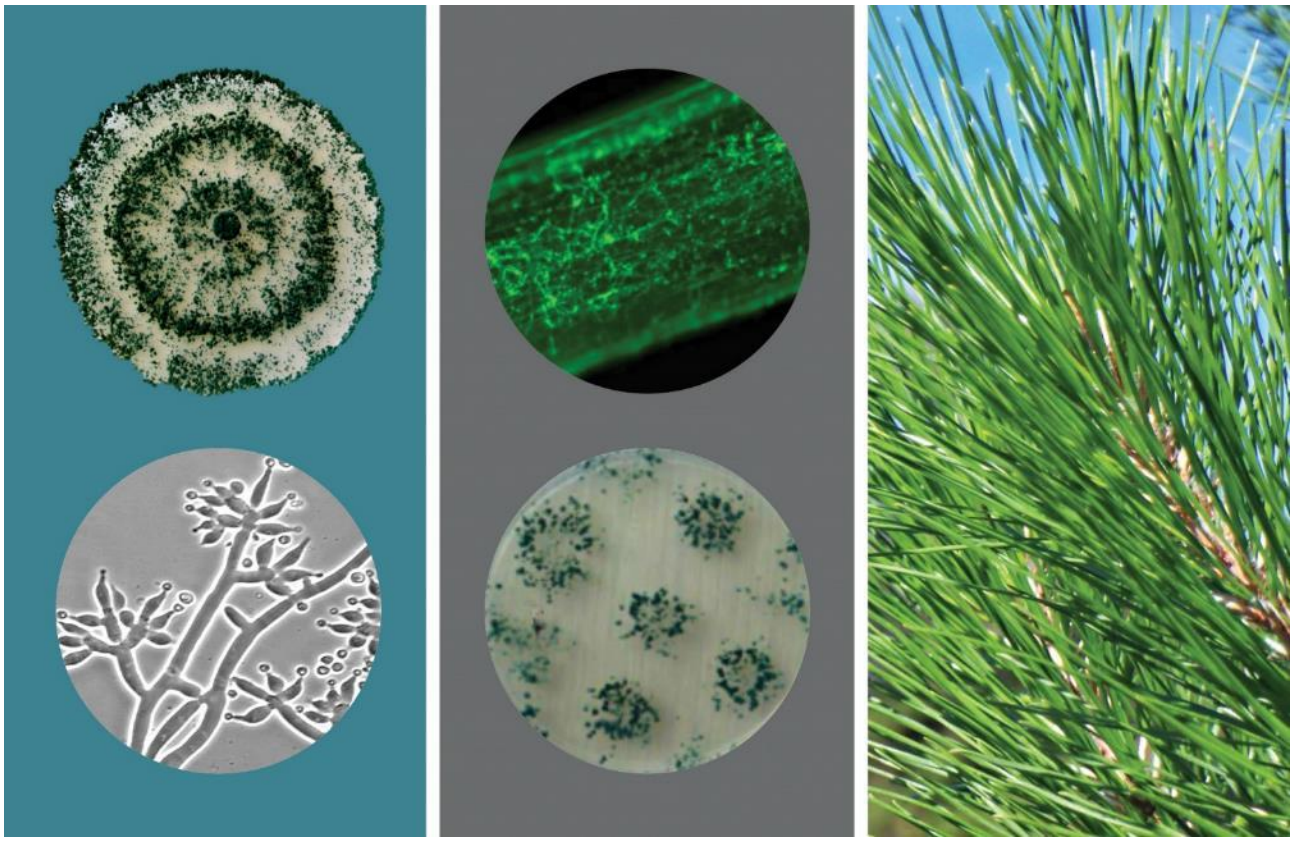
Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Persistence of *Trichoderma* in Nursery and Forest Plantations

Authors:
Nicholas Cummings and Robert Hill, Bio-Protection Research Centre, Lincoln University



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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. A major question arising from this work is whether the applied strains are able to persist under field conditions to provide long-term growth benefits and protection from disease.

Trichoderma species are ubiquitous in the environment, and application of molecular techniques is necessary to allow differentiation of biocontrol stains from natural fungal populations and obtain information on long term persistence following field application. Specific PCR assays are now available for two *Trichoderm*a strains currently under evaluation in plantation trials. Characterisation of *Trichoderma* species that occur naturally in plantations will provide important prerequisite information for the development and testing of further molecular tracking systems for additional strains.

In this project we examined the persistence of applied *Trichoderma* strains through isolation of cultures from the roots of treated plants. Cultures were examined using specific PCR primers and direct sequencing. Isolates were also obtained from untreated plants and identified by sequence analysis to determine the background diversity of *Trichoderma* species that are naturally present in plantations.

Key Results

- Previously developed strain-specific primers confirmed persistence of *T. atroviride* strain LU633 at one site three years after planting
- Species specific primers were developed for *T. atrobrunneum* and were able to successfully detect this species in isolates from plantation and nursery plants
- Background populations of *Trichoderma* were characterised in plantation sites and were found to represent at least 16 species including *T. atrobrunneum*, *T. austrokoningii, T. atroviride*, *T. citrinoviride*, *T. crassum*, *T. hamatum*, *T. harzianum*, *T. longipile*, *T. polysporum, T. spirale, T. tomentosum, T. trixiae*, *T. virens*, and three currently undescribed taxa.

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.

Trichoderma species are commonly found in all environments where plants are present, and efforts to determine the field persistence and activity of biocontrol strains need techniques capable of distinguishing the strains of interest from background populations. However, currently there is no available data on *Trichoderma* species that occur naturally in *P. radiata* plantations. Specific primer-based PCR assays were previously developed to allow detection of two *Trichoderma* strains currently used in plantation trials (report FOA-BIO-T002). Further development and utilisation of additional molecular assays for other trial strains may be streamlined by gaining an understanding of the background *Trichoderma* diversity existing in forestry plantations. *Trichoderma* is now considered to include over 250 named species, most of which require DNA sequence data for unambiguous identification to species level. Analysis of sequence data from the translation elongation factor 1α (*tef1*) gene is generally used for routine identification as analysis of this region is capable of differentiating all known *Trichoderma* species (Bissett et al. 2015).

