Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Production of *Trichoderma* inoculum for treatment of plant material

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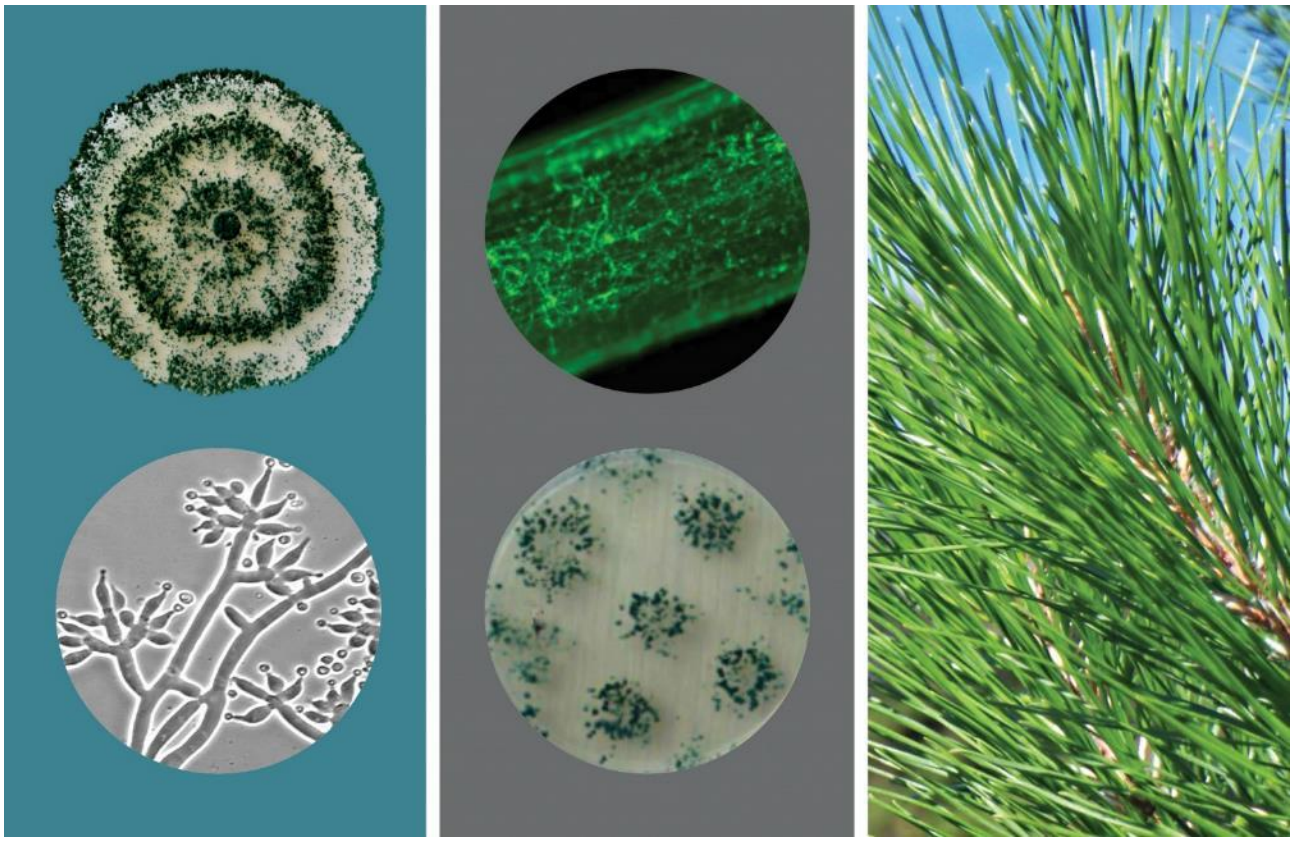

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EXECUTIVE SUMMARY

The main goal of the *Bioprotection for Foliar Diseases and Disorders of Radiata Pine* programme is to induce systemic resistance against foliar diseases through the use of beneficial endophytes and elicitors. As part of this project, a number of plantation trials have been established at sites around NZ to examine the effects of specific endophytic *Trichoderma* strains on *P. radiata* growth and disease resistance.

