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

Dr Robert Hill [1], Dr Michael Brownbridge [2], Dr Steve Reay [3], Tracey Nelson [2], B Gicquel [2], D Wright [2] J Swaminathan and Prof. Alison Stewart [1].

1. **Bio-Protection Research Centre (CoRE), Lincoln University, Lincoln**
2. **AgResearch Ltd, AgResearch Science Centre, Lincoln**
3. **Silver Bullet Forest Research Ltd, Auckland**

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Project Leaders: Dr Robert Hill (robert.hill@lincoln.ac.nz)
Bio-Protection Research Centre
PO Box 84
Lincoln University
Lincoln 7647

Dr Michael Brownbridge *
AgResearch Ltd
Cnr Springs Road and Gerald Street
Private Bag 4749
Christchurch 8140

* note Dr Michael Brownbridge left AgResearch in August 2009
please contact:
Dr Steve Reay (s.d.reay@xtra.co.nz) re insect biocontrol

Contents

Summary

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.

Working closely with industry (PF Olsen & Co Ltd), methods for applying a commercialized Trichoderma product (ArborGuardTM) were optimized, resulting in improved growth and health in forest nurseries and in over one-third less mortality for Armillaria disease in forest plantations where treated vs untreated seedlings have been used. This was achieved with a single nursery-applied treatment. Novel protectant microbial formulations also increased tree growth in the forest nursery by >30%, without the need for any fungicide sprays. with support from FRST, Technology NZ, the FBRC and individual forestry companies, the Trichoderma-based product ArborGuardTM was developed. A seed coating kit for forest nurseries to apply beneficial microbes has been developed.

Previous industry and FRST supported research on improving the success rate for P. radiata cuttings (2003-2007), has shown that an improvement in survival of healthy rooted cuttings of up to 20% can be achieved with beneficial microbe/natural product formulations.

Novel entomopathogenic fungi have been identified that may be used to reduce damage from bark beetles to radiata pine seedlings. A cost-effective delivery system is need for industry. Some fungal isolates will persist in the rhizosphere for an extended period, and soil populations are maintained at levels which are effective to insects. The opportunity exists to apply both Trichoderma and entomopathogenic fungal biocontrol agents to the soil/roots of radiata pine to protect seedlings against pest and disease damage.

In Malaysia a *Trichoderma* bioprotection project on *Acacia mangium* in the Planted Forest Zone of Sarawak has increased healthy tree production by about 40% compared with fungicide-sprayed and unsprayed controls. The nursery system is now being changed to inoculate all Acacia seedlings with *Trichoderma* (30 – 50 million trees / year).

Objective 1 : (FBRC) Development of beneficial microbe application kit for forest nurseries

Development of beneficial microbe application kits for forest nurseries:

A prototype kit, suitable for coating Pinus radiata seed with selected beneficial microbes by forest nurseries, has been developed and tested. ArborGuardTM was successfully applied to the 2008/09 seed crop sown at the PF Olsen Nursery (Waiuku). Several different Trichoderma isolates were applied to pine seed for trials at the Timberlands Te Ngae Nursery (Rotorua). A concrete mixer was used for coating large batches of stratified seed (c10 kg) and a small hand operated mixer for smaller quantities (1 – 5 kg). Very small quantities (several grammes) were also coated using polythene bags. The suitability and compatibility of mixtures of selected Trichoderma isolates and isolates of entomopathogenic fungi are currently being evaluated, applied as seed coatings.

Seed inoculation protocol (for ArborGuard™ and other beneficial microbes) on P.radiata

Ingredients:
1 kg of stratified *Pinus radiata* seed
2.5 g ArborGuard™formulation
PVA (interior) adhesive
Food dye (optional)
c 15g Talcum powder

Procedure:

- Drain any excess water from stratified pine seed.
- Mix in a concrete mixer at a slow speed
- A well mixed slurry of water and interior grade PVA, 27ml and 5 ml respectively, followed after at least two minutes of mixingt to distribute the flurry all over the seed.
- ArborGuard™ slurry (2.5 g ArborGuard™ , 10 ml H_2O and 4 ml of food dye - optional) or other beneficial microbes.
- Rinse the remaining ArborGuard™ slurry.
- Mix for at least a further 2 minutes to ensure good coverage before adding enough talcum powder (approx 15 g), to ensure the seed does not stick to the walls of the concrete mixer.
- Mix for about 10 minutes from start to finish, sufficient to give an even coverage of the respective ingredients to all of the seed.
- Disperse seed evenly and thinly (1-2 seeds deep) over a suspended piece of nylon shade cloth and leave to air-dry (c 2 hours) or use a fan.

Objective 2 : (FBRC) Enhancing root initiation, health and vigour of P.radiata cuttings

The Bio-Protection Centre research group has screened over 60 Trichoderma isolates from the Trichobank culture collection, for their ability to enhance root development and establishment of cuttings. This work aims to develop a microbial product which will benefit the forestry, industry. Willow was chosen as an initial model system, primarily because it produces roots easily and quickly. Root development was compared to untreated controls and various commercially available rooting products. Three Trichoderma isolates gave outstanding promotion of roots compared to any of the other treatments and a number of other isolates also provided promising results. A selection of the best isolates is now being tested further examining their potential to enhance rooting and health of cuttings in *Pinus radiata*. Nursery trials with c 100,000 cuttings were established at PF Olsen & Co Ltd (containerised), Timberlands, Te Ngae and the Rangiora Nursery (soil bed) in April 2009. this work will be the major focus for 2009/10 CoRE FBRC –traded research.

The best isolates from this screening trial will now be tested on P radiata cuttings at the PF Olsen and Te Ngae nurseries in 2009/10.

Entomopathogenic fungi for bark beetle biocontrol (Appendix 1)

(see Appendix 1)

Armillaria disease forestry trials:

The *Trichoderma* vs *Armillaria* forest plantation trials at Rotoiti (established in 2007) and Kaingaroa (established in 2001), were assessed for tree health, mortality and for growth in June 2009.

Rotoiti Forest Trial

In May 2008 over 7,000 trees in this trial were assessed for health and mortality (see Table 1, Appendix 2). The ArborGuard™ treatment had the highest health score and lowest mortality from *Armillaria* disease.