In this project, *Trichoderma* cultures were isolated from surface-sterilised roots collected from treated and untreated plants at several trial sites to a) examine persistence of applied strains and b) characterise the diversity of background *Trichoderma* populations. Isolates were identified to species level following PCR and phylogenetic analysis of partial tef1 gene sequences. Treated plants were also screened with previously developed primers for strains LU633 and 584. A species-level specific primer was developed for the recently described *T. atrobrunneum,* as several strains used in trials represent this species. The *T. atrobrunneum*-specific primer was used to screen a set of isolates obtained from plantation trial plants and nursery seedlings.

METHODS

Sample collection & *Trichoderma* isolation

Roots were collected from treated and untreated *P. radiata* trees at plantation sites with collection details in Table 1. Samples were stored in moistened zip-lock bags and stored at 4°C before processing. Roots were also sampled from nine month-old nursery seedlings (20 plants) supplied by PF Olsen that had been inoculated with four *Trichoderma* treatments at sowing. For each sample, roots were washed thoroughly under running tap water to remove soil and other debris. Roots were then cut into approximately thirty 0.5-10mm segments. Under aseptic conditions, 25 root pieces were surface-sterilised by submersion in a deep-well petri dish containing ~50 ml of freshly prepared 1% Virkon (Antec) for 10 minutes. After 10 minutes, root pieces were rinsed by transferring to a deep well dish containing ~50 ml of sterile reverse-osmosis water and gently agitated. Using flamed forceps, root pieces were removed and placed onto sterile filter paper and briefly air-dried to remove excess water. Five surface-sterilised root pieces were placed onto each of five plates of MRB medium (malt extract 1.0%, yeast extract 0.1%, quintozene 0.02%, rose bengal 0.015%, agar 2%) amended with 100mg/L chloramphenicol. Plates were incubated at 22°C for 7-12 days... *Trichoderma* colonies were subcultured to plates of MYE agar (malt extract 1.0%, yeast extract 0.1%, agar 2%).

DNA extraction

For DNA extraction, a loopful of conidia from pure cultures was spread with a glass spreader onto a plate of 1% malt extract agar overlaid with sterile colourless cellophane. Plates were incubated for two days to allow a thin layer of mycelium to cover the plate. Approximately 50 mg of mycelium was harvested with a flamed scalpel into a sterile 1.5 ml Eppendorf tube and frozen at -20°C. Genomic DNA was extracted from mycelium using a Geneaid Genomic DNA Plant Mini Kit and stored at -20°C.

Screening with specific primers

To confirm the presence of strains LU584 and LU633 in the isolates using conventional PCR, a set of DNA extracts from cultures isolated from treated trees in plantation trials (Table 1) was examined. These included plants inoculated with LU633 and LU584 (included as part of Mix A) and other treatments serving as negative controls. Quality of DNA extracts was confirmed by initial PCR of the ITS region with primers ITS4 and ITS5. DNA extracts were then screened in separate PCRs using the specific primer pairs RM1/RM2 (LU584-specific) and RM5/RM6K (LU633-specific). All PCR amplifications were performed in a total reaction volume of 20 µl including 2µL of 10X reaction buffer, 200 dNTPs, 0.4 µM of each primer, 1U DNA polymerase (Intron iStar taq) and 2 µl template DNA. PCR reactions were initiated with a denaturing step of 95°C for 4 min followed by 35 cycles of 95°C (1min), 55°C (1 min),and 72°C (45 sec); with a final extension step of 72°C for 7 min. Positive (DNA from LU584 and 633 respectively) and negative (water) controls were included in each assay. PCR products were visualised by staining with RedSafe (Intron) following electrophoresis of 10 µl of each product in 1.5% agarose gels.

Tef1 sequences from several isolates used in trial treatments and identified as *T. atrobrunneum* (see previous technical report FOA-BIO-T003) were used to develop species-level specific primers. Forward and reverse primers were designed and using Primer-blast (NCBI)**.** Primer sequences selected were Atrb1 (forward): 5' CACCGTAAGTTGCACCCTATATT-3' and Atrb3 (reverse): 5'3-CTAGCTCGCAGTTGCACCAT-3'. Specificity was confirmed by testing with DNA extracts from isolates previously identified to species level from *tef1* sequences. PCR conditions follow those described above for RM5/6 and RM1/RM2 except for the annealing temperature which was changed to 56°C. A set of DNA extracts from cultures obtained from treated plants in planation trials as above was screened in order to detect *T. atrobrunneum.* DNA extracts from 20 cultures obtained from nursery seedlings as above were also screened using these primers*.*

Sequencing and phylogenetic analysis

For each isolate an approximately 900 base pair (bp) fragment from the 5' end of the tef1α gene was amplified using the primer pair tef71f (C AAA ATG GGT AAG GAG GAS AAG AC) and tef997R (CA GTA CCG GCR GCR ATR ATS AG) (Shoukouhi and Bissett 2009). All PCR amplifications were performed in a total reaction volume of 20 µl including 0.4 µM of each primer, 200 uM dNTPs, 2. µl 10X reaction buffer, 2.5 mM MgCl2, 2 µl template DNA and 1U Taq Polymerase. PCR conditions followed a procedure described in Cummings et al. 2016. DNA was initially denatured for 6 min at 95°C, followed by 4 cycles each of 1 min at 95°C, 90 s at 70°C, 90 s at 72°C, followed by 26 cycles with the annealing temperature decreasing from 68°C to 55°C (a gradient of 0.5°C reduction per cycle), followed by 12 cycles with the annealing temperature at 55°C and a final extension of 7 min at 72°C. PCR products were visualised by staining with RedSafe (Intron) following electrophoresis of 5 µl of each product in 1% agarose gels. PCR products were cleaned using a commercial cleanup kit (Zymo Clean & Concentrator-5) and sequenced in both forward and reverse directions using the internal sequencing primers tef85f (AG GAC AAG ACT CAC ATC AAC G) and tef954r (AGT ACC AGT GAT CAT GTT CTT G) (Shoukouhi and Bissett 2009) at the Bio-Protection DNA Sequencing Facility (Lincoln University).Consensus sequences were assembled from forward and reverse sequencing chromatograms using ChromasPro v. 1.7.6. Following BLAST searching to identify species afilliations, sequences were incorporated in datasets with representative sequences from NZ and overseas *Trichoderma* collections (including type or authentic specimens). Sequence alignments were performed using MUSCLE implemented in MEGA7 Phylogenetic analyses were conducted in MEGA 6 using the neighbour-joining method (Kimura 2-parameter) with branch support from bootstrap analysis (1000 replicates).

