Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Monitoring establishment and long-term persistence of a model *Trichoderma* strain (LU633) in *Pinus radiata* plantation trials using qPCR

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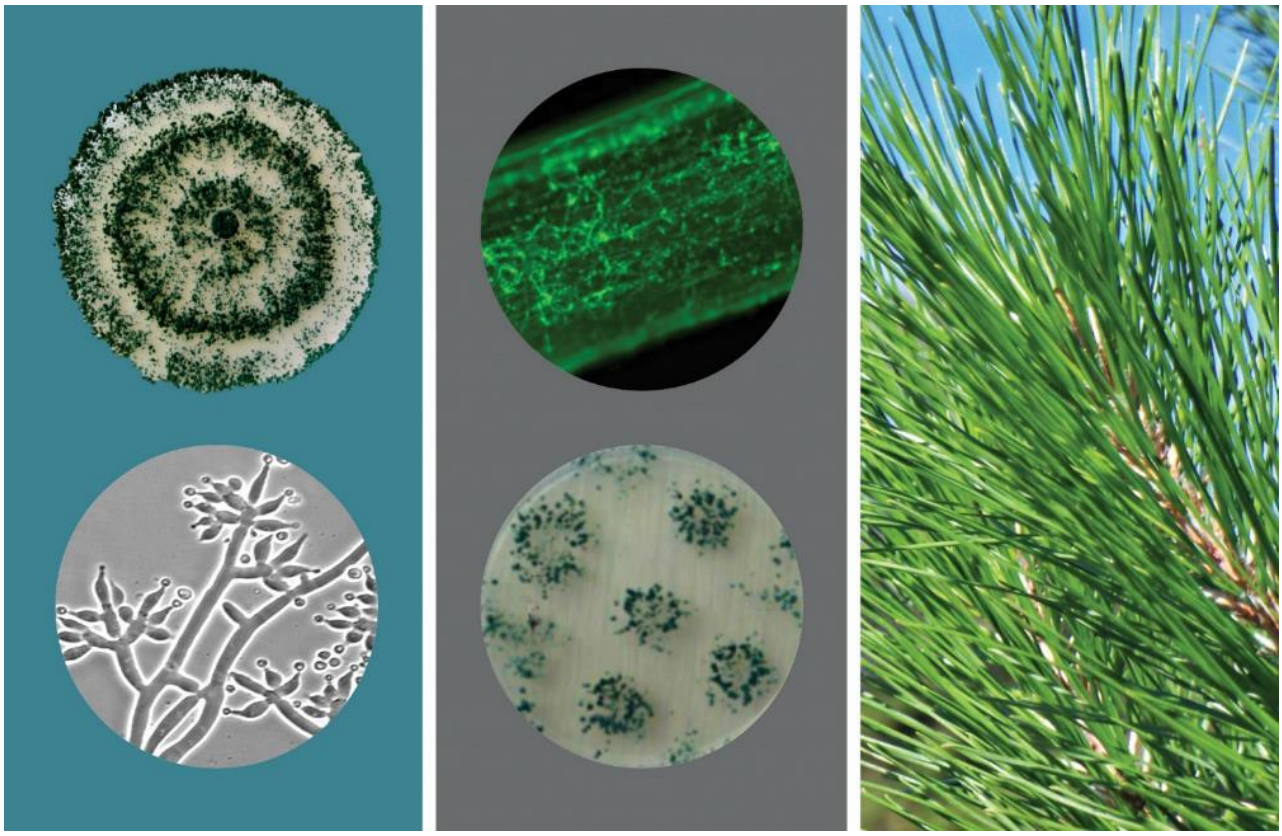

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FOREST GROWERS RESEARCH

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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 New Zealand to examine the effects of specific endophytic *Trichoderma* strains on *Pinus radiata* growth and disease resistance. An important question about deployment of these strains is whether they can establish and persist (*i.e.* continue to exist) under field conditions to increase their chances of providing long-term growth benefits and protection from disease.

This report follows on from previous greenhouse *P. radiata* seedling work looking at colonisation and persistence of *Trichoderma* strains used in our forestry bioprotection trials. A sensitive, strain-specific real-time polymerase chain reaction (qPCR) assay was used to determine the persistence of one of the *Trichoderma* bioprotectant strains, LU633, in six bioprotection trials planted 3.5 years ago.

Key Results

- The majority of samples (47 out of 52) showed presence of *T. atroviride* LU633 in the singleplex reaction qPCR assay using LU633 primers and probe, with nine samples from the Golden Downs (Kohatu block), Harakeke and Waipaoa plantations having abundant LU633 DNA material, according to cycle threshold (Ct) values.

- *P. radiata* root samples treated with *T. atroviride* LU633 were shown to have LU633 DNA levels ranging from 0 to 2.6 ng/µL with the highest levels in the Golden Downs (Kohatu block) plantation at an average of 0.39 ng/µL.

INTRODUCTION

Endophytic *Trichoderma* fungi show great potential for improving health of forest trees. The main goal of the *Bioprotection for foliar diseases and disorders of radiata pine* research programme is to induce systemic resistance against foliar diseases through the use of beneficial endophytes and elicitors. Initial research in this programme isolated a large number of *Trichoderma* strains which were screened for the ability to promote growth and suppress disease in laboratory and nursery trials. The most effective strains were applied as nursery seed treatments and form the basis for *Pinus radiata* planation trials at sites in New Zealand. For these specific strains to be used as beneficial biocontrol agents with long-lived forest trees, they must colonise and persist over the long term in the host root system.

