatments



One possible reason for the loss could be leaching of conidia from the bags with increased watering required over the five to nine month period that coincided with the warmer months of November through March. The other alternative is that these two isolates are endophytic in nature, so that long term survival in soil is not a naturally occurring state for the conidia. The three seedlings that did still have *Beauveria* present in soil from the root zone after nine months were all root-dipped with F647, and two of these seedlings also had the fungus present in the needles.

RECOMMENDATIONS

- Superior coating materials that preserve the viability of fungal conidia on seeds and seed radicles to deliver conidia to the soil root zone have been identified; their ability to deliver more than one microbe to provide a cocktail of beneficial fungi to the pine seedling root zone should now be investigated.
- Delivery of endophytic *Beauveria* to *Pinus radiata* seedlings via seed and root coating with methylcellulose has been demonstrated, but with a less than 50% success rate using standard plating techniques to re-isolate the fungus. Molecular techniques to

detect the *Beauveria* in plant tissue should be investigated to determine whether they might provide a higher detection rate or verify the success rate of endophyte infection.

- The potential use of these coatings for application of *Trichoderma* spores needs to be tested, with the ultimate goal of co-inoculating pine seeds using this technology to provide both protection against

5. *Trichoderma* bio-protection of *Acacia mangium* in Sarawak, Borneo

Trichoderma application and production systems have been established at the Samarakan Nursery, Malaysia. A single application of *Trichoderma* has now replaced all fungicides as the new standard nursery practice. In order to validate long-term benefits of *Trichoderma*-treated *Acacia* trees, three pilot-scale plantation trials were set up in 2009 within the Planted Forest Zone. Assessments within the first 18 month after planting out into the forest show reduced mortality of *Trichoderma*-treated trees by on average 30% (**Figure 5.1**). The mortality rates presented do not include non-disease related causes of death such as waterlogging or erosion.

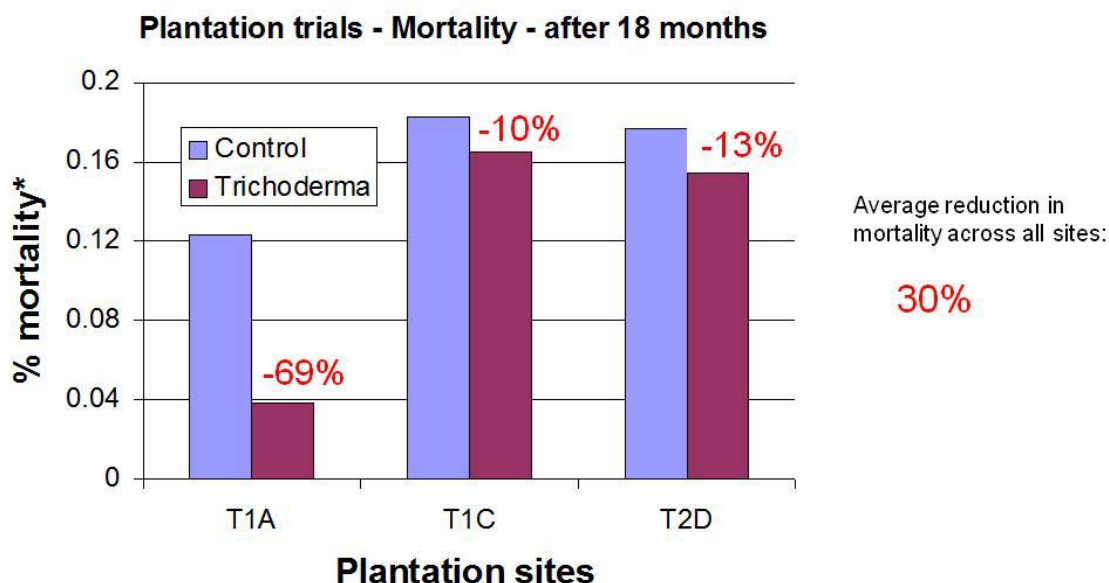


Figure 5.1 Mortality rate assessments at plantation sites T1A, T1C and T2D after 18 months comparing *Trichoderma*-treated *Acacia* trees with the untreated control.

