Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Bioprotection for foliar diseases and disorders of radiata pine
Quantitative PCR methods developed for LU633/584 (Milestone 2.5)
LU633/584 primers tested on forest and controlled environment samples (Milestone 2.7)

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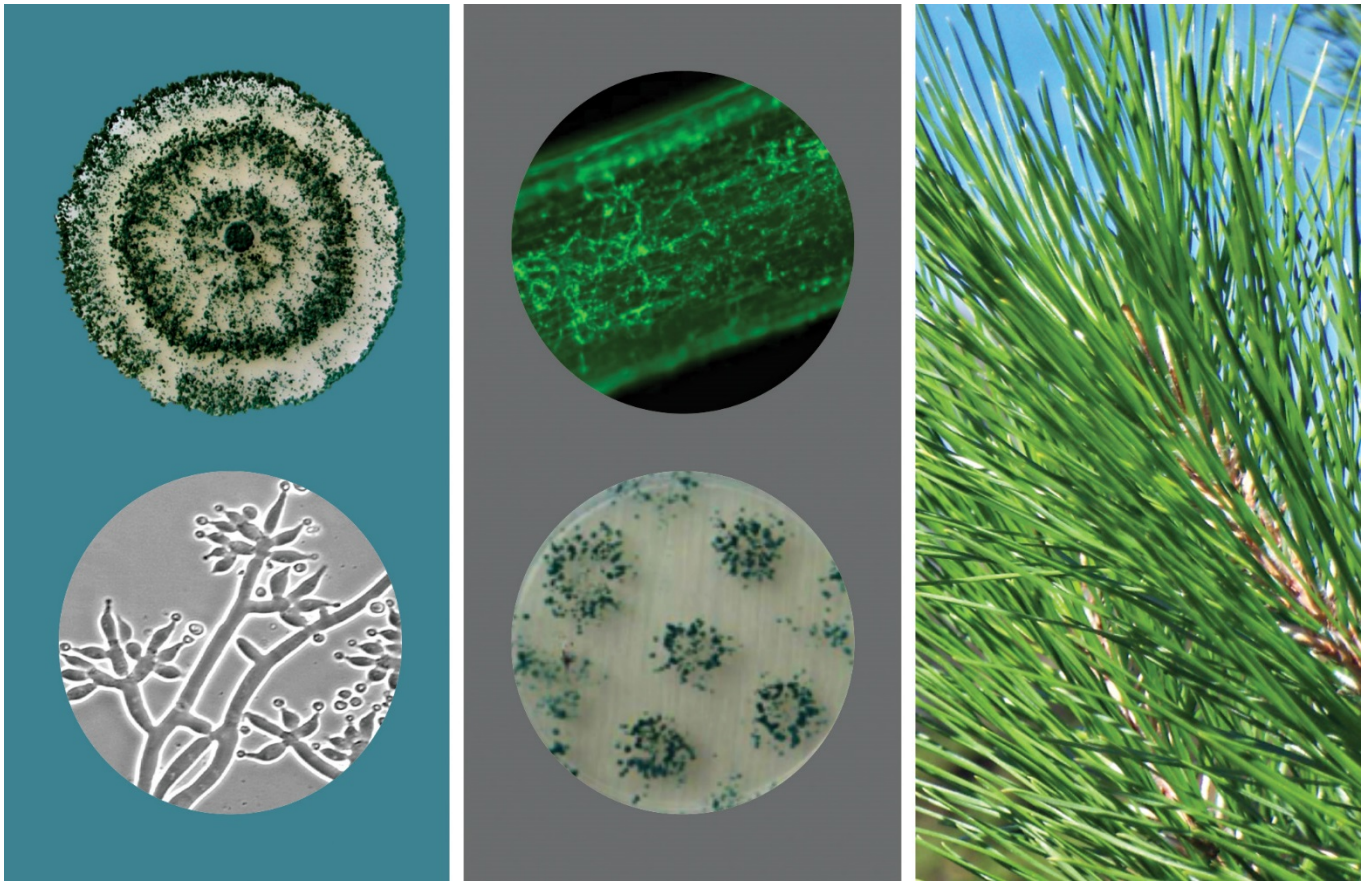

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EXECUTIVE SUMMARY

Trichoderma fungi show great promise for improving health of forest trees. The *Bioprotection for foliar disorders and disorders of radiata pine* research programme aims to identify and test the bio-protection ability of endophytic fungi including specific strains of *Trichoderma* species.