Previous research focus has been on colonisation and persistence of selected biocontrol *Trichoderma* strains in *P. radiata* seedlings. *Trichoderma* strains have been found to colonise roots at an early stage of seedling growth, at or before seedlings emerge from the soil (Hill *et al.,* 2016). In a second study, *Trichoderma* hyphae were found to proliferate in growing seedling roots and were extensively developed at 8 months of age (Whelan and Hill, 2017). At least 60% of the root pieces were colonised, with one *T. atrobrunneum* strain FCC327 having infected all the root pieces analysed. There is limited information about the persistence of these bioprotectant *Trichoderma* strains in established plantation tree roots.

Trichoderma species are ubiquitous in the environment and application of molecular techniques is necessary to allow differentiation of biocontrol strains from natural *Trichoderma* populations resident in soil and plant roots. Kabir *et al.* (2015) developed a polymerase chain reaction (PCR) assay with specific primers that could detect genomic DNA sequences unique to the *T. atroviride* LU633 strain, one of the strains currently under evaluation in the New Zealand plantation trials. This assay was further developed by Mellow *et al.* (2015) where the use of a specific probe, in addition to the specific primers, made the assay sensitive and specific for detection of the LU633 strain in field *P. radiata* root samples where the concentration of LU633 DNA material is likely to be low.

The primary aim of Task 2.1 was to investigate, using the Mellow e*t al*. (2015) PCR assay, the presence of *T. atroviride* LU633 strain in 3.5-year-old plantation tree roots to obtain information on long-term persistence following field planting. A secondary aim of Task 2.1 was to quantity the amounts of LU633 present in the samples.

METHODS

Sample collection

Roots were collected from five to twelve *P. radiata* trees in each of six *Trichoderma* bioprotection plantation trials (Table 1). Trees were selected from Treatment 1 that had *Trichoderma* strains LU633, LU132, LU140 and LU584 (Bio-Protection Research Centre culture collection, Lincoln University, New Zealand) applied, in equal proportions, as a seed-coat. Roots sampled were generally small feeder roots within the top 300 mm from ground level. Roots were moistened and stored in zip-locked bags at 4°C, then washed

thoroughly in running tap water and frozen at -20°C, for a maximum of 9 months, until DNA extraction.

Table 1: Root sample collection and treatment details in this study

Region	Location	Name of Forest Company and Plantation Name	Locality Code	No. of trees sampled	Treatment
Nelson	Kohatu	Nelson Forests Ltd, Golden Downs (Kohatu block)	Ko	12	+ *T. atroviride LU633*
Nelson	Kikiwa	Nelson Forests Ltd, Golden Downs (Kings Ridge block)	KR	10	+ *T. atroviride LU633*
Gisborne	Inland Tokomaru Bay	Ernslaw One Ltd, Waiau	W	10	+ *T. atroviride LU633*
Gisborne	Inland Whatatutu	Ernslaw One Ltd, Waipaoa	Wp	10	+ *T. atroviride LU633*
Manawatu-Whanganui	Ohakune	Ernslaw One Ltd, Karioi	Ka	5	+ *T. atroviride LU633*
Manawatu-Whanganui	Whanganui	Ernslaw One Ltd Harakeke	H	5	+ *T. atroviride LU633*
Canterbury	Kaituna Valley	small tree lot	UT1	1	- *Trichoderma*
Canterbury	Motukarara	small tree lot	UT2	1	- *Trichoderma*
Canterbury	Rakaia	small tree lot	UT3	1	- *Trichoderma*
Nelson	Kohatu	Nelson Forests Ltd, Golden Downs (Kohatu block)	UT4	1	- *Trichoderma*
Nelson	Kikiwa	Nelson Forests Ltd, Golden Downs (Kings Ridge block)	UT5	1	- *Trichoderma*
Gisborne	Inland Tokomaru Bay	Ernslaw One Ltd, Waiau	UT6	1	- *Trichoderma*
Gisborne	Inland Whatatutu	Ernslaw One Ltd, Waipaoa	UT7	1	- *Trichoderma*

DNA extraction and probe-based qPCR

Each sample of frozen *P. radiata* root fragments was cut aseptically into approximately 20 mm length and ground separately to a powder in a sterile motar, using liquid nitrogen. Ground tissue was transferred to a sterile 1.7 mL tube and kept on ice until DNA extraction or alternatively stored at -80°C without thawing.

For fungal positive and negative controls, *T. atroviride* strain LU633 and *T. virens* (Bio-Protection Research Centre culture collection, Lincoln University) spores (1×10^3 / 5 mL) were placed in sterile potato dextrose broth (24 g/L, Difco) at 28°C and shaken at 150 rpm for 48 hours. Mycelia was filtrated and ground in liquid nitrogen. An additional negative control of double distillate sterile water was included.