The next assessments of these sites will be conducted in August 2011.



***Acacia mangium* seedlings at the Samarakan Nursery, Sarawak**
All *Trichoderma* – inoculated
Now standard practice replacing fungicide sprays





***Acacia mangium* plantation site E2E with *Trichoderma* 12 months canopy closed minimal weeds**



***Acacia mangium* plantation site E2E with no *Trichoderma* 12 months thin canopy note weed growth**

A new *Trichoderma* bioprotection project in Sumatra, Indonesia, is giving similar results to those obtained in Sarawak

6. Publications, outputs and awards

- Hohmann P, Jones EE, Hill RA, Stewart A (2011). Understanding *Trichoderma* in the root system of *Pinus radiata*: Associations between rhizosphere colonisation and grow promotions for commercially grown seedlings. *Fungal Biology* (in press). DOI:10.1016/j.funbio.2011.05.010.
- Hohmann P, Clouston A, Hill RA (2011). Improved *Trichoderma* formulations for forest nurseries. FOA/MAF 9th Annual Forestry Biosecurity workshop.
- Hill RA, (2010) Bayer Innovators Award for Agriculture and Environment in recognition of outstanding achievement in innovation, significant contribution to industry and positive impact on New Zealand and globally from this work.
- Hill RA, Stewart S, Hohmann P, Braithwaite M, Clouston A, Minchin R, Agabayani F (Grand Perfect Sdn Bhd) and Ambrose A, (Sarawak Forestry Corporation) (2010). Enhancing growth and health of *Pinus radiata* in New Zealand and *Acacia mangium* in Malaysia with selected *Trichoderma* isolates.
- McLean K, Dodd S, Stewart A, Hill RA (2010). Non Target impacts of the biocontrol agent *Trichoderma atroviride* on two native ecosystems in New Zealand. 11th International *Trichoderma* and *Gliocladium* workshop: *Trichoderma* – molecular mechanisms and applications of biocontrol in agriculture.
- Hill RA, Ambrose A, Sajali NA, Yatim M, Valdez RB, Agbayani F, Bungang J, Minchin R, Stewart A. Bioprotection of *Acacia mangium* using *Trichoderma* in Malaysia. In: S.M. Zydenbos & T.A. Jackson editor(s). *Microbial Products: Exploiting Microbial Diversity for Sustainable Plant Production*. New Zealand Plant Protection Society Inc; p51-56. 2010
- Stewart A, Brownbridge M, Hill RA, Jackson TA (2010). Utilising soil microbes for biocontrol. In: Soil Microbiology and Sustainable Crop Production. G.R Dixon & E. Tilston (Eds) Springer, Dordrecht. 315-371.
- Clouston AM, Hill RA, Minchin R, Braithwaite M, Stewart A. (2010). A bioassay screening *Trichoderma* isolates for enhancement of root development in *Impatiens walleriana* cuttings. NZ Plant Protection, 63: 33-38
- Ambrose A, Hill RA, Valdez RB, Okang AE, Ahmed N 2010: *Trichoderma*; A biological control tool to improve *Acacia mangium* growth and to control seedling diseases in the nursery. International Symposium on Forestry and Forest Products 2010 (ISFFP), Kuala Lumpur, Malaysia, October 5-7.
- Hill RA, Ambrose A, Okang AE, Valdez RB, Ajang L 2010: Protocol for mass production of *Trichoderma* inoculum at Samarakan. Industry report to Grand Perfect Sdn Bhd, Malaysia, August 24.
- Reglinski T, Rodenburg N, Taylor JT, Northcott GL, Spiers TM, Hill RA 2010: *Trichoderma atroviride* promotes growth and enhances systemic resistance to *Diplodia pinea* in radiata pine (*Pinus radiata*) seedlings. *Forest Pathology* (in press). DOI:10.1111/j.1439-0329.2010.00710.x
- Hill RA, Stewart A, Hohmann P, Braithwaite M, Clouston A, Minchin R 2010: Enhancing growth and health of *Pinus radiata* in New Zealand and *Acacia mangium* in Malaysia with selected *Trichoderma* isolates. 11th International workshop on *Trichoderma* and *Gliocladium*, Haifa, Israel, October 12-15.
- Hill RA, Stewart A, Agbayani F, Bungang J, Okang AE, Ambrose A, Ahmad N 2010: Productivity of