In June 2009 this trial was re-assessed. Unauthorised personnel had removed plot and marker posts for blocks 1 – 6. it was virtually impossible to be sure that trees were assessed according to treatment in these blocks. (Figures 9 – 10, Appendix 2). However, for Blocks 7 – 9 there was no difficulty in assessing the trees. The ArborGuard™ treatment (T3) was best for health and had the lowest mortality (less than 5%) (Figures 9 – 10, Appendix 2).

Kaingaroa Forest Trial

Tree mortality was virtually unchanged from the previous assessment.

Results for assessment of tree health height and DBH and the ratio of DBH / height are shown in figures 1 – 8 Appendix 2. Differences between ArborGuard™ and control treatments and ‘containerised’ and ‘bare root’ stock were small.

Bio-Protection of Acacia mangium using Trichoderma in the Planted Forest Zone, (PFZ) Sarawak:

The success of the New Zealand forestry bio-protection research has led to a collaborative partnership between the Bio-Protection Research Centre, Lincoln University, Sarawak Planted Forests Sdn Bhd and Grand Perfect Sdn Bhd in the Planted Forest Zone of Sarawak, Malaysia. Dr Hill has been contracted as a consultant to lead a two-stage project: firstly to enhance Acacia mangium seedling establishment, growth and health in the forest nursery and secondly to control Ganoderma root rot disease in plantation forests using selected local Trichoderma isolates.

This project involves a collaborative partnership between the Bio-Protection Research Centre, Lincoln University, Sarawak Planted Forests Sdn Bhd and Grand Perfect Sdn Bhd. Dr Robert Hill has been contracted as a consultant to lead the project, working closely with Grand Perfect Research and Development and Sarawak Forestry Corporation staff.

Using selected local Trichoderma isolates, twelve forest nursery trials, with up to 12 treatments per trial, were established at the Samarakan nursery between August 2008 and August 2009. The best Trichoderma-treatments increased seedling growth and vigour and reduced disease incidence, without the use of any fungicide sprays. The average number of seedlings meeting specification for planting out into the forest (108 days after sowing) per tray was; *Trichoderma* (mean) 41, untreated control (mean) 29, fungicide control (standard nursery practice, mean) 26. Seedling height followed a similar pattern: best Trichoderma, mean 38.3 cm; untreated control mean 30.6 cm and fungicide sprayed mean 27.8 cm.


left, T5 untreated control; right, T1 Trichoderma inoculated

A large-scale validation of the best Trichoderma treatments has been completed in the forest nursery (in the first quarter of 2009), and pilot-scale forestry plantation trials (vs Ganoderma disease), at three sites in the PFZ were established in August/September 2009.

Grand Perfect and the Sarawak Forestry Corporation have decided to change their nursery production system at Samarakan to treat all the Acacia seedlings with *Trichodermas* isolated in this project (30 – 50 million / year) and to discontinue fungicidal sprays.

The Planted Forests Project has been named as one of the six most important research projects in the world by Discover Magazine.

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Appendix 1 : Entomopathogenic fungi for bark beetle biocontrol

agresearch Farming, Food and Health. First
Te Ahuwhenua, Te Kai me te Whai Ora. Tuatahi

New Bioprotection Tools for Forestry

M. Brownbridge[1], S.D. Reay[2], T.L. Nelson[1], B. Gicquel[1], D. Wright[1] & J. Swaminathan[2].

[1]AgResearch Ltd
Lincoln Science Centre
Private Bag 4749
Christchurch 8140

[2]Silver Bullet Forest Research
Auckland

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New Zealand's science. New Zealand's future.

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CONTENTS

1. EXECUTIVE SUMMARY

- Entomopathogenic fungi are important natural mortality factors in bark beetle populations; to effectively utilize these agents in a bioprotection strategy, cost-effective delivery systems are needed

- Some isolates are rhizosphere-competent; delivery of these biocontrol agents to the seedling root zone of radiata pine via seed coatings would be simple to implement by the forestry industry.

- This project thus had the following objectives:
    - to assess survival of selected *Beauveria* and *Metarhizium* spp. isolates in seed coatings applied to stratified seed; and
    - to determine the compatibility of *Beauveria bassiana* and *Trichoderma* spp.; as the fungi could be co-inoculated onto seeds, compatibility is essential to ensure the biocontrol agents function effectively

- In general, *B. bassiana* conidia survived better on the seed, irrespective of the coating material and method used, than *M. anisopliae*

- Survival of fungal conidia was better in xanthan, methylcellulose and poly-ethylene oxide coatings; spray coating was a superior method of application

- Vegetative compatibility of the fungi was demonstrated; germination of *B. bassiana* conidia was inhibited in the presence of metabolites produced by one of the *Trichoderma* isolates tested

- Superior seed coating materials that preserve the viability of fungal conidia on seeds have been identified; their ability to deliver microbes into the pine root zone now needs to be confirmed.

- Compatibility between two (or more) biocontrol agents is an important selection criterion that must be considered in future development work.

2. BACKGROUND

Economic losses from fungal diseases and insect pests cost New Zealand's plantation forestry industry over $100M each year, mainly as a consequence of reduced growth but also from tree mortality. The primary disease-causing organisms are *Dothistroma*, *Cyclaneusma* and *Armillaria*, although *Nectria* flute canker has increased in importance in recent years. Maturation feeding by adult bark beetles can significantly impact seedling establishment and growth, and in severe cases seedlings will die. Our forests are under constant threat from new pest and disease incursions. New biocontrol agents are needed to provide the industry with effective and sustainable pest and disease management options.

Entomopathogenic fungi are important natural mortality factors in bark beetle populations (Brownbridge et al 2009, Reay et al 2008). Several *Beauveria* and *Metarhizium* spp. isolates, obtained from NZ field sites, are active against *Hylastes* and *Hylurgus* bark beetles (Fig 1). In planting bag trials using radiata pine seedlings, isolates persisted at levels which infected and killed insects for over 6 mo. To effectively utilize entomopathogenic and other beneficial fungi in a bioprotection strategy, cost-effective delivery systems are required for industry.