Plantation field trials have necessitated the development of efficient methods for producing large amounts of *Trichoderma* spore inoculum for nursery application. Previous research identified seed treatment as the most effective delivery system for establishing *Trichoderma* colonisation in the roots of *P. radiata* seedlings. However, this method is dependent on use of a highly purified spore product. A new capability for large-scale production of high quality *Trichoderma* inoculum using state-of-the art spore extraction technology was developed at the Bio-Protection Research Centre (BPRC) with the purchase of a Mycoharvester spore separator. This equipment was used to prepare purified *Trichoderma* spores which were supplied to PF Olsen and Te Ngae nurseries in 2014 and 2015 for application as seed treatments to provide inoculated seedlings for plantation trials.

Initial work using the Mycoharvester found that although it could effectively extract spores from the standard peat/wheatbran growth medium used for *Trichoderma* production, total spore yield was much lower than expected due to the physical characteristics of the substrate. Following an earlier BPRC-funded summer scholarship project which identified substrates and incubation conditions resulting in improved spore yields using this equipment, a new method of producing *Trichoderma* spore on a rice-based substrate was investigated. This procedure has now been adopted as the preferred method for production of *Trichoderma* spores for research trials. In addition to providing high yields of extracted spores, the method has been optimised to provide a labour-efficient, highly practical system for large-scale production of *Trichoderma* inoculum. These techniques are likely to be directly applicable to future commercial manufacture of *Trichoderma* biocontrol products for the NZ forestry sector.

INTRODUCTION

Foliar diseases and disorders are the largest cause of economic loss for the New Zealand forestry industry. A major aim of the *Bioprotection for Foliar Diseases and Disorders of Radiata Pine* research programme is to establish long term symbiotic relationships between *Pinus radiata* and endophytic *Trichoderma* species, which have been shown to induce systemic resistance and provide protection from foliar diseases in other plants. Initial research in this programme isolated a large number of *Trichoderma* strains which were screened for the ability to promote growth and supress disease in laboratory and nursery trials. The most effective isolates were applied as nursery seed treatments and form the basis of plantation trials at sites around the country.

Biocontrol products based on *Trichoderma* and other beneficial fungi are formulated from living spores (conidia) which germinate upon application and proliferate in soils or on plant roots. Large-scale production of spore inoculum for field trials or commercial applications generally involves the use of solid-state fermentation techniques based on organic substrates. A critical step in the preparation of inoculum is the extraction of spores from the production substrate. Ideally this process needs to maximise yield and provide large numbers of undamaged, viable spores.

Prior to 2014, *Trichoderma* researchers at the BPRC relied on a lengthy process for spore extraction which involved substrate washing, centrifugation, sedimentation and air drying of harvested spores. While providing high concentrations of purified spores in the final product, this method is highly labour-intensive and may significantly reduce spore viability and shelf-life as a result of the initial wetting of spores in the first stage of the technique. In late 2013 the BPRC purchased Mycoharvester spore processing equipment to improve formulation quality and provide a more efficient alternative for spore extraction. The Mycoharvester was specifically designed to facilitate large-scale and cost-effective extraction of high quality spore preparations for microbial product formulation and has been used successfully in several large biological control projects worldwide. The main advantage of this technology is that it allows extraction of dried spores directly from the production substrate, avoiding the multiple steps used in previous methods, including the problematic washing stage.

Mycoharvester equipment was used to prepare purified spores for nursery seed treatments in September 2014 and 2015, with extraction of spores for 2016 sowings now in progress. Over this period, large-scale production techniques were optimised to allow maximum recovery of spores using the Mycoharvester. Inoculum production is now based on a rice medium which replaces the peat/wheatbran substrate that was previously used. The current protocol for spore production represents a significant improvement over earlier methods and uses a two-stage system, combining liquid and solid culture, for efficient production of large quantities of high-quality dried *Trichoderma* inoculum.