forest nursery in Borneo boosted by indigenous trichodermas. 9th International Mycological Congress, Edinburgh, Scotland, August 1-6.

Reay SD, Brownbridge M, Giquel, B, Cummings, NJ, Nelson TL **2010**. Isolation and characterization of endophytic *Beauveria* spp (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. *Biological Control* 54:52-60.

Hohmann, P. (**2010**). Understanding Trichoderma bio-inoculants in the root ecosystem of *Pinus radiata* seedlings. PhD thesis. Bio-Protection Research Centre, Lincoln University, New Zealand.

Brownbridge M, Reay SD, Nelson, TL, Gicquel, B, Wright, D, Swaminathan, J. **2009**. New Bioprotection Tools for Forestry. Report for Lincoln University Subcontract Agreement A14171 NZ Forest Owners' Association, Client report number SC110/2009/67.

Hohmann, P., Jones, E.E., Hill, R.A., Stewart, A. (**2009**). Understanding Trichoderma bio-inoculants in the root system of *Pinus radiata*. Proceeding of the 17th Biennial Australasian Plant Pathology Society Conference, Newcastle, Australia, September 29 - October 1, p 102.

Hohmann, P., Jones, E.E., Hill, R.A., Stewart, A. (**2009**). Ecological studies of Trichoderma bio-inoculants in the soil ecosystem of *Pinus radiata*. Proceedings of the IUFRO International Forest Biosecurity Conference, Rotorua, New Zealand, March 16-20, p 195.

7. References

Posada, F and Vega, E 2005. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia* 97(6): 1195-1200.

Johnson, JA, Whitney, NJ. 1989. A study of fungal endophytes of needles of balsam fir (*Abies balsamea*) and red spruce (*Picea rubens*) in New Brunswick, Canada, using cultural and electron microscope techniques. *Canadian Journal of Botany* 70: 1754-1757.

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9. Appendices

Appendix 1 - PF Olsen Seedling Trial October 2010

Materials

Isolate inoculum containers (WT3, PBI, IT3)
 Arboguard spore powder
 1 L Fulvic Acid
 Muslin cloth
 3 L measuring cylinder
 4 x pair gloves Large
 2 x 1 L Difco agar bottles
 Large plastic funnel
 Scissors, spatula, paper towels, ethanol

Marker pens, tape, pens, paper,
 notebook, calculator
 Plastic bags for rubbish/general
 Venetian blind labels
 Large tape measure
 1 pack 50 mL Falcon tubes
 3 x 25 (?) L Knapsack sprayer
 4 x large pieces of cardboard for
 Treatment barrier

Method

Sow *P. radiata* seeds in root-pruning containers. Randomised block design with 12 blocks (= 12 replicates) in total (**Figure A1.1**). Treatments applied to 4-tray-plots >> 216 cuttings/plot (4 x 54 cuttings/tray) x 12 blocks = 2592 cuttings/treatment (20736 cuttings in total). Apply treatments to potting mix as per schedule using a knapsack sprayer @ ~7 mL/pot (15 L/treatment) calibrated @ ~1 L/90 s >> ~4 s/row/tray or ~2 min/4 trays. Apply *Trichoderma* treatments 1, 2 and 3 @ 10^7 spores/pot >> knapsack concentration is $\sim 1.4 \times 10^6$ spores/mL or $\sim 3 \times 10^{10}$ spores/20 L. ArborGuard (AG) treatments 4, 5, and 6 @ 5×10^6 spores/pot (AG10 = 10g/10,000 seedlings), 1.5×10^6 spores/pot (AG3) and 5×10^5 spores/pot (AG1) >> 2.9 g of AG (AG10), 1.4 g of AG (AG3) and 0.3 g of AG (AG1) in 20 L.