An important question about deployment of specific strains of *Trichoderma* is whether they are able to persist and become established in the soil and/or on roots in the forest, potentially providing long-term protection. Because *Trichoderma spp.* are ubiquitous, to answer this question it is necessary to develop molecular tools that can identify specific species and strains shown to have a bio-protective effect amongst the *Trichoderma* population. In milestone 2.4 (report FOA-BIO-T002), tests for identification of *Trichoderma atroviride* strains LU633 and LU584 were developed and validated using conventional PCR. In milestones 2.5 and 2.7 the aim was to extend this work to provide a sensitive, quantitative and specific probe-based real-time PCR assay for these strains and to trial use of these assays with forest and laboratory samples.

Task 2.5: Quantitative PCR methods were developed for LU633/584

One of the LU633 PCR primers that existed prior to this project had a sequence error, so new primers were designed and used. Following initial tests with SYBR Green quantitative PCR detection a probe-based detection and quantification system was developed that shows high efficiency, sensitivity and specificity for LU633. The assay is run in parallel with detection of a pine gene, *CAD,* (cinnamyl alcohol dehydrogenase), which allows normalisation of the results between different samples. The LU584 probe-based assay showed lower specificity and needs more optimisation.

Task 2.7: LU633/584 primers were tested on forest and controlled environment samples

Four DNA extraction methods were compared and one (Geneaid kit method) was selected for use on the basis of its reliablity in yielding clean, high-quality DNA suitable for real-time PCR, and ease of use with large sample numbers. DNA was extracted from *Trichoderma*-treated root samples from forests, nurseries and laboratories (PFR, Massey, Treelab). Levels of *T. atroviride* LU633 were low but significantly above control levels in some of the samples. Further optimisation of the assay will be carried out to increase sensitivity.

The work completed in these tasks shows the feasibility of using a PCR-based assay to quantify a specific strain of *T. atroviride* in pine root samples and provides a framework for development of other diagnostic tools.

INTRODUCTION

The main aim of this project is to determine whether bioprotectant treatments with specific endophytic fungi can reduce disease levels in the forest. Specific strains of *Trichoderma* spp. have been used in treating pines but it is not known for how long these strains persist in the rhizosphere or on pine roots. *Trichoderma atroviride* strains LU633 and LU584 are present in *Trichoderma* 'Mix A' that has been deployed in forest trials, and development of a strain-specific diagnostic assay had commenced prior to this project.

This report follows on from report FOA-BIO-T002, which describes validation of *T. atroviride* strain specific primers for LU584 and LU633 (Task 2.4). An error was discovered in one of the original LU633 sequences used for initial development of the diagnostic PCR test, so new primers were designed to the correct sequence.

The aim of Tasks 2.5 and 2.7 was to extend the work to develop a quantitative PCR-based diagnostic tool for LU633 and LU584 and to determine whether this technology is sufficiently sensitive to determine levels of these specific *T. atroviride* strains in bioprotectant trials and in the forest. The work will provide a pathway for development of tools for identification of other useful bioprotectant strains in future.

Specific objectives:

2.5 Quantitative PCR methods developed for LU633/584

2.5.1 Standard curves produced using SYBR green.

2.5.2 Probes designed and validated.

2.5.3 DNA from other non-target species and strains tested.

2.5.4 Final report on validation and use of assays (2.4,2.5,2.7) completed. (This document, and please see report no. FOA-BIO-T002 for Milestone 2.4).