Genomic DNA was extracted from plant and fungi using a mini plant genomic DNA extraction kit (Geneaid, New Taipei, Taiwan), following the manufacturer's instructions, and then quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Presence and abundance of *Trichoderma* strain LU633 was determined using real-time PCR, using fluorescently labelled probes and dyes to measure the progression of DNA

amplification during the reaction. Real-time PCR was carried out with both *Trichoderma* (LU633) and *P. radiata* (CAD) primer sets using *P. radiata* DNA templates. Primers and fluorescent probes were previously designed (Mellow, *et al*., 2015; Table 2). Singleplex PCR was used to detect the presence of LU633 DNA using only LU633 primers and probe. Duplex PCR was used to amplify both LU633 and *P. radiata* DNA simultaneously in the same reaction tubes, by adding both LU633 and *P. radiata* (CAD) primers and probes. Duplex reactions theoretically allow normalisation (account for differences in the starting concentration of total DNA) of, thus accurate quantification of LU633 DNA. The fungal and plant probes have different fluorescent tags so can be distinguished from one another in the duplex qPCR reactions. The 5' and 3' ends of LU633-specific TrichKa probe were labelled with 6-carboxyfluorescein (FAM) and quencher dye BHQ-1, respectively. The 5' and 3' ends of the *P. radiata* cinnamyl alcohol dehydrogenase (CAD) gene probe were labelled with HEX and BHQ-1, respectively (Chettri *et. al.,* 2012). Real-time PCR with probes was carried out using the qPCR protocol of Chettri *et al.* (2012) and the reaction was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems™).

Table 2. Primers and probes sequences used

Target	Primer/probe name	Sequence (5' to 3')	Amplicon size (bp)
P. radiata	Primer CAD918 Primer CAD1019 Probe 945	CAGCAAGAGGATTTGGACCTA TTCAATACCCACATCTGATCAAC HEX-TGTGAACCATGACGGCACCC-BHQ1	101
LU633	Primer RM5Ka Primer RM6Ka Probe TrichKa	GCAAGTTGGATACAGTTG CCAGTAACTAGAATCGCA 6FAM-TTGTATTAGTCCCACTCTATCAAG-BHQ1	72

The reactions used PerfeCTa qPCR ToughMix, ROX (2x) (Quanta Biosciences) where each 10 µL singleplex reaction contained: 5 µL of 2× qPCR ToughMix, 1 µL 10× primer probe mix, 2 µL PCR grade water and 2 µL DNA template. For duplex reactions, each 10 µL of reaction contained: 5µL of 2× qPCR ToughMix, 2 µL 10× *Trichoderma* LU633 primer probe mix, 1µL *P. radiata* CAD primer probe mix and 2 µL DNA template. The 10× primer probe mix consisted of *T. atroviride* LU633 TrichKa probe, and/or *P. radiata* CAD probe 945, each at 2 µM, along with LU633 specific primers (RM5ka & RM6ka) or *P. radiata* specific primers CAD918, CAD1019, each at 4 µM.

PCR was conducted with pre-incubation at 95^{o} C for 10 minutes followed by 55 cycles of: denaturation at 95°C for 10 seconds, annealing at 58°C for 15 seconds, extension at 72°C for 20 seconds, followed by cooling at 40°C for 10 seconds**.** Positive (DNA from LU633) and negative (water and DNA from *T. virens*) controls were included in each real-time PCR run and reactions were run in triplicate.

A standard curve was prepared with 6 replicate samples of nine-fold serially diluted LU633 DNA (concentrations used were 5 ng/µL, 1.25 ng/µL, 0.3125 ng/µL, 0.078125 ng/µL, 0.0195 ng/uL, 0.00489 ng/uL, 0.00122 ng/µL, 0.0003 ng/µL, and 0.0009 ng/µL) spiked with *P. radiata* root DNA at 10 ng/µL.

RESULTS AND DISCUSSION

A sensitive, species-specific qPCR assay, capable of detecting *T. atroviride* LU633 strain DNA, was used to determine the presence and abundance of this bioprotectant strain in *P. radiata* tree roots sampled from six planation locations in the North and South Island of New Zealand. Nearly all samples (47 out of 52) had amplification (i.e. presence) of *T. atroviride* LU633 genetic material in the singleplex reaction assay using LU633 primers and probe, with nine samples from the Golden Downs (Kohatu block), Harakeke and Waipaoa plantations having abundant LU633 DNA material (Table 3; Figure 1). This, and previous studies, have indicated that not only is this bioprotectant strain capable of establishing strongly in seedling roots when applied as a seed-coat (Hill *et al.*, 2016; Whelan and Hill, 2017), but also able to persist once the seedlings have been planted in the field, at least until the trees are 3.5 years old. Presence of LU633 in roots implies provision of growth benefits and protection from disease. Confirmation of growth and health benefits can be made by field measurements of tree growth parameters (e.g. height and diameter) and disease presence. In the New Zealand *Trichoderma* bioprotectant trials, average height and diameter of young trees have been measured at up to 20% and 15% greater in treated, compared to untreated, trees (Hill and Whelan, 2017).

As the tree matures, the bioprotectant may be exposed to environmental change, including population changes in rhizoplane, rhizosphere and root endophytic micro-organisms, tree resource competition and climate change, particularly at canopy closure. Further monitoring work is required to determine if environmental stress/change affects the persistence, and potentially the effectiveness, of the LU633 strain, and therefore the long-term bioprotection of older plantations.

Five of the treated trees in this study did not have presence of applied LU633 and may have been because the LU633 strain:

- was at an undetectable level using the current assay,
- did not colonise the seedling roots during seedling growth,
- did not survive during tree establishment and was outcompeted by other microbe-organisms (including one or more of the original co-bioprotectant strains applied as part of the seed-coat).

Strains of *T. atroviride* that occur naturally in New Zealand *P. radiata* plantations appear to be at relatively low levels. Cummings and Hill (2016) found *T. atroviride* in 6% of samples harvested from eight plantations, while Hill *et al*. (2017) found it present in one out of nine plantation sites. In the current study, the absence of *Trichoderma* strain LU633 in the seven untreated control *P. radiata* trees (Table 3; Figure 2) was expected because the chance of this specific *T. atroviride* LU633 strain naturally infecting tree roots was very low in these plantation environments.