Treatments:

- | | |
|--|------------------------------------|
| (1) WT3 (3 isolates, from willow trial) | (5) AG0.3 (5 isolates, ArborGuard) |
| (2) PBI (4 isolates, pasture mix) | (6) AG0.1 (5 isolates, ArborGuard) |
| (3) IT3 (3 isolates, from impatiens trial) | (7) Untreated control |
| (4) AG1 (5 isolates, ArborGuard) | (8) Untreated control |

Figure A1.1 Schematic illustration of the experimental design.

	Block I				Block II				Block III				Block IV			
Row 1	5	4	7	2	6	1	3	5	3	7	4	2	4	3	7	2
	8	3	6	1	7	8	2	4	5	6	1	8	6	5	8	1
	Block V				Block VI				Block VII				Block VIII			
Row 2	3	1	8	2	8	3	5	1	6	2	4	5	1	5	8	6
	7	6	5	4	2	6	4	7	3	8	7	1	7	2	3	4
	Block IX				Block X				Block XI				Block XII			
Row 3	4	8	7	3	5	2	6	7	8	1	6	4	6	1	8	2
	2	1	5	6	4	1	8	3	3	2	5	7	5	3	4	7

Appendix 2 – Commercial validation trials using *P. radiata* cuttings

Experimental Design PF Olsen Cutting Trial May 2010

Materials

Isolate inoculum containers (WT3, PBI, IT3)
 Arboguard spore powder
 1 L Fulvic Acid
 Muslin cloth
 3 L measuring cylinder
 4 x pair gloves Large
 2 x 1 L Difco agar bottles
 Large plastic funnel
 Scissors, spatula, paper towels, ethanol

Marker pens, tape, pens, paper,
 notebook, calculator
 Plastic bags for rubbish/general
 Venetian blind labels
 Large tape measure
 1 pack 50 mL Falcon tubes
 2 x 25 L Brewers container
 2 x 15 L Knapsack sprayer
 4 x large pieces of cardboard for
 Treatment barrier

Method

Set *P. radiata* cuttings in root-pruning containers. Randomised block design with 12 blocks (= 12 replicates) in total (**Figure A1.2**). Treatments applied to 4-tray-plots >> 216 cuttings/plot (4 x 54 cuttings/tray) x 12 blocks = 2592 cuttings/treatment (31104 cuttings in total). Apply treatments to foliage and potting mix as per schedule using a knapsack sprayer @ ~7 mL/cutting (15 L/treatment) calibrated @ ~1 L/90 s >> ~4 s/row/tray. Apply *Trichoderma* treatments (1, 2, 3, 4, 6, 7, 8, 9) @ 10^7 spores/cutting >> knapsack concentration is $\sim 1.4 \times 10^6$ spores/mL or 2.6×10^{10} spores/15 L. Apply fulvic acid treatments (5, 8, 9) @ 1:300 which equals ~50 mL/15 L.

Treatments:

- | | |
|---|-----------------------------------|
| (1) WT3 @ setting (3 isolates, from willow trial) | (7) WT3 @ 4 months |
| (2) PBI @ setting (4 isolates, pasture mix) | (8) FA @ setting + WT3 @ 2 months |
| (3) AG @ setting (5 isolates, ArborGuard) | (9) FA @ setting + WT3 @ 4 months |
| (4) LU996 @ setting | (10) Untreated control |
| (5) FA @ setting (fulvic acid) | (11) Untreated control |
| (6) WT3 @ 2 months | (12) Untreated control |

Figure A1.2 Schematic illustration of the experimental design.