Some fungal isolates are known to be rhizosphere-competent, and will persist in the root zone of the plant for an extended period. The opportunity exists to apply these biocontrol agents to the soil/roots of radiata pine to protect seedlings against pest and disease damage. Delivery of these microorganisms to the seedling root zone via simple seed coatings would be easy to implement and be of great utility within the forestry industry.

This project thus had the following milestone objectives:

1. To determine the survival of selected *Beauveria* and *Metarhizium* spp. isolates in up to four seed coatings on stratified seed.
2. To assess the *in vitro* compatibility of bark beetle-active strains of fungi (*Beauveria* and *Metarhizium* spp.) with *Trichoderma* spp. (ArborGuard).

Figure 1. a. *Hylastes ater*; b. *Hylurgus ligniperda* infected with *Metarhizium anisopliae*.

ii. Spray coating
Four coatings were prepared by adding dry ingredients to 25ml aliquots of *Beuveria* or *Metarhizium* spore suspensions (0.1% xanthan; 2% methylcellulose; 10% PVA; 5% polyethylene oxide). A fifth coating was prepared by adding dry conidia to 25ml aqueous Shellac latex prepared using methods described in Chang et al (1990).

Fifty gram batches of seed were placed in a rotating Erweka coating pan (45°angle, 200rpm) and 25ml coating material applied via a spray gun at 4bar pressure. Where seeds started clumping unheated forced air was applied by a hairdryer to break clumps. A small quantity of talc was required to maintain Polyethylene oxide-coated seeds in free flow. After coating, seeds were removed from the pan and placed onto trays until dry in appearance prior to packaging into TGT bags (6 bags per 50g treatment). Bags were held at 4 and 20℃. Spore loadings on the seeds wer e determined 0, 1 and 5 days after preparation by cfu counts. The entire experiment was replicated three times.

iii. Spore loadings and seed viability
For each coating and fungus, 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 at each sample time. Seeds were allowed to soak for 30 mins 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 repli-plates were prepared for each dilution. Plates were incubated at 20℃ and cfus co unted after 10 days.

To determine whether the coatings affected seed viability, 10 seeds from each coating were placed on moist filter paper in a Petri dish (85 mm diam.). Plates were sealed with Parafilm and incubated in the dark. Germination was assessed after five days.

3.2 *In vitro* compatibilty of entomopathogenic fungi and *Trichoderma* spp.

3.2.1 Vegetative compatibility

If beneficial microbes are to be used together within the same environment, compatibility is essential to enable each to function effectively. Vegetative compatibility between *Trichoderma* strains used to suppress diseases in pine seedlings and *Beauveria* strains used for insect biocontrol was thus assessed. Three *Trichoderma* strains (T633, T668, T297) and four *Beauveria* isolates F647, F624, F643 and E97 were included in the evaluation.

The assays were set up on 10% PDA plates. A sterile filter paper disc (5mm diam) was first inoculated with 10µL of a *B. bassiana* spore suspension containing ca. 5.10^6 viable spores/mL 0.01% Triton X-100, and then placed on one half of the PDA plate. Plates were incubated at 20℃ for 10 days to allow time fo r the colony to develop. After 10d, a filter paper disc inoculated with *Trichoderma* spores was placed on the other side of the plate. *Trichoderma* has much faster radial growth than *B. bassiana*, so this time interval was required to prevent overgrowth of the entire plate by the *Trichoderma* strain before the *Beauveria* colony had had time to develop. The *Beauveria* colony measured 15-20mm in diameter by the time the *Trichoderma*-inoculated discs were placed onto the plates. Three plates per *Beauveria*/*Trichoderma* combination were produced. Colony diameter was measured every 3 days and inhibition zones along colony margins measured if observed.

3.2.2 Effect of fungal metabolites on spore germination

Both *Beauveria* and *Trichoderma* strains are known to produce a range of metabolites, some of which have antibiotic properties. Selected isolates were thus produced in liquid culture and effects of metabolites excreted into the growth medium on spore germination assessed. Four isolates were used: *B. bassiana* F624 and E97, and *Trichoderma* spp. T633 and T668.

Fungi were produced in a defined liquid medium (Jaronski and Jackson 2008) in 250 mL Erlenmeyer shake flasks. Each flask contained 50mL fermentation broth, and two production flasks were assigned to each isolate. Flasks were inoculated with 1mL spore suspension containing ca. 10^8 spores/mL 0.01% Triton X-100 (harvested from 10-d old cultures grown on quarter-strength PDA) and were incubated at 25℃, 220 rpm for 72h.

Cultures were pre-filtered through cheesecloth to remove coarse particulates, and the broth was then passed through a Whatman #1 filter paper to remove additional fungal material (blastospores, mycelia). The resulting filtrate was filter-sterilized using a syringe filter (0.22µm) into a sterile 50mL Falcon tube and stored at 4℃ until needed.

Germination assays were carried out in multi-well tissue culture plates. Treatments were set up to determine whether germination was affected as a result of the metabolites in the medium, or whether effects were primarily due to a lack of available nutrients. Sabouraud dextrose broth (SDB) was mixed with broth filtrate to provide nutrients. Germination was thus assessed in each of 3 filtrate treatments (Table 2); treatment 3 (dilute SDB) served as a positive control, providing a measure of 'potential' germination under the described conditions.

Treatment number	Vol. 10X SDB (µL)	Vol. filtrate (mL)	Vol. sterile water (mL)	Final % filtrate concentration
1	0	5	0.0	100
2	50	4.95	0.0	99
3	50	0	4.95	0

Table 2. Filtrate/SDB combinations for germination tests.

Stock treatments were prepared as detailed in Table 2 for each fungal filtrate and 0.5 mL of each added to 8 wells in a sterile 24-well plate (i.e., 2 replicate lanes/filtrate treatment). Spore suspensions of each isolate (F624, E97, T668, T633) were prepared at ca. $1x10^8$ spores/mL sterile 0.01% Triton and 10µL of each suspension were added to two replicate wells of each filtrate treatment; thus germination of each isolate was assessed in each filtrate treatment, which included a self (filtrate): self (spore) combination. Inoculated plates were placed on a rotary lab shaker at ca. 80 rpm, 25°C for 18 h. The process was repeated for the second replicate flask.