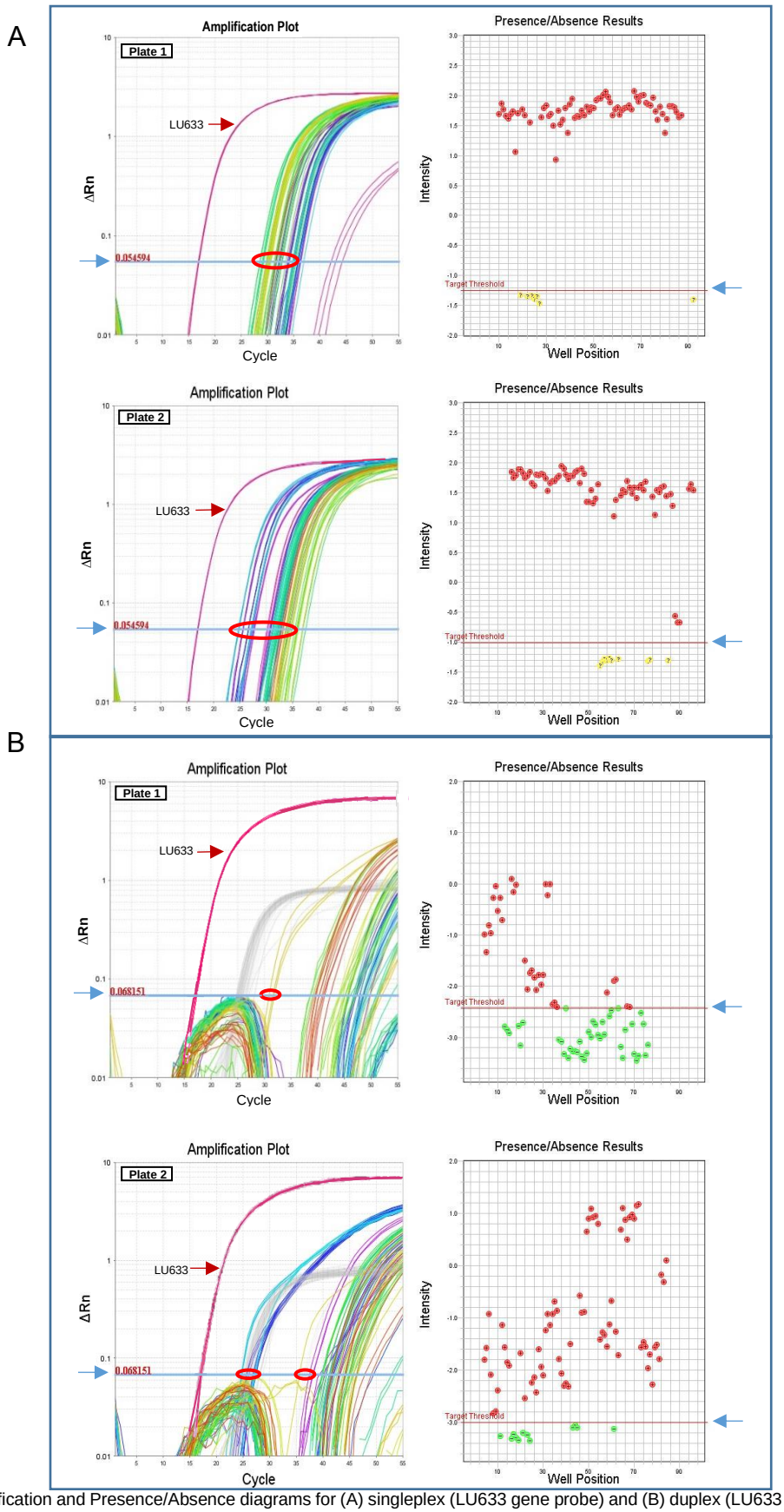

Figure 1: Amplification and Presence/Absence diagrams for (A) singleplex (LU633 gene probe) and (B) duplex (LU633 and *P. radiata* CAD gene probe) qPCR reactions of root samples from six plantation sites in two PCR plates. Amplification diagrams show the variation of fluorescence (log (ΔRn)) and PCR cycle number. ΔRn is Rn minus the reaction baseline and Rn is the fluorescence of the probe reporter dye normalised by dividing into the fluorescence of the passive reference dye. The Ct value (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the target threshold (blue arrow) and values are presented in Table 3. Red ovals indicate samples with positive reactions indicative of moderate or abundant amounts of target DNA. Grey lines in the duplex reaction diagram indicate the fluorescence signature of the *P. radiata* samples. All replicate samples are presented.
For the presence/absence diagrams, red circles indicate amplification of LU633 DNA above the threshold, yellow circles indicate amplification but at levels below the threshold and green circles indicate no amplification.