METHODS

Large-scale spore production using peat/wheatbran substrate

Equal volumes of peat, wheatbran and water were mixed thoroughly. For each tray, 1.5 litre (~700g) of substrate was dispensed into small autoclave bags which were in enclosed larger bags and autoclaved at 121°C for one hour. Substrate was stored at 4°C after autoclaving. For inoculation,*Trichoderma* isolates were streaked onto plates of MYE agar (malt extract 1.0%, yeast extract 0.1%, agar 2%) and incubated for 1-2 weeks at room temperature. Spore suspensions for inoculating substrate were prepared by pouring ½ strength potato dextrose broth (PDB, Difco) onto sporulating colonies on MYE agar plates and harvesting conidia by scraping with a glass spreader. Each bag of substrate was inoculated with 50 ml of spore suspension and mixed thoroughly by kneading to ensure the spore suspension was evenly distributed through the medium. Each inoculated bag of substrate was transferred aseptically into sterile 5 litre polypropylene trays and spread to form an even layer approximately 2 cm deep. Trays were covered in large plastic bags with the ends folded closed and sealed with tape. Trays were incubated at 23˚C under ambient light conditions until prolific sporulation was observed (e.g. 10-21 days).

Figure 1. *Trichoderma* production using peat/wheatbran substrate in 5 litre trays.

Two-stage fermentation system for large-scale spore production

Liquid cultures were prepared in 500 ml Erhlenmeyer flasks containing 200ml of ½ strength PDB. Flasks were inoculated with a loopful of conidia from sporulating agar cultures prepared as above. Cultures were incubated at 23˚C on a rotary shaker set at 120 rpm for 2-3 days (Fig. 2).

Medium grain brown rice was weighed into 500g amounts into small (250 X 400 mm) autoclave bags. 250 ml of tapwater was added to each bag and left to soak overnight. Bags were autoclaved for 15 minutes at 121°C. After autoclaving each bag was inoculated with 5 ml of liquid culture prepared as above and mixed. Bags were incubated at 23˚C under ambient light conditions for 1-2 weeks (Fig. 3). Bags were massaged daily to break up mycelial clumps and encourage sporulation.

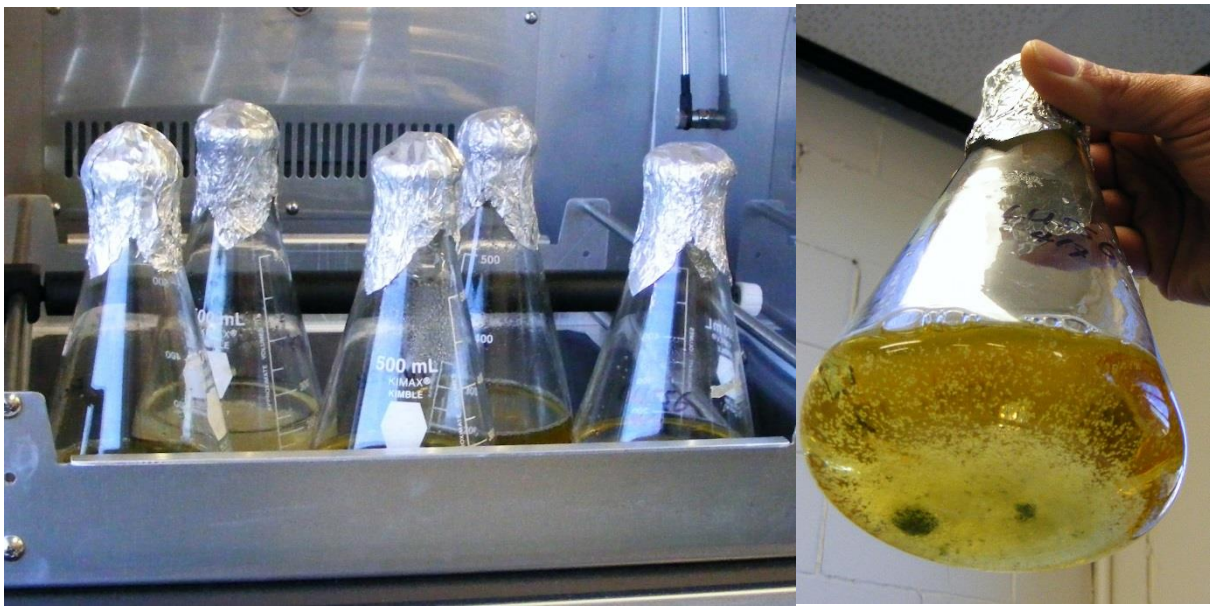

Figure 2. Liquid shake cultures of *Trichoderma* used to inoculate solid (rice) substrate.