Germination was halted after 18h by adding 30µl of lactophenol cotton blue stain to each well. Plates were then held at 4℃ until samples c ould be processed. To check germination, a 25µl sample from each replicate well was carefully transferred onto a microscope slide, overlaid with a cover slip and examined under a microscope. Counting 3x100 spores for each sample (total 300 per treatment), the number of germinated spores were recorded, allowing the mean per cent germination rate to be calculated for each fungus/filtrate treatment combination.

4. RESULTS AND DISCUSSION

4.1 Seed coatings

Coating	Fungus	Mixing	Spray-coat
Xanthan	*B. bassiana*	3.85×10^6	2.51×10^6
Methylcellulose	*B. bassiana*	4.70×10^6	3.27×10^6
Polyethylene oxide	*B. bassiana*	4.29×10^6	6.33×10^6
Shellac	*B. bassiana*	4.35×10^6	5.03×10^5
PVA	*B. bassiana*	9.80×10^5	6.44×10^6
Xanthan	*M. anisopliae*	1.55×10^6	1.82×10^6
Methylcellulose	*M. anisopliae*	1.37×10^6	1.48×10^6
Polyethylene oxide	*M. anisopliae*	1.10×10^6	1.11×10^6
Shellac	*M. anisopliae*	1.12×10^6	5.47×10^5
PVA	*M. anisopliae*	5.03×10^5	1.80×10^6

Table 3. Initial spore loadings per seed by coating type and application method

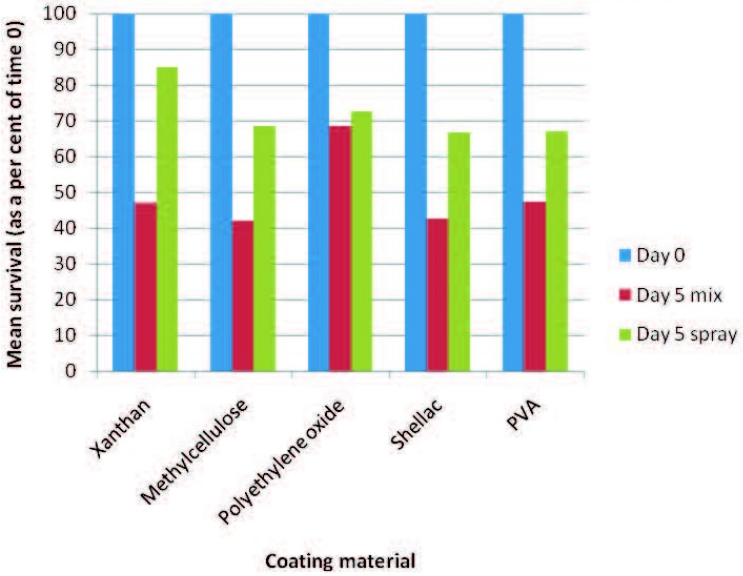
Comparable spore loadings were obtained with xanthan gum, methylcellulose or polyethylene oxide for both fungi, irrespective of the application method used (mixing or spray-coating) (Table 3). The lowest loadings were obtained by mixing seeds with spores encapsulated in PVA, irrespective of the fungal species; whereas spray-coating with shellac provided the lowest initial loadings, irrespective of the fungus used.

In general, *B. bassiana* conidia survived better on the seed, irrespective of the coating material and method used, than *M. anisopliae* (Figs 2 and 3). For *B. bassiana*, viable spore loadings dropped to between ca. 40-70% of the initial level after 5 days at room temperature when applied by mixing, ca. 65-85% when spray-coated (Fig 2). Overall, higher numbers of viable *B. bassiana* conidia were recovered when spray-coated onto the seed, for all of the coating materials tested. Polyethylene oxide appeared to be the best material for coating *B. bassiana* onto seed by mixing. Differences in survival among coatings applied by spraying were not significant.

Viable spore loadings dropped to between 20 to 40% of the initial level after 5 days for *M. anisopliae* when applied by mixing (Fig 3); similar levels of survival were obtained in methylcellulose, xanthan and polyethylene oxide, whereas viability was not maintained as well in shellac and PVA. Survival was generally higher when spores were applied to seeds using a spray-coating technique, falling to between 10 to 60% of initial levels. Xanthan, methylcellulose and polyethylene oxide again provided superior protection. Shellac and PVA did maintain spore viability as well as these materials.

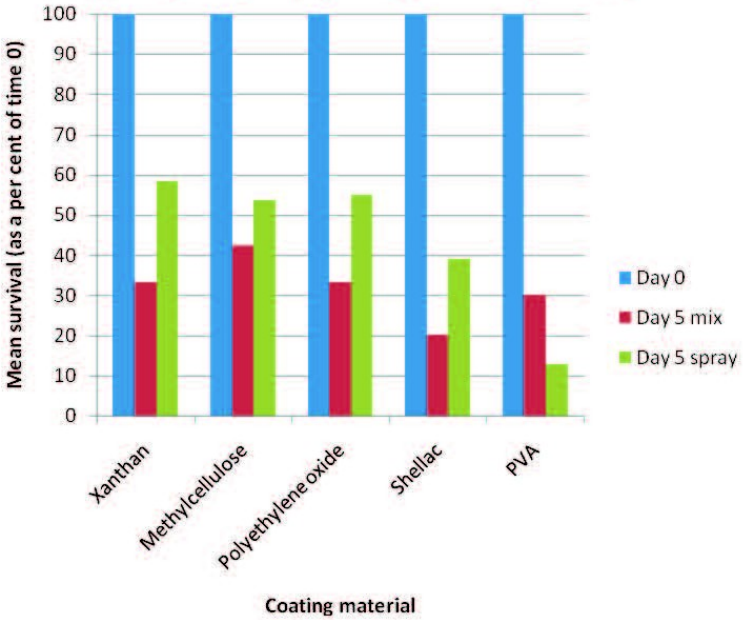
Seed viability was unaffected by the coatings applied, with >97% germination across all replicates and treatments after 5 days.