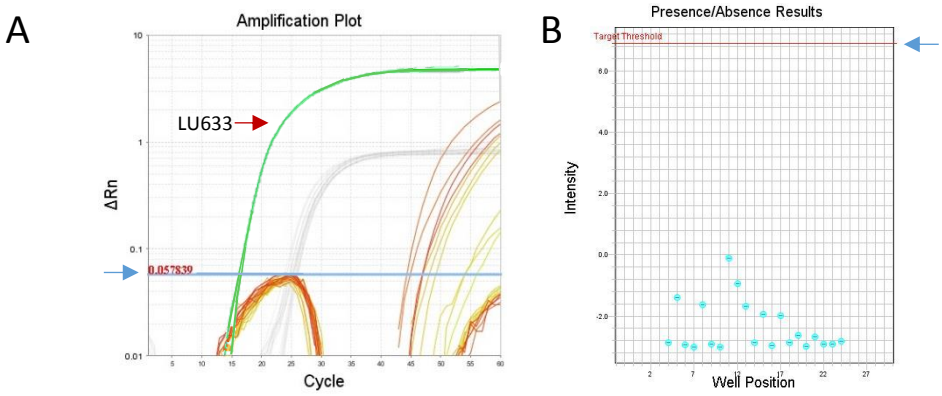

Figure 2: Amplification (A) and Presence/Absence (B) diagrams for duplex reaction qPCR of untreated/control root samples. Amplification diagrams show the variation of fluorescence (log (ΔRn)) and PCR cycle number. ΔRn is Rn minus the reaction baseline and Rn is the fluorescence of the probe reporter dye normalised by dividing into the fluorescence of the passive reference dye. The Ct value (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the target threshold (blue arrow) and values are presented in Table 3. Grey lines in the duplex reaction diagram indicate the fluorescence signature of the *P. radiata* samples. All replicate samples are presented.
For the presence/absence diagram, blue circles indicate no amplification.

Table 3: Amplification results of singleplex and duplex reactions using LU633/ *P. radiata* CAD probe-based qPCR system in LU633 treated and untreated *P. radiata* samples collected from different plantations in New Zealand.

	SINGLEPLEX REACTION			DUPLEX REACTION				
Location	**Sample**	**Ct value [a] LU633**	**LU633 detection [b]**	**Ct value LU633**	**LU633 detection**	**Ct value *P. radiata***	***P. radiata* detection**	**Concentration LU633 (ng/µL)**
	Water	>55.0 ± 0.0	Negative control	>55.0 ± 0.0	Negative control	>55.0 ± 0.0	Negative control	-
	T. virens	NT	Negative control	>55.0 ± 0.0	Negative control	>55.0 ± 0.0	Negative control	NT
	LU633	17.0 ± 0.1	Positive control	16.6 ± 0.3	Positive control	NT	NT	NT
INLAND TOKOMARU BAY	W1	33.1 ± 0.2	+	42.9 ± 1.2	+	28.6 ± 0.0	+	0.0125 ± 0.0015
	W2	33.8 ± 0.1	+	46.7 ± 3.2	+	30.5 ± 0.1	+	0.0081 ± 0.0004
	W3	35.2 ± 1.6	+	44.0 ± 3.4	+	29.4 ± 0.1	+	0.0044 ± 0.0035
	W4	34.6 ± 0.3	+	45.0 ± 1.0	+	28.6 ± 0.2	+	0.0049 ± 0.0011
	W5	49.3 ± 9.8	-	>55.0 ± 0.0	-	28.3 ± 0.0	+	0.0000 ± 0.0000
	W6	>55.0 ± 0.0	-	>55.0 ± 0.0	-	*	*	0.0000 ± 0.0000
	W7	34.8 ± 1.0	+	55.0 ± 0.0	-	30.8 ± 0.3	+	0.0048 ± 0.0026
	W8	35.8 ± 0.3	+	46.1 ± 0.6	+	28.1 ± 0.2	+	0.0023 ± 0.0004
	W9	35.9 ± 1.0	+	44.3 ± 0.5	+	30.4 ± 0.1	+	0.0024 ± 0.0015
	W10	34.8 ± 2.5	+	41.6 ± 1.0	+	29.0 ± 0.0	+	0.0079 ± 0.0079
KOHATU	Ko1	32.2 ± 0.2	+	40.6 ± 1.1	+	28.2 ± 0.2	+	0.0222 ± 0.0026
	Ko2	31.3 ± 0.1	+	45.1 ± 0.8	+	28.3 ± 0.1	+	0.0405 ± 0.0035
	Ko3	32.4 ± 0.7	+	44.1 ± 1.5	+	29.0 ± 0.1	+	0.0209 ± 0.0082
	Ko4	31.7 ± 0.1	+	53.8 ± 1.1	-	27.4 ± 0.1	+	0.0319 ± 0.0020
	Ko5	31.9 ± 0.3	+	39.8 ± 0.1	+	28.1 ± 0.2	+	0.0281 ± 0.0047
	Ko6	27.3 ± 0.1	+	27.2 ± 0.1	+	29.4 ± 0.2	+	0.5395 ± 0.0365
	Ko7	24.8 ± 0.0	+	24.7 ± 0.1	+	27.6 ± 0.0	+	2.6065 ± 0.0819
	Ko8	32.6 ± 0.1	+	41.5 ± 0.3	+	28.7 ± 0.1	+	0.0172 ± 0.0016
	Ko9	31.3 ± 0.3	+	41.9 ± 1.3	+	26.7 ± 0.2	+	0.0415 ± 0.0089
	Ko10	32.5 ± 0.3	+	41.6 ± 0.3	+	27.5 ± 0.1	+	0.0182 ± 0.0030
	Ko11	26.7 ± 0.0	+	26.9 ± 0.1	+	27.7 ± 0.1	+	0.7906 ± 0.0064
	Ko12	27.3 ± 0.1	+	27.3 ± 0.2	+	28.5 ± 0.0	+	0.5230 ± 0.0394
WHANGANUI	H1	25.6 ± 0.0	+	25.6 ± 0.2	+	26.4 ± 0.1	+	1.5507 ± 0.0460
	H2	30.9 ± 0.3	+	42.4 ± 0.6	+	27.8 ± 0.2	+	0.0535 ± 0.0091
	H3	33.9 ± 0.8	+	44.7 ± 0.5	+	28.0 ± 0.1	+	0.0084 ± 0.0050
	H4	27.7 ± 0.1	+	40.0 ± 0.3	+	27.7 ± 0.0	+	0.4005 ± 0.0208
	H5	30.5 ± 0.4	+	38.0 ± 0.4	+	27.0 ± 0.2	+	0.0701 ± 0.0152