2.7 LU633/584 primers tested on forest and controlled environment samples

2.7.1 Best methods for DNA extraction established.

2.7.2 Quantitative PCR trialled with DNA samples.

2.7.3 LU633/584 primers tested on forest and controlled environment samples

METHODS

DNA extraction and basic PCR methods

T. atroviride LU584 and LU633 strains were obtained from Lincoln University. The isolates were grown in potato dextrose broth (PDB) at 28°C, with shaking at 150 rpm, for 48 h. Genomic DNA was extracted using a mini plant genomic DNA extraction kit (Geneaid, New Taipei, Taiwan) following the manufacturer's instructions, then quantified using a QUBIT fluorometer (Carlsbad, CA, United States of America). Real-time PCR was done using a LightCycler480 instrument (Roche Applied Sciences, Penzberg, Germany). PCR primer sequences are shown in Table 1. The SYBR green real-time PCR was performed using a LightCycler® 480 SYBR Green Master mix (QARTA BIO; www.quartabio.com) where each 10 µl of reaction volume contained 2 µl of 5 × qPCR mix, 4 µl PCR grade water, 1 µl of forward primer (10 µm) 1 µl of reverse primer (10 µm) and 2 µl DNA template (known and standard). PCR was conducted with pre-incubation at 95^{o}C for 15 min followed by 45 cycles of denaturation at 95^{o}C for 10 s, annealing at 58^{o}C for 30 s, extension at 72^{o}C for 30 s followed by cooling at 40^{o}C for 10 s.

Primers and fluorescent probes for probe-based real-time PCR were designed using AlleleID® software (http://www.premierbiosoft.com). The 5' and 3' ends of TrichKa (LU633-specific) and Lu584Pc (LU584-specific) probe were labelled with 6-carboxyfluorescein (FAM) and quencher dye BHQ-1 respectively; 5' and 3' ends of the *Pinus radiata* cinnamyl alcohol dehydrogenase (CAD) gene probe were labelled with HEX (5') and BHQ-1 (3') respectively. Real-time PCR with probes was carried out using the quantitative PCR (qPCR) protocol of Chettri et al. (2012). The reaction was performed using a LightCycler® 480 Probes Master kit (Roche Applied Science) where each 10 µl of reaction contained: 5 µl of 2 × Probe master mix, 1 µl 10 × primer probe mix, 2 µl PCR grade water and 2 µl DNA template (known and standard). The 10 × primer probe mix consisted of *T. atroviride* LU633 or LU584 probes (TrichKa or Lu584Pc), or pine *CAD* probe 945, each at 2 µM, along with LU633 specific primers (RM5ka & RM6ka) or LU584 (Lu584PcF & Lu584PcR), or pine specific primers *CAD*918, *CAD*1019, each at 4 µM. PCR was conducted with pre-incubation at 95^{o}C for 10 min followed by 55 cycles of: denaturation at 95^{o}C for 10 s, annealing at 58^{o}C for 15 s, extension at 72^{o}C for 20 s followed by cooling at 40^{o}C for 10 s.

Table 1. Primers and probes used for *Trichoderma* biomass quantification

Target	Primer name	In-house primer number	Primer/probe sequence (5' to 3')	Amplicon size (bp)
Primers for conventional and SYBR green real-time PCR				
LU584	RM1	1014	CACTTCCAACTTATTTTCTGCC	95
	RM2	1015	CCGTACATGATGCCTCACA	
LU633	RM5	1018	GCAAGTTGGATACAGTTGGCT	72
	RM6K	1621	GCCAGTAACTAGAATCGCAT[1]	
ITS control[2]	ITS1F	13	CTTGGTCATTTAGAGGAAGTAA	550
	ITS4	17	TCCTCCGCTTATTGATATGC	
Primers for probe-based real-time PCR				
P. radiata	CAD918	935	CAGCAAGAGGATTTGGACCTA	101
	CAD1019	936	TTCAATACCCACATCTGATCAAC	
	Probe 945		HEX-TGTGAACCATGACGGCACCC-BHQ1	
LU633	RM5Ka	1619	GCAAGTTGGATACAGTTG	72
	RM6Ka	1620	CCAGTAACTAGAATCGCA	
	Probe TrichKa		6FAM-TTGTATTAGTCCCACTCTATCAAG-BHQ1	
LU584	Lu584PcF	1622	CGTTCAACTAACAAATTGG	81
	Lu584PcR	1623	CCAGTAAGTAGAATGGTATG	
	Probe Lu584Pc		6FAM-TTTGTATGAGTCCCACTCTATCAAGAT-BHQ1	

[1]This primer sequence is the corrected sequence version (see Technical report task 2.4).
[2]Universal ribosomal spacer region primers (White et al., 1990)
See previous report (FOA Massey Technical Report Task 2.4; FOA-BIO-T002 for *T. atroviride*-specific primers.