Figure 2. Survival of *Beauveria bassiana* conidia on wheat seed in different materials when applied by mixing or spray-coating, 0 and 5 days after preparation



Considering initial coating levels and survival of spores on the seed, xanthan, methylcellulose and polyethylene oxide appear to be the most suitable coating materials, whether applied by hand-mixing or spray-coating, for both fungal species. Spray-coating could be readily adapted to nurseries; no specialised equipment was used in the current trials, and it provided a fast and efficient method of applying spores to the seeds.

Figure 3. Survival of *Metarhizium anisopliae* conidia on wheat seed in different materials when applied by mixing or spray-coating, 0 and 5 days after preparation



Although spore numbers declined, significant loadings remained on the seeds after 5 days and the reduction observed is unlikely to be biologically significant. In a nursery where seeds would be planted within 5 days, the cooler soil conditions would be more conducive to spore survival than those used in the current study. Future studies will quantify root colonisation after planting; previously, we have shown that these entomopathogens are rhizosphere-competent. The suitability of the coating techniques for *Trichoderma* need to be determined, with the potential for co-application of both fungi to provide disease and insect protection for pine seedlings.

4.2 Compatibility

4.2.1 Vegetative compatibility

Beauveria and *Trichoderma* colonies grew together without significant evidence of inhibition, i.e., clear zones along colony margins were not observed (Fig 4). However, spore formation appeared to be inhibited at the intersection between the fungal colonies for some *Beauveria*/*Trichoderma* combinations (Fig 5). *Trichoderma* spores were observed several days after they were first seen along the rest of the colony margin, suggesting some inhibition of the sporulation process. This may have been a result of competition between the fungi for resources (nutrition) in the media which slowed fungal development, or through growth inhibition caused by secreted fungal metabolites.

Figure 4. Compatible growth between *Trichoderma* T633 (left) and *B. bassiana* F643; note spore formation by T633 along the entire colony margin..

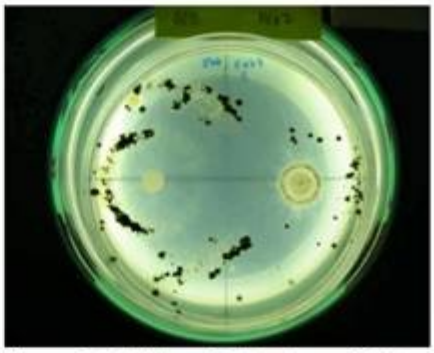


Figure 5. Inhibition of *Trichoderma* T668 (left) sporulation by *B. bassiana* F647 along the intersection between the two developing colonies

4.2.2 Effects of metabolites on spore germination

i. Germination assessed in B. bassiana *E97 broth culture filtrate*

	mean % germination (3x100spores for each combination)		
Spores	Treatment 1	Treatment 2	Treatment 3
E97	0	48	88
F624	27	74	95
T633	11	85	68
T668	32	90	90

Table 4. Mean per cent germination of *Beauveria* and *Trichoderma* spores in *B. bassiana* E97 broth culture filtrate

Poor germination rates were obtained in *B. bassiana* E97 culture filtrate alone (Treatment 1), for all of the test fungi (Table 4). The highest germination rates were observed in the dilute broth (Treatment 3), except for T633. However, germination was generally comparable between Treatments 2 (culture filtrate supplemented with SDB) and 3, suggesting that metabolites produced by E97 have little effect on germination of spores produced by the other test fungi. Interestingly, the greatest effect was observed with spores from strain E97, which did not germinate at all in the spent broth alone, and only reached 48% in the supplemented filtrate compared to 88% in the dilute SDB. For all other isolates, the low levels of germination observed in Treatment 1 compared to Treatment 2 suggest that inhibition is due to lack of nutrients rather than due to specific effects of any metabolites present in the broth filtrate.

ii. Germination assessed in B. bassiana *F624 broth culture filtrate*

	mean % germination (3x100spores for each combination)		
Spores	Treatment 1	Treatment 2	Treatment 3
E97	0	18	88
F624	20	87	95
T633	19	91	68
T668	25	94	90

Table 5. Mean per cent germination of *Beauveria* and *Trichoderma* spores in *B. bassiana* F624 broth culture filtrate

Similar results were obtained in the *B. bassiana* strain F624 culture filtrate (Table 5). The lowest levels of germination were obtained in the pure culture filtrate (Treatment 1) and the highest in the dilute SDB (Treatment 3). Germination of E97 was again low in the supplemented culture filtrate (Treatment 2), but germination rates for the remaining isolates were comparable to those obtained in the dilute broth.

iii. Germination assessed in Trichoderma *T633 broth culture filtrate*

	mean % germination (3x100spores for each combination)		
Spores	Treatment 1	Treatment 2	Treatment 3
E97	0	0	88
F624	1	0	95
T633	1	16	68
T668	1	2	90

Table 6. Mean per cent germination of *Beauveria* and *Trichoderma* spores in *Trichoderma* T633 broth culture filtrate

Metabolites produced by *Trichoderma* T633 appeared to have an inhibitory effect on germination across the test isolates (Table 6). Less than 1% of the spores counted germinated in the neat culture filtrate and germination was strongly inhibited even in the supplemented filtrate. Germination was, effectively, totally inhibited for three of the four isolates tested; interestingly, germination of T633 spores was <25% of the observed rate in the dilute broth. Results suggest that factors present in the culture filtrate, rather than a lack of nutrients, were primarily responsible for the observed response. *Trichoderma* isolates are known to produce a range of metabolites with antibiotic properties. Additional testing is needed to determine whether effects on germination are temporary (i.e., germination is slowed) or inhibited altogether. In the assays to determine vegetative compatibility, T633 was not antagonistic to any of the test strains with the exception of *B. bassiana* E97, where growth inhibition was observed.

iv. Germination assessed in Trichoderma *T668 broth culture filtrate*

	mean % germination (3x100spores for each combination)		
Spores	Treatment 1	Treatment 2	Treatment 3
E97	1	42	88
F624	11	62	95
T633	4	82	68
T668	1	69	90

Table 7. Mean per cent germination of *Beauveria* and *Trichoderma* spores in *Trichoderma* T668 broth culture filtrate

Results again suggested that lack of nutrients was the primary factor in the inhibition of spore germination across the test isolates. Poor germination was obtained in the pure culture filtrate. Germination of E97 was again relatively low in the supplemented culture filtrate (Treatment 2) compared to the dilute broth, 42 vs 88%, respectively. Germination rates for the remaining isolates were more comparable to those obtained in the dilute broth. Based on these data, T668 appears to be more compatible with the *Beauveria* isolates than T633.