[a] Ct values indicates the PCR cycle number at which PCR cycle amplification became exponential. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, where:
Cts ≤ 29 are strong positive reactions indicative of abundant target nucleic acid in the sample,
Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid,
Cts ≥ 38 are weak reactions indicative of minimal or zero amounts of target nucleic acid.
Mean of three replicates.

[b] + indicates amplification, - indicates no amplification, NT = not tested, * = value outside expected range.

Table 3: Continued.

Location	SINGLEPLEX REACTION			DUPLEX REACTION				
	Sample	Ct value [a] LU633	LU633 [b] detection	Ct value LU633	LU633 detection	Ct value *P. radiata*	*P. radiata* detection	*Concentration* LU633 (ng/µL)
INLAND WHATATUTU	Wp1	29.9 ± 0.1	+	41.6 ± 0.7	+	27.3 ± 0.0	+	0.0997 ± 0.0055
	Wp2	30.5 ± 0.2	+	39.9 ± 0.4	+	27.2 ± 0.0	+	0.0685 ± 0.0068
	Wp3	30.4 ± 0.1	+	40.2 ± 0.7	+	27.4 ± 0.0	+	0.0724 ± 0.0068
	Wp4	30.2 ± 0.4	+	51.7 ± 0.4	-	27.0 ± 0.1	+	0.0830 ± 0.0241
	Wp5	31.5 ± 0.3	+	31.4 ± 0.5	+	33.8 ± 0.0	+	0.0358 ± 0.0059
	Wp6	35.0 ± 0.6	+	50.3 ± 1.6	-	30.0 ± 0.1	+	0.0040 ± 0.0017
	Wp7	32.9 ± 0.6	+	44.7 ± 0.8	+	28.3 ± 0.2	+	0.0153 ± 0.0050
	Wp8	28.7 ± 0.2	+	44.5 ± 0.6	+	27.0 ± 0.1	+	0.2103 ± 0.0291
	Wp9	32.1 ± 0.7	+	45.5 ± 0.3	+	27.1 ± 0.0	+	0.0248 ± 0.0101
	Wp10	29.2 ± 0.1	+	38.7 ± 0.4	+	27.4 ± 0.0	+	0.1542 ± 0.0083
OHAKUNE	Ka1	>55.0 ± 0.0	-	52.9 ± 3.7	-	28.5 ± 0.0	+	0.0000 ± 0.0000
	Ka2	34.6 ± 1.2	+	48.0 ± 0.6	+	27.8 ± 0.0	+	0.0057 ± 0.0032
	Ka3	35.5 ± 0.6	+	>55.0 ± 0.0	-	28.2 ± 0.1	+	0.0030 ± 0.0012
	Ka4	36.2 ± 0.1	+	48.3 ± 2.0	-	29.2 ± 0.2	+	0.0016 ± 0.0019
	Ka5	43.1 ± 1.1	+	>55.0 ± 0.0	-	31.1 ± 0.1	+	0.0000 ± 0.0000
KIKIWA	KR1	30.2 ± 0.1	+	46.7 ± 0.4	+	27.9 ± 0.2	+	0.0838 ± 0.0077
	KR2	35.3 ± 0.6	+	52.5 ± 2.2	-	26.9 ± 0.1	+	0.0033 ± 0.0011
	KR3	35.7 ± 1.1	+	52.8 ± 3.8	-	26.7 ± 0.0	+	0.0027 ± 0.0015
	KR4	>55.0 ± 0.0	-	>55.0 ± 0.0	-	27.9 ± 0.0	+	0.0000 ± 0.0000
	KR5	>55.0 ± 0.0	-	54.6 ± 0.7	-	28.7 ± 0.2	+	0.0000 ± 0.0000
	KR6	35.7 ± 0.7	+	51.9 ± 2.7	-	27.5 ± 0.1	+	0.0025 ± 0.0011
	KR7	35.8 ± 0.0	+	49.0 ± 0.2	-	27.4 ± 0.0	+	0.0022 ± 0.0000
	KR8	35.5 ± 0.7	+	49.7 ± 1.6	-	27.5 ± 0.0	+	0.0028 ± 0.0013
	KR10	34.4 ± 0.5	+	47.1 ± 0.6	+	28.0 ± 0.2	+	0.0055 ± 0.0015
	KR11	32.4 ± 0.3	+	46.1 ± 0.7	+	26.9 ± 0.1	+	0.0197 ± 0.0040
UNTREATED ROOTS	UTI	>55.0 ± 0.0	-	>55.0 ± 0.0	-	27.3 ± 0.1	+	0.0000 ± 0.0000
	UT2	>55.0 ± 0.0	-	>55.0 ± 0.0	-	28.0 ± 0.0	+	0.0000 ± 0.0000
	UT3	>55.0 ± 0.0	-	>55.0 ± 0.0	-	27.9 ± 0.1	+	0.0000 ± 0.0000
	UT4	>55.0 ± 0.0	-	>55.0 ± 0.0	-	26.6 ± 0.1	+	0.0000 ± 0.0000
	UT5	>55.0 ± 0.0	-	>55.0 ± 0.0	-	28.5 ± 0.0	+	0.0000 ± 0.0000
	UT6	>55.0 ± 0.0	-	>55.0 ± 0.0	-	28.3 ± 0.0	+	0.0000 ± 0.0000
	UT7	>55.0 ± 0.0	-	>55.0 ± 0.0	-	28.0 ± 0.2	+	0.0000 ± 0.0000

[a] Ct values indicates the PCR cycle number at which PCR cycle amplification became exponential. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, where:
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Cts ≥ 38 are weak reactions indicative of minimal or zero amounts of target nucleic acid.
Mean of three replicates.
[b] + indicates amplification, - indicates no amplification, NT = not tested.