	Block I						Block II						Block III						Block IV					
Bed 1	4	8	7	2	12	9	4	6	11	3	12	10	12	8	3	6	7	2	9	11	5	4	7	12
	6	3	1	10	5	11	5	2	9	1	8	7	10	11	1	4	5	9	8	1	2	6	10	3
Bed 2	Block VIII						Block VII						Block VI						Block V					
	6	7	10	2	4	9	7	10	12	2	9	5	7	6	3	12	11	4	9	5	6	7	12	1
	3	11	12	8	5	1	8	4	6	1	11	3	2	8	1	9	5	10	2	4	11	3	8	10
Bed 3	Block IX						Block X						Block XI						Block XII					
	8	11	1	7	9	4	9	5	7	4	8	1	2	11	8	7	5	9	1	10	8	11	3	9
	3	10	6	12	2	5	6	11	12	2	10	3	4	3	10	12	1	6	5	12	2	4	7	6

Experimental Design Rangiora Cutting Trial May 2010

Materials

Isolate inoculum containers (WT3, PBI, IT3)
 1 L Fulvic Acid
 Muslin cloth
 3 L measuring cylinder
 4 x pair gloves Large
 2 x 1 L Difco agar bottles
 Large plastic funnel
 Scissors, spatula, paper towels, ethanol
 Marker pens, tape, pens, paper,
 notebook, calculator

Plastic bags for rubbish/general
 Venetian blind labels
 Large tape measure
 1 pack 50 mL Falcon tubes
 2 x 25 L Brewers container
 2 x 15 L Knapsack sprayer
 4 x large pieces of cardboard for
 Treatment barrier