Compatibility between two (or more) biocontrol agents is essential for them to perform effectively when co-applied. This is an important selection criterion that must be considered in future development work. Results from these trials will be valuable in guiding future research towards the integration of bioprotection strategies for plantation forestry.

5. RECOMMENDATIONS

- Spray coating provides a fast and efficient method of applying spores to seeds requiring no specialised equipment; the utility of this technique should now be assessed under nursery conditions.
- Superior seed coating materials that preserve the viability of fungal spores on seeds have been identified; their performance in terms of delivering microbes into the pine root zone now needs to be confirmed.
- 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.
- Application of this technology to deliver beneficial microbes to pine cuttings is worthy of investigation.
- Compatibility between two (or more) biocontrol agents is an important selection criterion that must be considered in future development work.

6. ACKNOWLEDGEMENTS

This research was funded, in part, by FRST Contract LINX0804, subcontract no. A12389 'Entomopathogens for protection of *radiata* pine seedlings from bark beetles'. Support of the Forest Owners' Association is also acknowledged through the FBRC, subcontract no. A14171 'New bioprotection tools for forestry'.

7. REFERENCES

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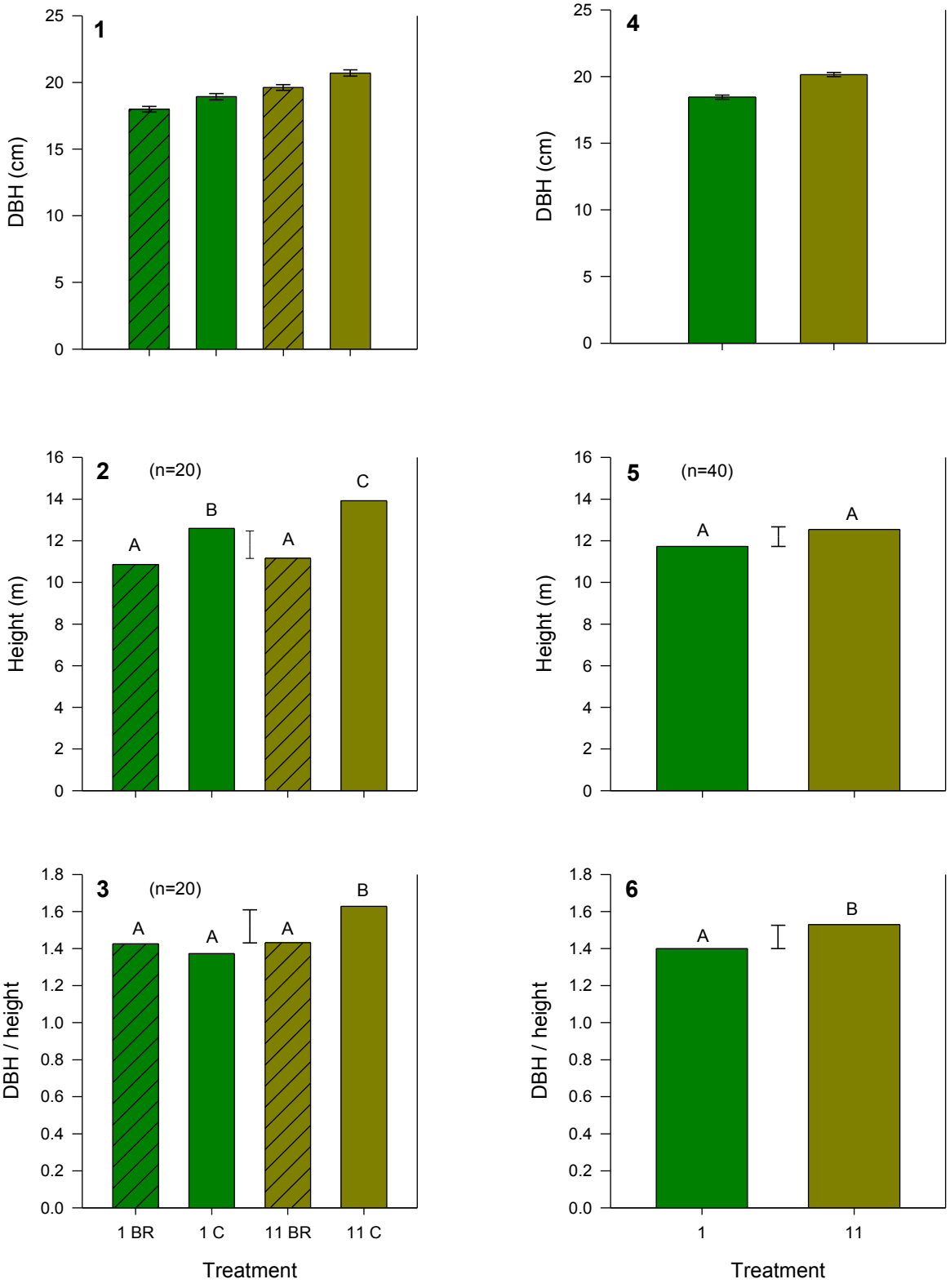
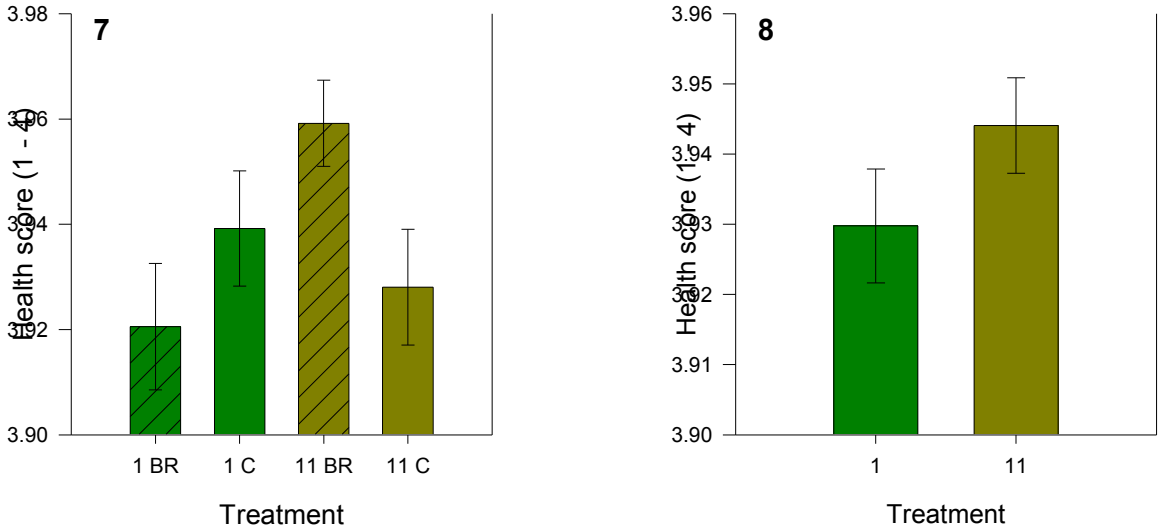
Reay SD, Brownbridge M, Cummings NJ, Nelson TL, Souffre B, Lignon C, Glare TR 2008. Isolation and characterization of *Beauveria* spp. associated with exotic bark beetles in New Zealand *Pinus radiata* plantation forests. Biological Control 46:484-494.