RESULTS & DISCUSSION

Table 1. Collection details for *Trichoderma* isolates obtained from plantation sites with species identification based on analyses of *tef1* sequences.

Isolate	*Trichoderma* species	Collection locality	Treatment
P1	*T. hamatum*	Moutere, Nelson	Untreated
P2	*T. harzianum*	Moutere, Nelson	Untreated
P3	*T. harzianum*	Moutere, Nelson	Untreated
P4	*T. longipile*	Kohatu, Nelson	Untreated
P5	*T. spirale*	Kohatu, Nelson	Untreated
P6	*T. harzianum*	Kings Ridge, Nelson	Untreated
P8	*T. hamatum*	Blenheim	Untreated
P9	*T. harzianum*	Kohatu, Nelson	Untreated
P10	*T. spirale*	Kohatu, Nelson	Untreated
P12	*T. atroviride*	Blenheim	Untreated
P13	*T. aff. deliquescens*	Blenheim	Untreated
P14	*T. trixiae*	Wharerata Forest, Gisborne	Untreated
P15	*T. harzianum*	Kings Ridge, Nelson	Untreated
P22	*T. hamatum*	Pearse Valley Nelson	Untreated
P23	*T. tomentosum*	Moutere, Nelson	Untreated
P25	*T. polysporum*	Wharerata Forest, Gisborne	Untreated
P27	*T. hamatum*	Wharerata Forest, Gisborne	Untreated
P29	*T. citrinoviride*	Blenheim	Untreated
P30	*T. aff. deliquescens*	Kaingaroa Forest, Bay of Plenty	Untreated
P31	*T. spirale*	Kaingaroa Forest, Bay of Plenty	Untreated
P32	*T. spirale*	Kaingaroa Forest, Bay of Plenty	Untreated
P33	*T. virens*	Kings Ridge, Nelson	Untreated
P35	*T. austrokoningii*	Pinnacles Forest, Rotorua	Untreated
P36	*T. "novaeharzianum"*	Pinnacles Forest, Rotorua	Untreated
P37	*T. tomentosum*	Pinnacles Forest, Rotorua	Untreated
P38	*T. cf. hamatum*	Pinnacles Forest, Rotorua	Untreated
P39	*T. tomentosum*	Pinnacles Forest, Rotorua	Untreated
P40	*T. crassum*	Kaingaroa Forest, Bay of Plenty	Untreated
P41	*T. crassum*	Kaingaroa Forest, Bay of Plenty	Untreated
P42	*T. trixiae*	Kaingaroa Forest, Bay of Plenty	Untreated
P43	*T. cf. hamatum*	Kaingaroa Forest, Bay of Plenty	Untreated
P44	*T. novaeharzianum*	Kaingaroa Forest, Bay of Plenty	Untreated
7a	*T. virens*	Wharerata Forest, Gisborne	Untreated control
7b	*T. crassum*	Wharerata Forest, Gisborne	Untreated control
9a	*T. virens*	Wharerata Forest, Gisborne	Mix A
9d	*T. trixiae*	Wharerata Forest, Gisborne	Mix A
18c	*T. spirale*	Kohatu, Nelson	PR1
22a	*t. simmonsii*	Kohatu, Nelson	PR3
23b	*T. "sp. 792"*	Kohatu, Nelson	PBI
27c	*T. trixiae*	Moutere, Nelson	PR1
27a	*T. harzianum*	Moutere, Nelson	PR1

Table 1 cont. Collection details for *Trichoderma* isolates obtained from plantation sites with species identification based on analyses of *tef1* sequences.

Isolate	*Trichoderma* Species	Collection locality	Treatment
28e	*T. atroviride*	Moutere, Nelson	Untreated control
A1	*T. hamatum*	Pinnacles Forest, Rotorua	Mix A
A2	n/a	Pinnacles Forest, Rotorua	Mix A
A3	n/a	Pinnacles Forest, Rotorua	Mix A
A5	*T. aff. deliquescens*	Pinnacles Forest, Rotorua	Mix A
8A	*T. atroviride*	Wharerata Forest, Gisborne	PR2
B1	n/a	Pinnacles Forest, Rotorua	Mix B
B2	*T. harzianum*	Pinnacles Forest, Rotorua	Mix B
C1	*T. aff. deliquescens*	Pinnacles Forest, Rotorua	Untreated control
D1	*T. crassum*	Pinnacles Forest, Rotorua	Mix A
J1	*T. crassum*	Pinnacles Forest, Rotorua	Mix A
H1	*T. crassum*	Pinnacles Forest, Rotorua	Mix B
H2	*T. crassum*	Pinnacles Forest, Rotorua	Mix B
E2	*T. aff. deliquescens*	Pinnacles Forest, Rotorua	Mix A
I1	*T. atroviride*	Pinnacles Forest, Rotorua	Mix A
I2	n/a	Pinnacles Forest, Rotorua	Mix A

n/a = identification not confirmed due to sequencing errors

Development of species-specific primer for *T. atrobrunneum*

Several isolates (FCC318, FCC319, FCC320, FCC368) used in plantation trials were previously identified as *T. atrobrunneum* based on phylogenetic analysis of the *tef1* gene region (see previous technical report FOA-BIO-T003). Species-specific primers for *T. atrobrunneum* were developed based on this region and tested against the closely related species *T. harzianum* and several other species including *T. atroviride*, *T. crassum*, *T. polysporum* and the undescribed endemic "species 792". Results produced the expected 581 bp band from *T. atrobrunneum*, which was not shown from the other species tested (Fig. 1).