Assessment of primer specificity and sensitivity in probe-based real-time PCR

To assess the specificity of the primers, real-time PCR amplification with the probe system was carried out as described above, using genomic DNA from ten strains of *T. atroviride* and from 13 other fungal species, including common forest fungi (Table 4). The quality of the DNA was first assessed using universal ribosomal space (ITS) primers in a conventional PCR. Then duplicate real-time PCR reactions were run using the specific primer/probe combination along with a positive control.

To assess the sensitivity of the probe-based PCR method, and to provide standard curves for quantification, *T. atroviride* strain LU633 standard curves with 10, 2, 0.4, 1, 0.08, 0.016, 0.0032, 0.00064, 0.000128 ng of fungal DNA, or strain LU584 with 10, 1, 0.1, 0.01, 0.001, 0.0001 ng of fungal DNA, were prepared from DNA extracted as described above. All reactions were run in triplicate or, for lower dilutions, with six replicates. For normalisation with pine DNA a standard curve was prepared with 100, 20, 4, 0.8, 0.16, 0.032 ng of pine DNA (in triplicate) extracted from root samples that were not inoculated with *Trichoderma* spp..

Development of a quantitative duplex real-time PCR assay

Because the samples we will test contain a mixture of pine and fungal tissue, duplex reactions were set up to assess the effect of mixtures of fungal and pine DNAs on the efficiency of the PCR reactions. Standard curves were prepared by mixing fungal and pine DNA in a ratio of 1:5, having 50, 10, 2, 0.4, 0.08, 0.016, 0.0032 ng of pine and 10, 2, 0.4, 0.08, 0.016, 0.0032, 0.00064 ng of *T. atroviride* LU633 DNA. Along with all the assay reactions a mixture containing water without DNA was included as a negative control. The efficiency and the *Error* value (mean squared error of the single data points fit to the regression line) for the mixed fungal + pine duplex reaction were compared to those of the standard curves obtained using purified fungal or pine DNA. All the standard curves, which show the relationship between concentration of DNA target and crossing point (Cp) in the qPCR reaction, were saved as external standard curves and used later for other quantification assays.

In order to be able to make comparisons between different samples, the concentration of *T. atroviride* strains as determined by qPCR was expressed relative to (ie. normalised by) the amount of pine DNA in the reaction. To do this, both fungal and plant genes were amplified in the same quantitative PCR reaction tubes by adding both fungal (LU633) and plant (*CAD*) primers and probes. The fungal and plant probes have different fluorescent tags so can be distinguished from one another in the qPCR reactions. The concentrations of fungal (LU633) and plant (pine) DNA in each sample were then determined from the standard curves (see above). A normalised value for LU633 abundance in each sample was determined as: concentration of LU633/concentration of pine (*CAD*).

Extraction of DNA from root tissue

Four methods were trialled to extract DNA from pine roots (appendix 2):

(1) CTAB (cetyltrimethylammonium bromide) extraction method is used to extract DNA from plant and fungal tissue containing high levels of polysacarides. Under high salt conditions the CTAB will bind to polysaccharides, allowing them to be removed from the solution. Originally developed by Doyle and Doyle (1987) and adapted by Stewart and Via (1993); also see report FOA-BIO-T002; task 2.4.

(2) Modified Qiagen kit method (developed by Tim Owen, 2010). This method was used to isolate DNA from single lesions of Dothistroma needle blight on radiata pine needles (Owen, 2010).

(3) Geneaid kit extraction method (Genomic DNA Mini Kit (Plant), Geneaid Biotech Ltd., New Taipei City, Taiwan). Root samples were prepared for extraction by first removing loosely associated soil (leaving closely associated soil attached). Roots were then ground in liquid nitrogen using a pestle and mortar. Approximately 50 mg of ground root tissue was weighed and transferred

to a 1.5 ml microcentrifuge tube. DNA extractions using the Geneaid kit extraction method were conducted according to manufacturer's instructions. An additional wash step using absolute ethanol was carried out if pigments remained on the DNA binding column after initial wash steps. DNA was eluted in 50 µl of the Elution Buffer provided with the kit.
(4) Power Plant Kit (MO BIO laboratories, Inc., Carlsbad, CA 92010, USA) method, used according to the manufacturer's instructions

To compare these extraction methods, root samples from sample set B (two biological replicates) were used for DNA extraction. DNA quantification and purity were assessed spectrophotometrically (OD_{260}/OD_{280} ratio) and using a QUBIT fluorometer as described above. PCR amplification of the extracted DNA was tested using pine *CAD* gene primers in both conventional and real-time PCR as described above. The selected method (Geneaid kit) was then used to extract DNA from samples listed in Table 2.