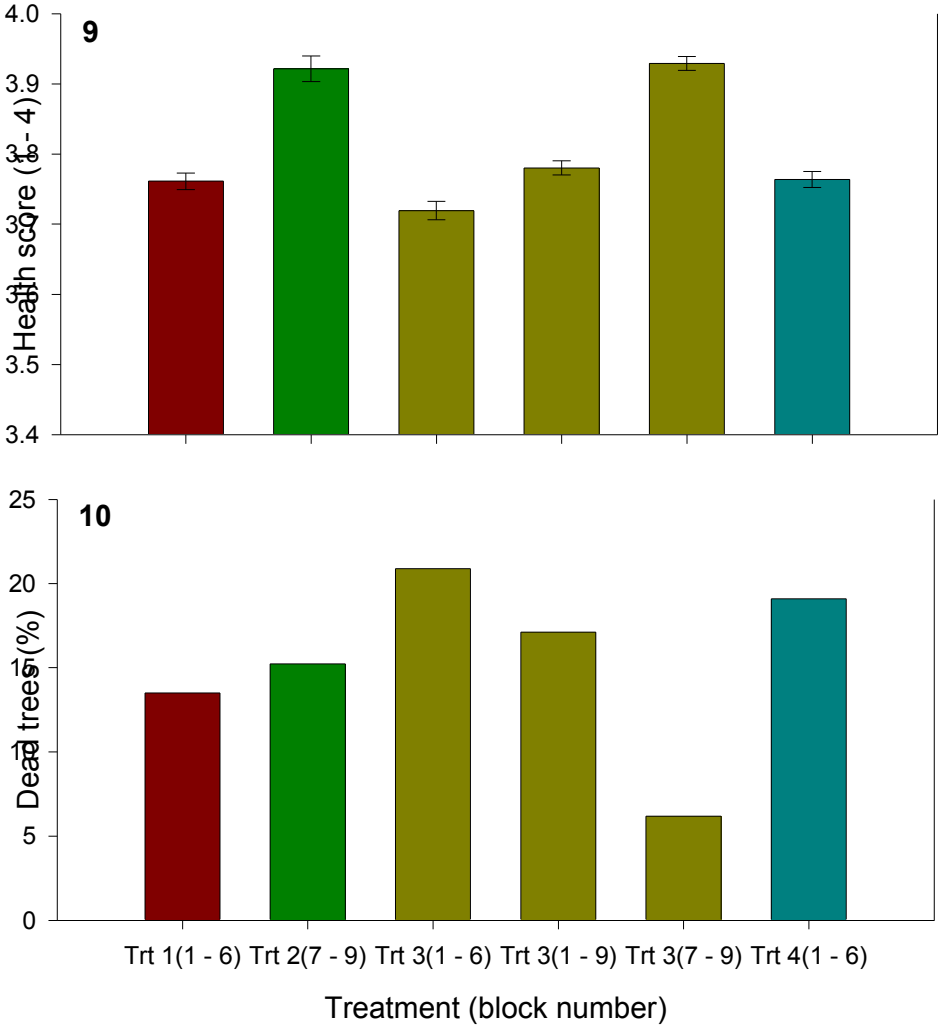
Appendix 2 : New Zealand forestry trials assessment Rotoiti Forest and Kaingaroa Forest June 2009



Figures 1 – 6 Kaingaroa forest data summary for the diameter breast height (DBH), height and DBH / height for treatments 1 and 11 with the relative measurements split between bareroot (BR) and containerised (C) seedlings (Figures 1 – 3) and the overall combined data (Figure 4 – 6). Error bars indicate the standard error of the mean (SEM) for Figures 1 and 4, while the means for Figures 2, 3, 5 and 6 respectively are separated using an unrestricted Lsd (*p* 0.05).



Figures 7 - 8 Kaingaroa forest data summary for the mean health scores for treatments 1 and 11 with the relative scores split between bareroot (BR) and containerised (C) seedlings (Figure 7) and the overall combined data (Figure 8). Error bars indicate the standard error of the mean (SEM) for each of the respective means.



Figures 9 - 10 Rotoiti forest data summary for the mean health score (Figure 9) and percent dead trees (Figure 10) for treatments 1 - 4. Independant variables in parenthesis indicate the respective block numbers measured for each of the treatments. Error bars indicate the standard error of the mean (SEM) for each for the respective health score means.