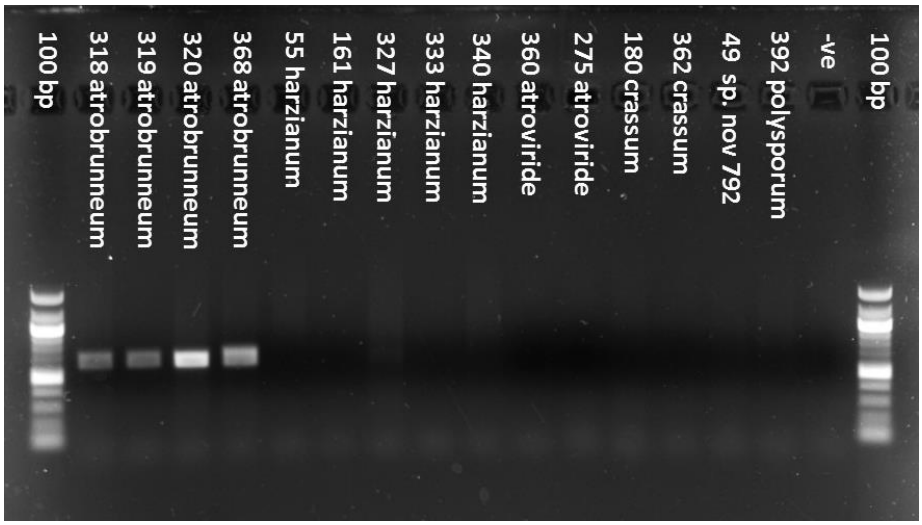

Figure 1. Verification of *T. atrobrunneum*-specific primers Atrb1f and Atrb3r.

Trichoderma atrobrunneum persistence in nursery seedlings

Primers Atrb1 and Atrb3 were used to screen DNA extracts from cultures isolated from nine month-old nursery seedlings which included plants treated with *T. atrobrunneum* strain FCC320 (Fig. 2) The expected size band was amplified from all isolates obtained from surface sterilised roots of seedlings that had been treated with *T. atrobrunneum* FCC320, showing that this isolate is capable of establishing as an internal root colonist following seed treatment.

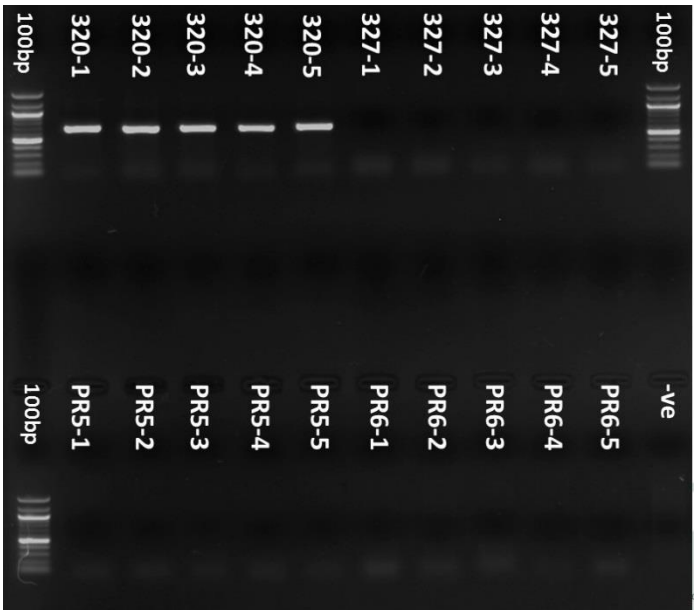

Figure 2. Screening of isolates from nursery seedlings with specific primers for *T. atrobrunneum.*

Trichoderma atrobrunneum persistence in plantation trials

Primers Atrb1 and Atrb3 were used to screen DNA extracts from cultures isolated from nursery seedlings which included *T. atrobrunneum* treatments (Fig 3). The species was only detected in one sample (8a), which was isolated from a plant inoculated with *T. atrobrunneum* strain FCC368 (applied as part of mixture PR2). Sequencing confirmed the isolate as *T. atrobrunneum*, however the sequence differed by several basepairs from that of FCC368, suggesting that the isolate is an environmental strain of *T. atrobrunneum*.

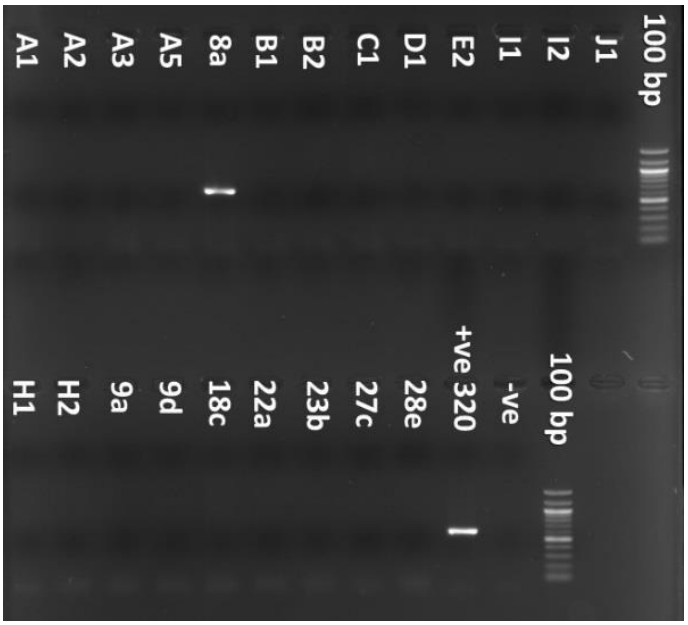

Figure 3. Screening of isolates from plantation trials with specific primers for *T. atrobrunneum*

Trichoderma atroviride persistence in plantation trials

Analysis using strain-specific primers RM5/RM6K detected LU633 in two cultures isolated from roots of the same treated tree at the Pinnacles forest plantation trial (I1 and I2, Fig. 4) . A *tef1* sequence was obtained from isolate I1 and showed 100% similarity match with a sequence from LU633 (Fig 5). The sampled tree was planted in 2012 and had been treated with "Mix A" (comprising Trichoderma strains LU132, 140, 584 and 633). Our result shows that at least in some cases LU633 is able to establish and persist for several years as an internal colonist of pine roots. Screening with specific primers for LU584 (RM1/2) was unable to detect this strain in the samples tested, and correspondingly none of the sequences examined matched with LU584. Two sequences originating from cultures isolated from untreated plants showed identical matches with LU140 and 132, indicating that this *tef1* haplotype does occur naturally in pine plantations, although apparently at low levels.