The assay was run in parallel with both singleplex (LU633) and duplex (LU633 and *P. radiata*) reactions. When comparing the LU633 amplification between the singleplex and duplex reactions, it was apparent that the detection of LU633 DNA in the duplex reaction was compromised with the majority of samples showing significantly higher Ct values compared to the Ct values in the singleplex reaction. These higher Cts often fell within a range of Cts that can be classed technically as false negatives. It is likely the efficiency and sensitivity of fungal amplification in duplex reactions may have been reduced (compared with singleplex reactions) due to the build-up of PCR inhibiting chemicals, competition for resources, or inactivated polymerases in the later reaction cycles. Inhibitors may prevent primers from annealing to DNA, bind to nucleic acids and interfere with DNA replication or bind essential co-factors like magnesium, reducing their availability by inhibiting enzyme-co-factor interaction. Mellow *et al.* (2015) found that the ratio of LU633 to *P. radiata* DNA, in field samples of LU633 treated trees, was approximately 1:500, with the concentration of LU633 and *P. radiata* DNA being approximately 0.02 and 10 ng/µL, respectively. The limited amplification resources may have preferentially detected the highly concentrated *P. radiata*, rather than the scarce fungal DNA material and reduced the efficiency of fungal amplification with LU633 primers.

Standard curves are used to determine the effective dynamic range of the amplification reaction across a concentration gradient. Mellow *et al.* (2015) developed standard curves based on set ratios of LU633 and *P. radiata* DNA concentrations. However, in the current study, the concentration of *P. radiata* DNA was found to be very similar in all samples, with minimal variation in Ct values (ranging from 26.4 to 30.8; Table 3; grey lines in Figures 1B and 2). An approach, different to that of Mellow *et al.* (2015), was used to develop a standard curve suitable for determining LU633 DNA concentration in singleplex reactions. A standard curve was developed using a set amount of *P. radiata* DNA (10 ng/µL) and a serial dilution series of LU633 DNA (ranging from 5 to 0.0009 ng/µL) to reflect the low and high level of variability in *P. radiata* and fungal DNA respectively (Figure 3). Using this standard curve, *P. radiata* root samples treated with *T. atroviride* LU633 were shown to have LU633 DNA levels ranging from nil to 2.6 ng/µL with the highest levels in the Golden Downs (Kohatu block) plantation (mean of 0.39 ng/µL).

This standard curve did not maintain the linear relationship and had more variability when LU633 DNA concentration was very low (<0.02 ng/µL; Figure 3), therefore measurement of low concentrations of fungal DNA in field samples was less accurate. A large number of replicates may be necessary in qPCR to overcome this limitation (Forootan *et al*., 2017) and further work is required to optimise this number of replicates.