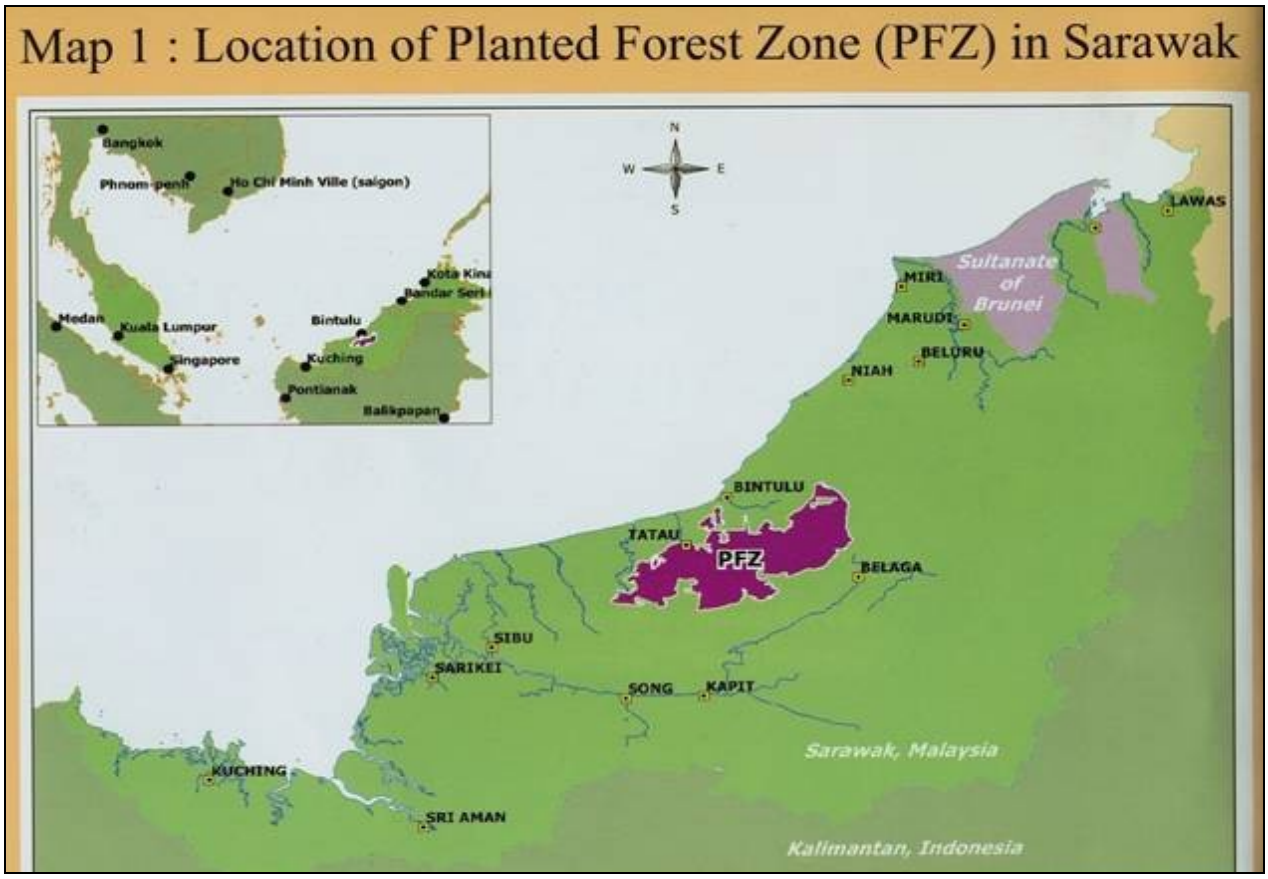
ROTOITI FOREST TRIAL SUMMARY OF 26/05/08 ASSESSMENT

Total number of trees included in this assessment = 7,129
Total number of dead trees = 38 (0.5%)
Average health score = 3.6 overall, on a scale of 0 (=dead) - 4 (= very healthy)

	Mean health score (%)	Mean mortality (%)
T 1 {BD1 bacterial mixture)	3.5	0.6
T 2 {BD T109}	3.7	0.8
T 3 {ArborGuardtm)	3.7	0.4
T 4 {Control, untreated}	3.5	0.7
Overall mean	3.6	0.5

Appendix 3 : Bio-protection of Acacia in PFZ, Sarawak

Location of the planted forest zone in Sarawak, Malaysia



Testimonial on PFZ Project

I write to thank you for the privilege of being able to witness the work that you are undertaking in the Planted Forest. It was inspiring. The project is a beacon of hope in a region that has suffered greatly from lack of planning and foresight.

The work that you and the Bio protection unit has undertaken has achieved startling results. In my time as a farmer and more latterly as a funder of agricultural sciences I am struggling to think of a more dramatic visible result. The increase in growth rates and the extra bloom of health in the acacia seedlings treated with your system were absolutely stunning. In addition to the obvious commercial benefits, here is an immediate impact of changing the nursery to a biological system. The large settling ponds at either end of the complex are being used for recreation as well as fishing and cresses for consumption. Being able to move away from the potpourri of herbicides, fungicides, etc has a fundamental human benefit. Absolutely consistent with the objectives to have production forestry, people and the natural forest coexist.

If possible can you, please convey my admiration to Alison and the Bio protection unit for being there. Not all of my country's efforts in Asia I am proud of. However, as a Kiwi I am very proud of this one.

The planted forest project that is attempting on a large scale to meet the needs of sustainable development is very deserving of whatever support that our country can muster.
I am sure apart from the philanthropic aspects of being involved I feel that the rewards for being at the cutting edge of an international effort such as this will provide good sustainable returns. I feel from my observations that the high quality mix of people that have been attracted to the overall concept will ensure success and an international focus on the art of the possible.
It was a great to witness real, practical science at the forefront. It was inspirational.

So thank you again Robert the time with you gave me valuable insights and enabled me to judge on the rest of my mission the impacts, practices and potential solutions with a little more clarity and balance.

I am in your debt.