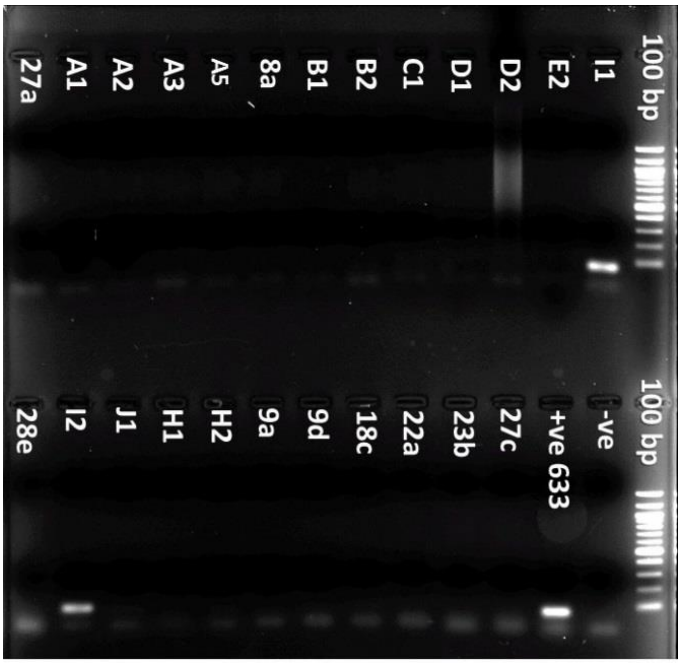

Figure 4. Screening of isolates from plantation trials with specific primers for strain LU633

Sequence-based identification of *Trichoderma* isolates

A wide range of *Trichoderma* species were identified from cultures isolated from the roots of treated and untreated *P. radiata* collected at plantation sites (Table 1, Figs 5-8). Isolates were distributed amongst 13 currently named species: *T. atrobrunneum*, *T. austrokoningii, T. atroviride*, *T. citrinoviride*, *T. crassum*, *T. hamatum*, *T. harzianum*, *T. longipile*, *T. polysporum, T. spirale, T. tomentosum, T. trixiae*, and *T. virens*. Two apparently undescribed taxa (designated as "sp. 702" and "novaeharzianum") were also present in our samples. These correspond with two endemic *Trichoderma* species which have also been previously isolated from soils and other substrates in NZ. Several other isolates formed a distinct group related to *T. deliquescens* that is likely to represent an additional novel species. However, due to a lack of available tef1 sequence data from closely related species their taxonomic position could not be reliably confirmed by phylogenetic analysis. Analysis of sequence data from other genes is required to fully clarify the relationships of this group within *Trichoderma.*

Although sequencing results appeared to confirm persistence of the applied strain LU633 in one case, isolates examined from *Trichoderma*-treated plants were generally identified as different species from those of the applied strains. This highlights the difficulty of using a direct culturing approach to accurately confirm persistence in plantation trial sites, especially in the presence of diverse background *Trichoderma* populations. Isolation methods are likely to select for faster-growing or otherwise more competitive *Trichoderma* species (e.g. *T. virens*) which may obscure the recovery of applied strains from isolation plates. Additionally, results may be particularly affected by sampling bias. Isolations from root material usually yield a large number of

Trichoderma colonies, however practical constraints permit examination of only a limited number of cultures, which may not be fully representative of all species/strains occurring in the roots.

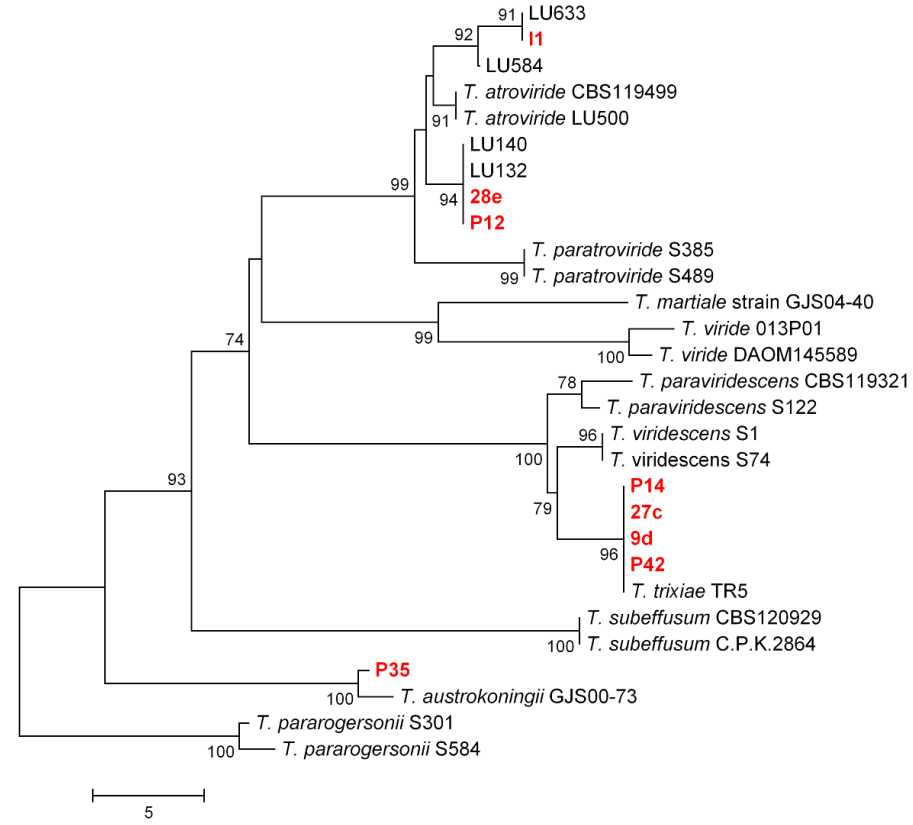

Figure 5. Neighbour-joining analysis of *tef1* sequences from isolates *T. atroviride* and related species.