Figure 3. Sporulating cultures of *Trichoderma* grown on a brown rice substrate.

Mycoharvester extraction

After incubation, sporulating *Trichoderma* cultures on peat/wheatbran or rice substrate were transferred to paper bags for drying. Bags were dried at room temperature on wire mesh racks for at least one week using domestic fans to provide air movement (Fig. 4). Bags were also shaken twice daily to further reduce drying times.

After drying, conidia were extracted using a MycoHarvester (v5, VBS Agriculture Limited) according to the manufacturers instructions (Fig. 4). To determine the concentration of spores in the final product, 10 mg of spore material was suspended in 10 ml of 0.01% Tween 80 and numbers of conidia/g determined from haemocytometer counts.

Figure 4. Drying bags of *Trichoderma* inoculum (left), Mycoharvester spore extractor (right).

RESULTS & CONCLUSIONS

Mycoharvester spore extraction equipment was used to prepare purified *Trichoderma* conidia for treatments of *P. radiata* seed at PF Olsen and Te Ngae nurseries in 2014 and 2015.Total amounts of material supplied are given in Table 1 below.

Table 1. *Trichoderma* inoculum supplied to nurseries in 2014 and 2015.

Treatment	Seed treated (kg)	Total spores supplied	Year
Mix A	100	2.5×10^{12}	2014
FCC320*	10	2.5×10^{11}	2014
FCC327*	10	2.5×10^{11}	2014
PR5	10	2.5×10^{11}	2014
PR6	10	2.5×10^{11}	2014
Mix A	100	2.5×10^{12}	2015
PR1	10	2.5×10^{11}	2015
PR2	10	2.5×10^{11}	2015

single isolate treatments, other treatments include four isolates

Spore inoculum prepared in 2014 was produced using a peat/wheatbran growth substrate incubated in 5 litre plastic trays. Although numbers of conidia varied considerably between different *Trichoderma* isolates, this method was found to produce up to 5×10^{11} conidia per tray. However, spore yields obtained from mycoharvester processing were generally reduced to less than 10% of this amount. Subsequently, an improved method using a brown rice medium for spore production was developed to give higher yields of extracted spore product (Table 2).

Table 2. Yields of spores obtained from Mycoharvester extraction of *Trichoderma* spores grown on brown rice production substrate

Isolate	Yield /500g rice	Extracted product conidia/g
LU132	1.12×10^{10}	9.33×10^{9}
LU140	$7.65E \times 10^{10}$	4.1×10^{10}
LU584	$1.30E \times 10^{11}$	4.9×10^{10}
LU633	2.45×10^{11}	5.2×10^{10}
FCC49	$6.06E\times 10^{9}$	9.08×10^{9}
FCC55	2.53×10^{11}	7.95×10^{10}
FCC362	8.20×10^{9}	8.5×10^{9}
FCC368	3.18×10^{11}	7.75×10^{10}
FCC13	2.08×10^{11}	3.9×10^{10}
FCC14	4.36×10^{10}	2.52×10^{10}
FCC15	2.25×10^{10}	2.15×10^{10}
FCC16	5.29×10^{9}	9.98×10^{9}
FCC180	6.69×10^{10}	2.51×10^{10}

A two-stage system using an initial liquid culture step followed by solid substrate fermentation on brown rice has now been adopted as the preferred method to produce spores for Mycoharvester extraction. In addition to allowing recovery of high yields of spores, the method has several other advantages. Compared with using the peat/wheatbran substrate, the brown rice medium requires much less time to prepare and autoclaving times are also reduced. Use of liquid cultures for inoculation of the rice substrate also saves considerable operator time during the inoculation

process and allows large quantities of inoculum to be prepared relatively quickly. As a simpler system with fewer practical steps, the potential for contamination of cultures is also reduced. The method may be easily upscaled for production of commercial quantities of *Trichoderma* inoculum for forestry biocontrol products developed in the future.

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