Table 2. Forest and laboratory samples

Sets of *P. radiata* samples, with or without *T. atroviride* LU633, that were tested:

Sample set	Source[1]	Date sent[2]	Treatment date (if known)	Trichoderma treatment[3]	Trichoderma root sample number	Control root sample number
A	PFR	April 2014	2013	LU633 & FCC320	12 (T1-T12)	12 (C1-C12)
B	Scion/Massey	May 2014	2013	Mix A	6	6
C1	P F Olsen main nursery crop	May 2014	2013	Mix A	2	2
C2	PF Olsen 2013 Trial	May 2014	May-Aug 13	Mix A	2 (T1)	2 (T51)
C3	Wharerata	May 2014	2012	MixA	1 (T1)	1 (T2)
C4	Pinnacles	May 2014	2012	MixA	5 sites (T1)	5 sites (T3)
D	PFR	Jan 2015	Oct 2014	LU633 & FCC320	12 (T1-T12)	12 (C1-C12)
E[4]	Tree Lab	June 2015	Mar-Jun 2015 (x2)	LU633	5	4

[1]Plant and Food Research (PFR) samples A and D from Tony Reglinski; forest samples C1-C4 from Robert Hill; sample E from Jenny Aitken.
[2]Samples were couriered to Massey and stored at -20°C or -80°C on arrival.
[3]Mix A contains LU633 and LU584 as well as two other *T. atroviride* strains.
[4]Samples were combined (Trichoderma or control) due to insufficient tissue for DNA extraction.

RESULTS AND DISCUSSION

Task 2.5 Quantitative PCR methods developed for LU633/584.

The current primer sets had only been used for conventional PCR prior to this study. The aim of this task was to develop a quantitative assay using real-time PCR to both distinguish and quantify the LU584 and LU633 *T. atroviride* isolates. The first question to address was whether the existing strain-specific primers, used in conventional PCR, would also function in real-time PCR. This was done using SYBR green, a dye that fluoresces when it binds to double-stranded DNA and can show whether PCR amplification has worked by showing an increase in fluorescence over time as the DNA concentration increases. The second step was to design a more robust and specific real-time PCR detection system by designing strain-specific probes that bind to amplified target DNA; these fluorescently-labelled probes serve the same purpose as SYBR green in enabling DNA detection but only target DNA molecules will be detected. The specificity, sensitivity and reproducibility of these probes needed to be verified before application of these to detection of LU633/LU584 in root and soil samples.

2.5.1 Standard curves produced using SYBR green.

Four sets of primers, as shown in Table 1, were used in qPCR using SYBR Green to test their ability to amplify DNA from *T. atroviride* LU584 or LU633 grown in culture. Three experiments were performed (A,B,C) with results shown in Appendix 1 (Figure A1) and summarised in Table 3.

Table 3. Test of LU584 and LU633-specific primers using SYBR Green qPCR

Experiment	DNA template	Ct points[1] using primers specific for			
		LU584 RM1/RM2	*T. atroviride* RM3/RM4	LU633 RM5/RM6	*T. atroviride* RM7/RM8
A	LU584	13	13	-	13
	LU633	-	-	25	-
B	LU633	**33**	19	26	19
C	LU584	15	15	**35**	15

[1]Ct points are 'crossing points', indicating the PCR cycle number at which PCR amplification became exponential. A lower Ct number indicates better amplification and/or higher starting concentration of template. High values of 33 and 35 indicate low, or no, amplification). Bold numbers indicate values where high (non-specific) Ct values were expected. See Appendix Figure A1 for amplification curves for A, B and C.