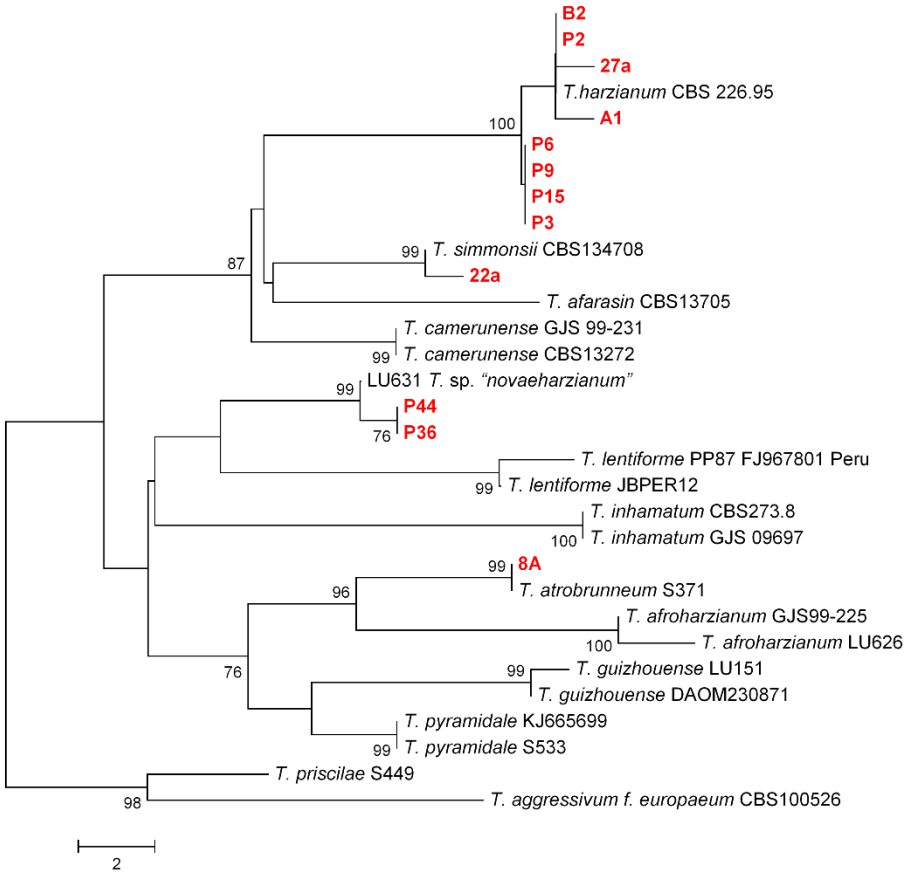

Figure 6. Neighbour-joining analysis of *tef1* sequences from isolates in the *T. harzianum* complex.

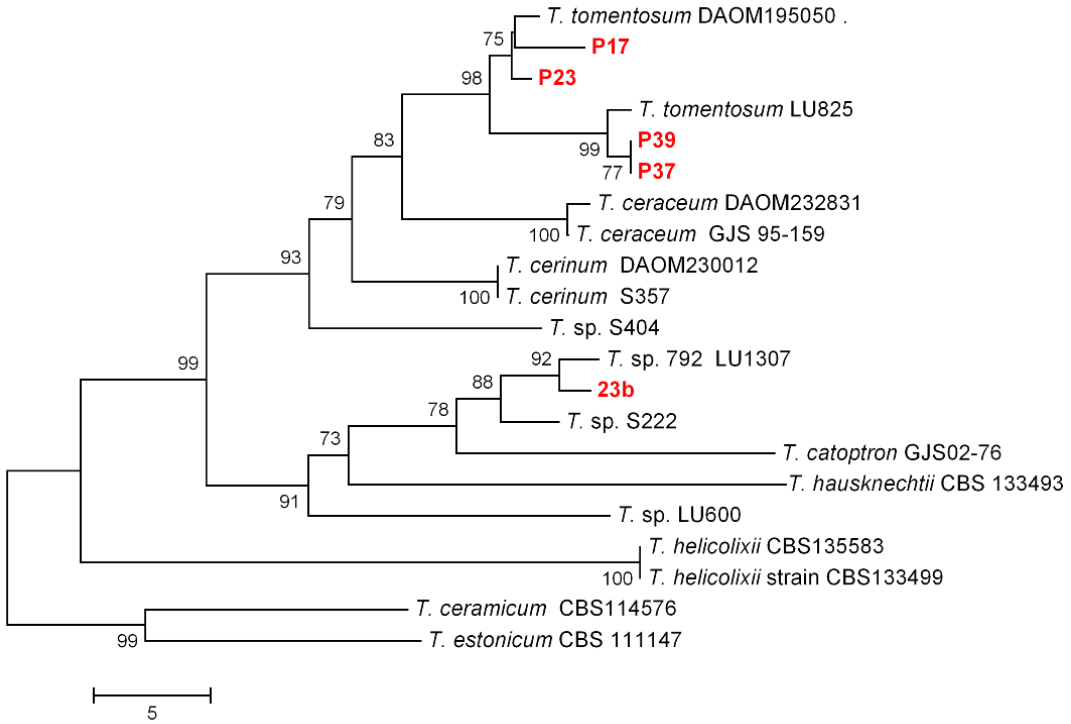

Figure 7. Neighbour-joining analysis of *tef1* sequences from *T. tomentosum* and related species.