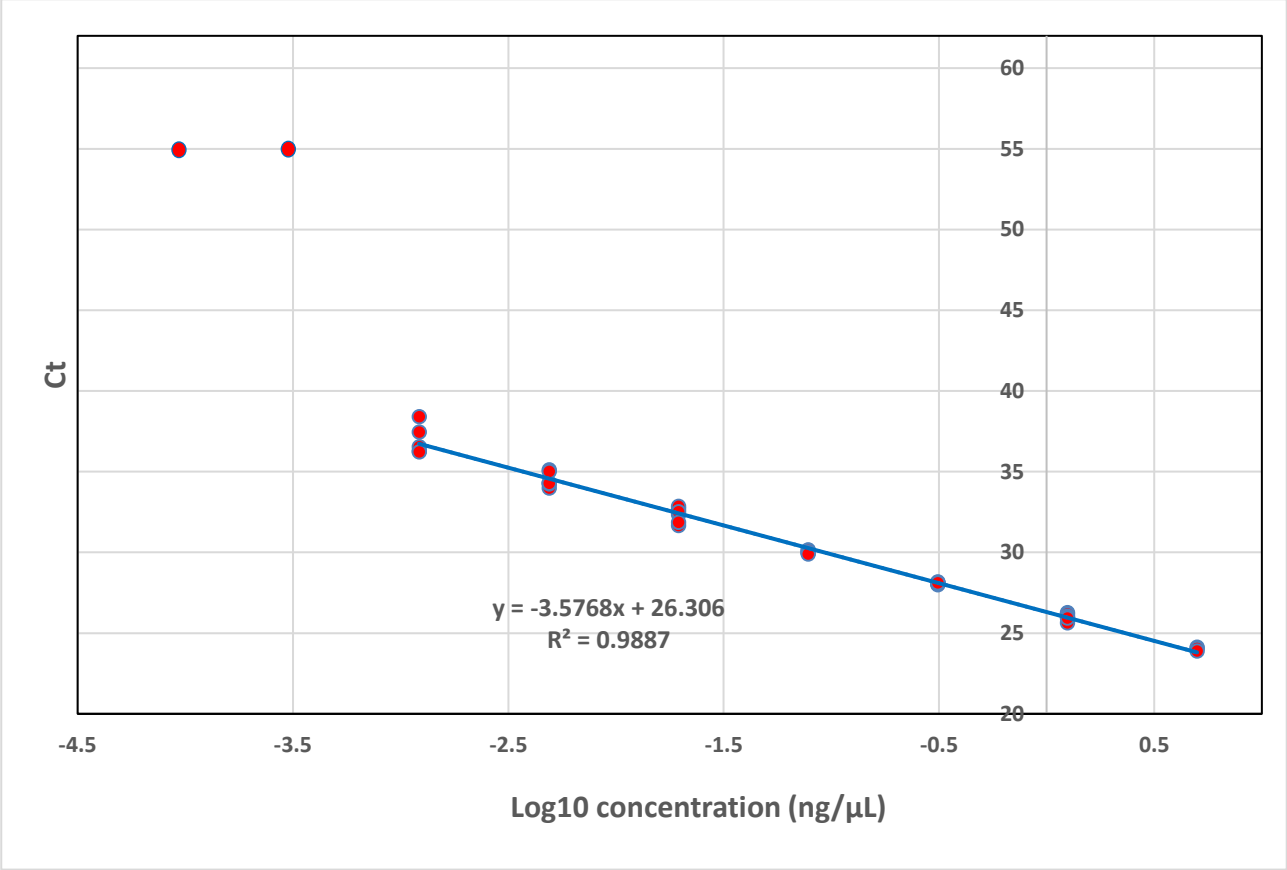

Figure 3: Standard curve used to determine the relationship between a set amount of *P. radiata* DNA (10 ng/µL) and variable concentrations of LU633 DNA (5 ng/µL, 1.25 ng/µL, 0.3125 ng/µL, 0.078125 ng/µL, 0.0195 ng/µL, 0.00489 ng/µL, 0.00122 ng/µL, 0.0003 ng/µL, and 0.0009 ng/µL) in singleplex reaction. The linear regression relationship between Ct values and LU633 concentration is indicated by the blue line with the limit of detection at approximately 0.001 ng/µL. Each LU633 concentration had six replicates.

Persistence of the bioprotectant treatment *T. atroviride* LU633 in New Zealand plantation trials has been confirmed in two additional studies. Cummings and Hill (2016) detected this strain in *Trichoderma* cultures isolated from roots collected from one three-year-old LU633-treated tree, using conventional PCR. The strain was not detected in any other trees, but the direct culturing method may have selected for faster-growing or otherwise more competitive *Trichoderma* species (e.g. *T. virens*) which may have obscured the recovery of the LU633 strains from isolation plates. Additionally, results may have been affected by sampling bias, where practical constraints limit the number of cultures examined by PCR and may not be fully representative of all *Trichoderma* strains occurring in the roots. Analysis with qPCR overcomes some of the limitations of conventional PCR by enabling fungal DNA material to be detected directly in plant material and in low concentrations. Mellow *et al.* (2015) detected applied T*. atroviride* LU633 in roots sampled from three out of eight plantation sites. Detection of LU633 in the other five sites may have been hindered due to possible sample storage and transport condition issues or the other strains within the bioprotectant mixture may have dominated over LU633.

In this study, only small roots near the ground surface were sampled because these were the easiest to recover. The section of root harvested may vary in quantity of fungal DNA. Hill *et al.* (2016) found higher number of *Trichoderma* colonies were isolated from proximal sections of young seedling roots. Further study of *Trichoderma* colonisation in different

sections of roots, in roots of larger size, and at greater depth may allow refinement to the root sampling method to improve the quality and quantity of DNA that can be extracted in qPCR, particularly in low fungal concentration situations.

Detection of low levels of LU633 in *P. radiata* root samples was achieved with qPCR in this study, with some modifications to the Mellow *et al*. (2015) method, including freeze-drying root tissue prior to DNA extraction to obtain higher DNA yields and addition of a larger volume of LU633 primer. These modifications may also have led to the fungal DNA yield being higher in this study compared to that found by Mellow *et al.* (2015).

More optimisation to the method will be made to improve reliability and sensitivity, particularly in samples with low fungal DNA concentrations. Confirmation of the duplex reaction assay limitations will be made by repeating the standard curves (with increasing ratios of LU633 to *P. radiata* DNA) in duplex reactions to allow PCR resource competition to occur. In addition, it will be determined if any PCR inhibitors affected the amplification of fungal DNA and resulted in false-negative results. Inhibitors could be detected by using PCR control reactions where defined amounts of DNA are added to the samples and analysed parallel to the target sequence (Schrader *et. al*., 2012).

CONCLUSION

A strain-specific, probe-based qPCR assay was used to detect the presence and abundance of the *Trichoderma* bioprotectant strain LU633 in a large number of 3.5-year-old plantation tree roots and contributed to information gain on the persistence of bioprotectants strains in forest environments.

Further work is required to determine the persistence of *Trichoderma* bioprotectant strains in plantations with different growing conditions to those sampled in this report. Further monitoring work is also required to determine the persistence over longer periods, particularly once canopy closure occurs and changes in the root environment are experienced. This work should be coupled with assessment of tree growth and disease parameters to determine the effectiveness of the bioprotectants over time. Further optimisation of the assay, to increase PCR product generation and improve reliability and sensitivity, may be beneficial in *P. radiata* root samples with low levels of DNA material.

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