Overall the results from the experiments summarised in Table 3 show that the LU633 and LU584 primers amplify their expected targets in the SYBR green real-time PCR system. LU584 DNA was amplified with both LU584-specific and *T. atroviride*-specific primers with low Ct (crossing point) values of 13-15, indicating rapid amplification. However the amplification of LU633 with LU633-specific primers was less efficient, with Ct = 25 (Table 2), despite good amplification of the same LU633 DNA with *T. atroviride*-specific primers. This poor strain-specific amplification with LU633 primers RM5/RM6 led to the hypothesis that there was a mismatch sequence in one of the primers,

a hypothesis that was supported by sequence analysis described in the previous report. Rather than optimising qPCR with SYBR Green the decision was made to order fluorescent probes and new specific primers that would give higher specificity.

2.5.2 Probes designed and validated.

The amplification efficiency and sensitivity using the new fluoresent probe and primers were determined using serial dilutions of DNA isolated from *T. atroviride* strains LU633 and LU584. The real time PCR standard curves obtained are shown in Figure 1. The amplified PCR product signals were detected in a linear range from 0.0001 to 10 ng of initial quantities of *Trichoderma* DNA (Figure 1A and 1B). Both showed good amplification efficiencies of 1.93; this is a measure of how much amplification has occurred in each cycle of the PCR, and this value is close to the expected value of 2.0 (the amount of target DNA is doubled in each round of PCR). The coefficients of determination (the mean squared error of the single data points fit to the regression lines shown in the figures) were 0.05 & 0.02 for LU633 and LU584 respectively, indicating a high correlation between the amount of fungal DNA and the corresponding Ct values. The limit of detection of the method within the linear range of the standard curve was 0.1 pg, or about 10 nuclei per reaction.

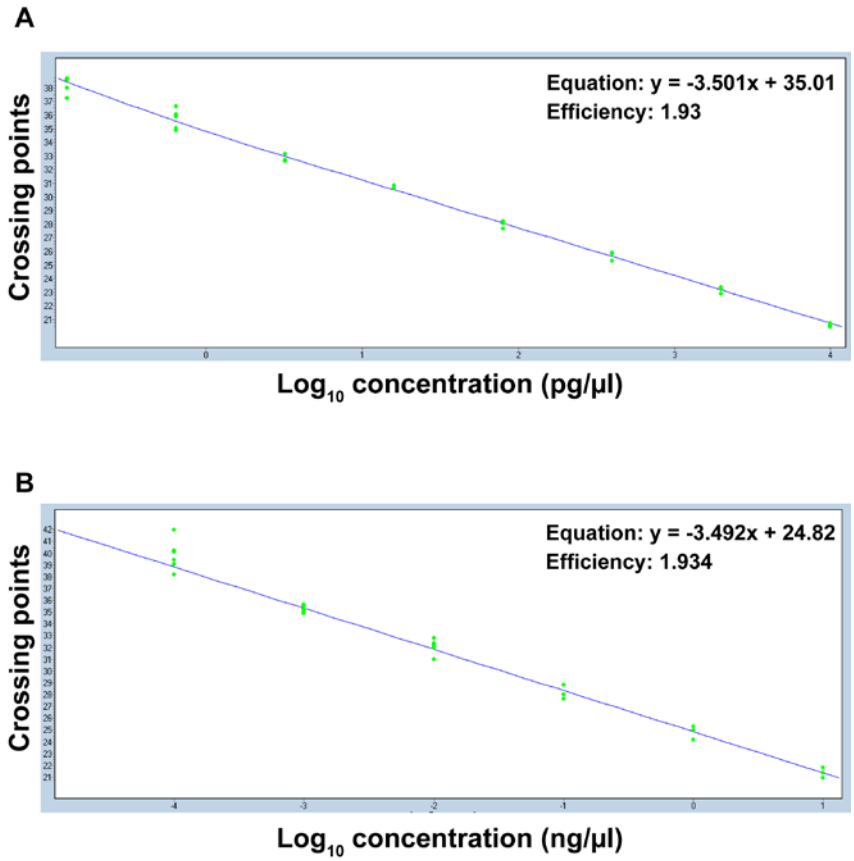

Figure 1: Quantification of serially diluted *Trichoderma atroviride* strains LU633 (A) and LU584 (B). Standard curves obtained by plotting the concentration of DNA versus the cycle number required to elevate the fluorescence signal above the threshold. The lowest limit of detection of fungal DNA within the linear range was ~0.1 pg. The equation shown is that of a straight line y=mx+c where y is the ct value on y axis, m is the slope, x is the log of DNA concentration and c is the y intercept.