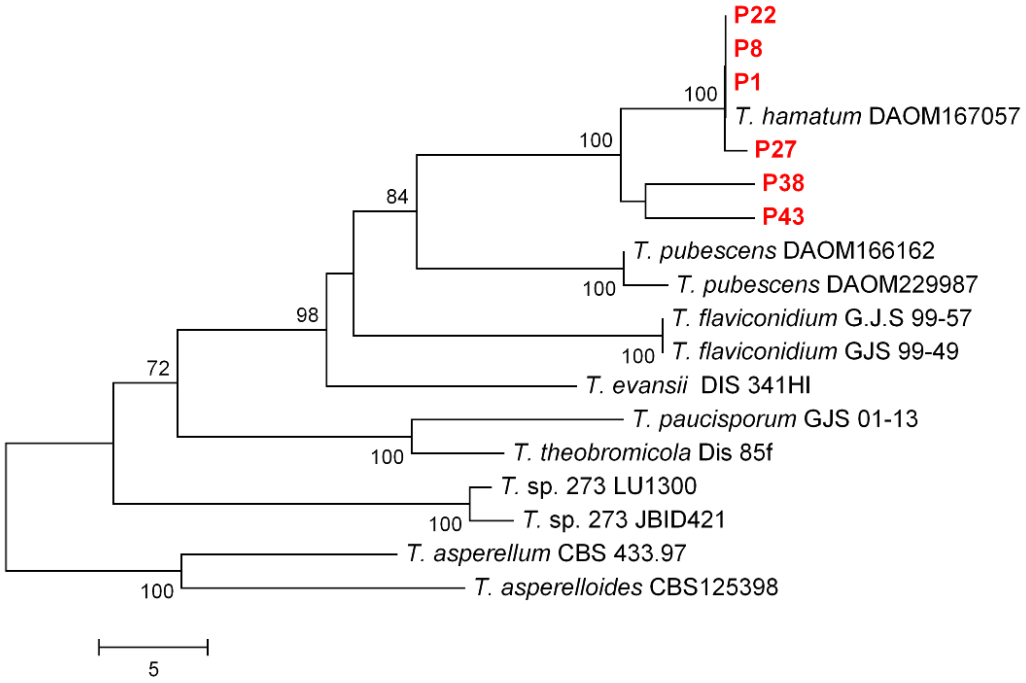

Figure 8. Neighbour-joining analysis of *tef1* sequences from *T. hamatum* isolates.

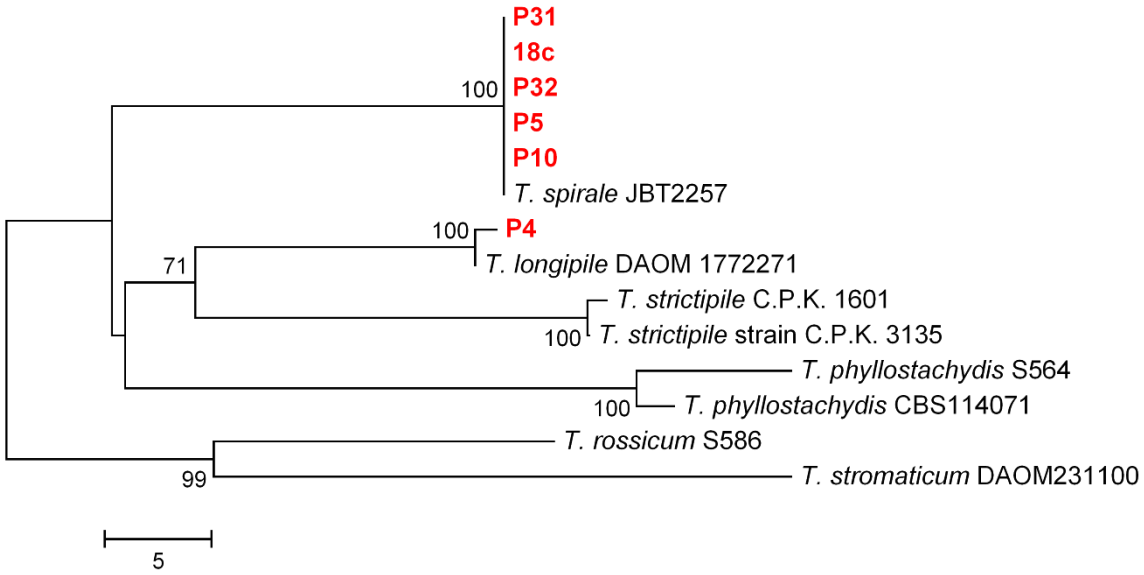

Figure 9. Neighbour-joining analysis of *tef1* sequences from *T. spirale and T. longipile* isolates.