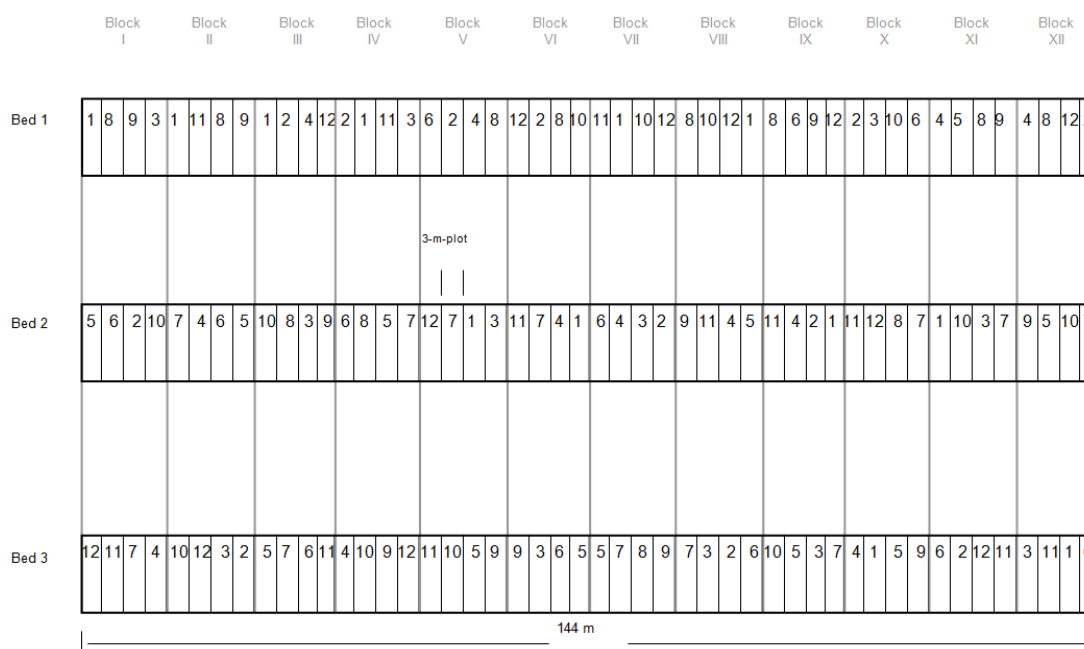
Method

Set bare-root *P. radiata* cuttings in 3 beds of 144 m length each. Randomised block design along the beds with 12 blocks (= 12 replicates) in total (**Figure A1.3**). Treatments applied to 3-m-plots >> 288 cuttings/plot (3 m x 8 rows x 12 cuttings/m) x 12 blocks = 3456 cuttings/treatment (41,472 cuttings in total). Apply treatments to foliage and soil as per schedule using a knapsack sprayer @ ~7 mL/cutting (25 L/treatment) calibrated @ ~1 L/90 s >> ~5 s/row/m. Apply *Trichoderma* treatments (1, 2, 3, 4, 6, 7, 8, 9) @ 10^7 spores/cutting >> knapsack concentration is $\sim 1.4 \times 10^6$ spores/mL or 3.5×10^{10} spores/25 L. Apply fulvic acid treatments (5, 8, 9) @ 1:300 which equals ~83 mL/25 L.

Treatments:

- | | |
|---|-----------------------------------|
| (1) WT3 @ setting (3 isolates, from willow trial) | (7) WT3 @ 6 months |
| (2) PBI @ setting (4 isoaltes, pasture mix) | (8) FA @ setting + WT3 @ 4 months |
| (3) Untreated control | (9) FA @ setting + WT3 @ 6 months |
| (4) LU996 @ setting | (10) Untreated control |
| (5) FA @ setting (fulvic acid) | (11) Untreated control |
| (6) WT3 @ 4 months | (12) Untreated control |

Figure A1.3 Schematic illustration of the experimental design.



Experimental Design Te Ngae Nursery Trial May 2010

Materials

Isolate inoculum containers (WT3, PBI, IT3)
 Arboguard spore powder
 1 L Fulvic Acid
 Muslin cloth
 3 L measuring cylinder
 4 x pair gloves Large
 2 x 1 L Difco agar bottles
 Large plastic funnel
 Scissors, spatula, paper towels, ethanol

Marker pens, tape, pens, paper,
 notebook, calculator
 Plastic bags for rubbish/general
 Venetian blind labels
 Large tape measure
 1 pack 50 mL Falcon tubes
 2 x 25 L Brewers container
 2 x 15 L Knapsack sprayer
 4 x large pieces of cardboard for
 Treatment barrier

Method

Set bare-root *P. radiata* cuttings in 3 beds of 144 m length each. Randomised block design along the beds with 12 blocks (= 12 replicates) in total (**Figure A1.4**). Treatments applied to 3-m-plots >> 288 cuttings/plot (3 m x 8 rows x 12 cuttings/m) x 12 blocks = 3456 cuttings/treatment (41,472 cuttings in total). Apply treatments to foliage and soil as per schedule using a knapsack sprayer @ ~7 mL/cutting (25 L/treatment) calibrated @ ~1 L/90 s >> ~5 s/row/m. Apply *Trichoderma* treatments (2, 3, 4, 5, 6, 7, 8, 9) @ 10^7 spores/cutting >> knapsack concentration is $\sim 1.4 \times 10^6$ spores/mL or 3.5×10^{10} spores/25 L. Apply fulvic acid treatments (1, 2, 3) @ 1:300 which equals ~83 mL/25 L.

Treatments:

- | | |
|--|--------------------------------|
| (1) FA @ setting (fulvic acid) | (7) Untreated control |
| (2) FA @ setting + WT3 @ 4 months | (8) AG @ 4 months (ArborGuard) |
| (3) FA @ setting | (9) LU996 @ 4 months |
| (4) WT3 @ 4 months (3 isolates, from willow trial) | (10) Untreated control |
| (5) Untreated control | (11) Untreated control |
| (6) PBI @ 4 months (4 isoaltes, pasture mix) | (12) Untreated control |

Figure A1.4 Schematic illustration of the experimental design.