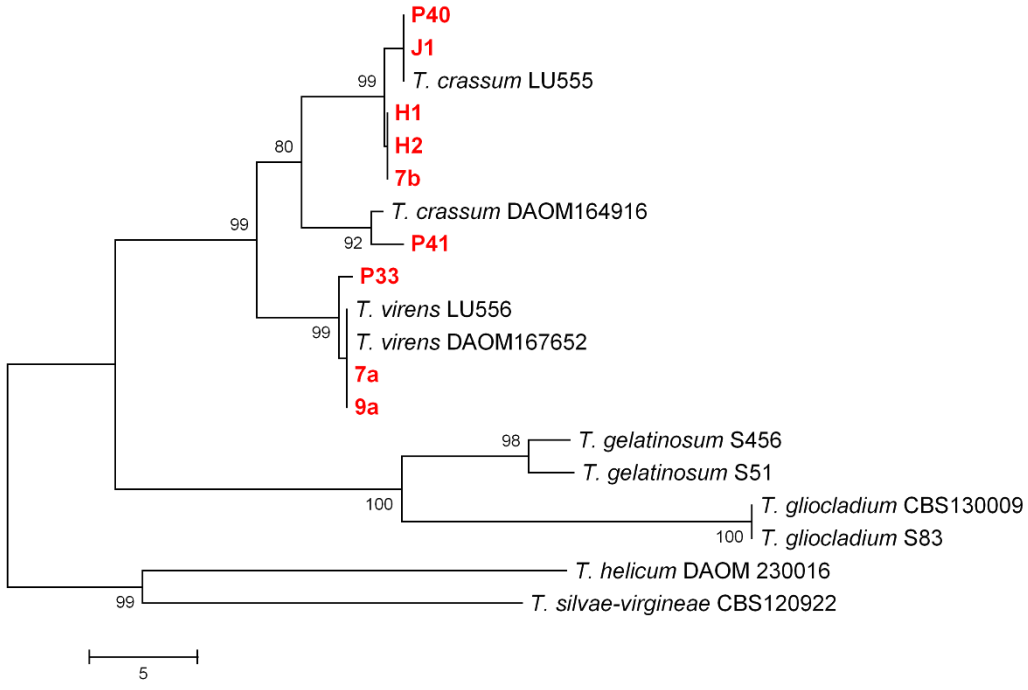

Figure 10. Neighbour-joining analysis of *tef1* sequences from *T. crassum* and *T. virens* isolates.

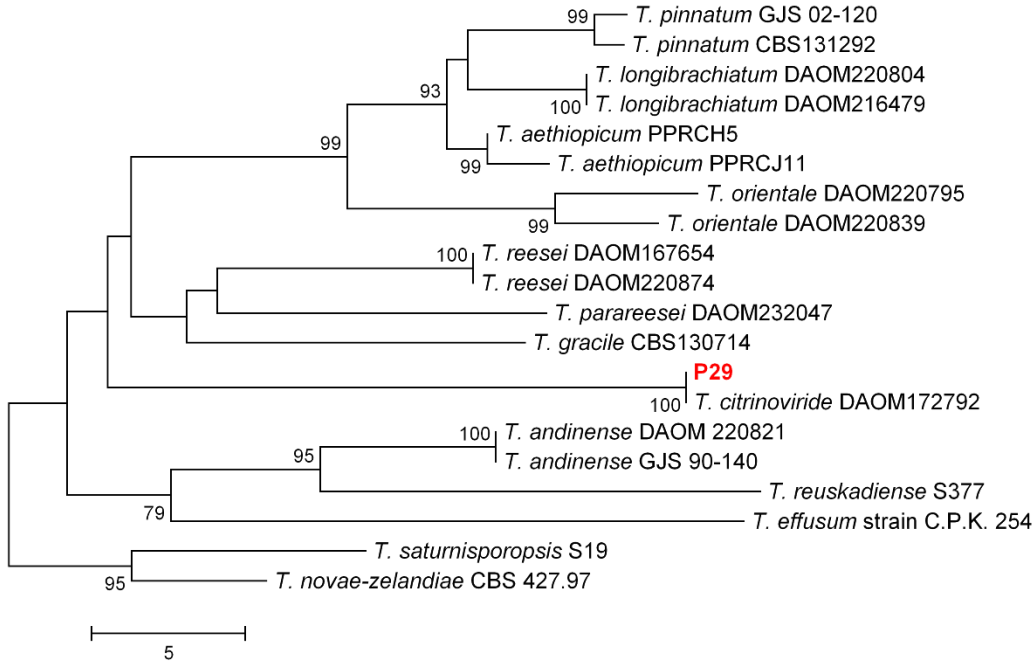

Figure 11. Neighbour-joining analysis of *tef1* sequence from the single isolate of *T. citrinoviride.*

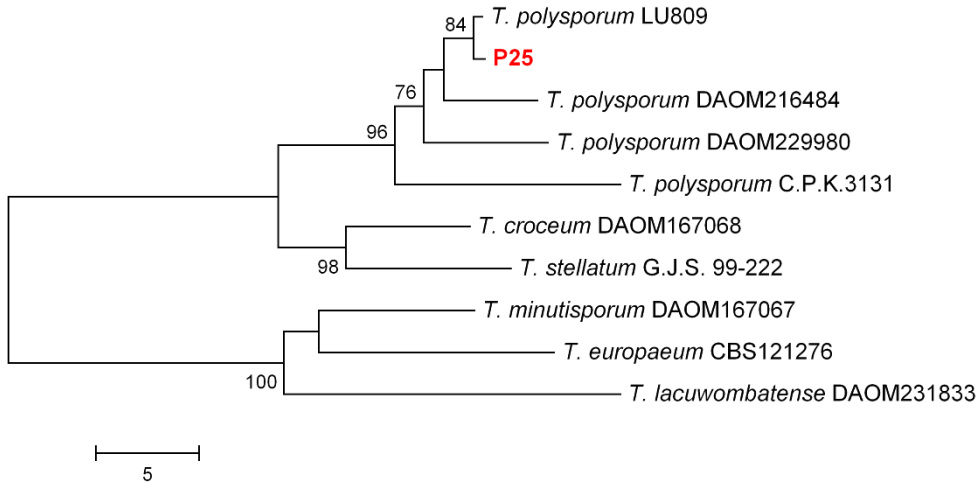

Figure 12. Neighbour-joining analysis of *tef1* sequence from the single isolate of *T. polysporum.*

CONCLUSIONS & RECOMMENDATIONS

- Strain-specific primers were able to confirm persistence of *T. atroviride* strain LU633 in cultures isolated from plantation trial sites.
- A species-specific primer was developed for *T. atrobrunneum* and was used to detect this species in isolates obtained from plantation trials and nursery plants.
- Sequence-based identifications revealed a diverse population of *Trichoderma* species in natural association with plantation trees, including at least 16 species
- Direct sequencing of the *tef1* barcoding region was also used to identify cultures isolated from plantation trials. However, in most cases isolates examined from inoculated trees represent *Trichoderma* species that occur naturally in plantation systems.
- While results show that re-isolation of applied *Trichoderma* strains from plantation trials can be achieved, practical considerations and the effects of sampling bias are likely to limit the value of this method for accurately determining isolate persistence.
- Further research efforts should focus on the development of additional strain-specific assays for direct detection of *Trichoderma* isolates from root tissues. Application of these methods will allow more efficient and cost-effective confirmation of persistence and additionally provide quantitative data.

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