To determine the specificity of the primers for LU633 and LU584, cross-template reactions (ie. LU584 template with LU633 primers/probe and vice-versa) were used in duplicate for each quantitative PCR run. They did not show any amplification, indicating that the primers and probes were able to distinguish between LU633 and LU584. The non-template control resulted in no visible signal until 55 cycles (Appendix 1 Figure A2).

2.5.3 DNA from other non-target species and strains tested.

PCR with primer pairs shown in Table 1 was carried out using genomic DNA extracted from a range of *T. atroviride* strains and other fungal genera. Although it is not possible to take into account all fungal species and strains that might be present on forest samples, by testing ten *T. aroviride* strains and 13 other species that included many known forest fungi, a good indication of specificity was obtained. The fungi tested are shown in Table 4: each of them tested positive with ribosomal ITS primers, indicating sufficient quantity and quality of DNA for PCR amplification, but negative with LU633-speciic primers.

The fluoresecent probe/primers expected to be specific to LU584 gave amplification products with several other *T. atroviride* strains (results not shown). Because many of the samples provided for testing at this stage were treated with LU633 and not LU584 (Table 2) it was decided to continue to optimise the LU633 assay only at this stage.

Table 4. Real-time PCR specificity test with LU633-strain specific primers and probe

Species (and strain)	LU633-specific (RM5Ka & RM6Ka) (real-time probe PCR)[1]	Positive control (ITS1/ITS4) (Standard PCR)
T. atroviride LU633	+	+
T. atroviride LU132	-	+
T. atroviride LU140	-	+
T. atroviride LU612	-	+
T. atroviride LU131	-	+
T. atroviride LU519	-	+
T. atroviride LU525	-	+
T. atroviride LU517	-	+
T. atroviride LU134	-	+
T. atroviride LU500	-	+
T. atroviride LU593	-	+
Alternaria alternata	-	+
Cyclaneusma minus	-	+
Cyclaneusma sp.	-	+
Fusarium graminearum	-	+
Glomerella cingulata	-	+
Lophodermium conigenum	-	+
Lophodermium pinastri	-	+
Mycosphaerella cryptica	-	+
Mycosphaerella intermedia	-	+
Mycosphaerella suberosa	-	+
Nectria fuckeliana	-	+
Phoma glomerata	-	+
Strasseria geniculata	-	+

[1] + indicates amplification; - indicates no amplification

Task 2.7 LU633/584 primers tested on forest and controlled environment samples

The specific primers developed in task 2.5 will be used to evaluate persistence of Trichoderma bio-inoculants in both laboratory and field-based trials. This task 2.7 involved optimisation of methods to extract and PCR amplify DNA from root samples, as well as developing an assay in which Trichoderma DNA can be quantified relative to pine DNA in the same sample (normalised) and providing preliminary data about the levels of Trichoderma DNA expected in various sample types.

2.7.1 Best methods for DNA extraction established.

Four different DNA extraction protocols were tested to determine which would be most reliable for obtaining DNA from root samples of sufficient quality for use in quantitative PCR. Ease of use and cost were also taken into account as a large number of samples will need to be processed. The results obtained are summarised here.

Methods 1 and 2 (CTAB and 'modified Qiagen' method) appeared to yield a high quantity of DNA although low OD260/280 ratios (1.62-1.68 and 1.72-1.86 respectively) indicated the DNA was not very pure (an OD260/280 reading of 1.8-2.0 indicates pure DNA). The quantitative PCR amplification efficiency for the CTAB method was 1.86 (optimum efficiency is 2.0). Further to this both methods 1 and 2 are quite time-consuming, so overall not the best choice for this project.

Methods 3 and 4 (Geneaid and Powerplant) involved use of commercial DNA extraction kits. Both methods took about the same length of time, but the Geneaid kit cost $4 per reaction whilst the Powerplant kit cost $7 per reaction. In several trials, a lower concentration of DNA was obtained using the Geneaid kit (up to 3-fold less than the Powerplant kit when using the same amount of starting material) but the quality score was much better (OD260/280 ratios of 1.82-1.90 for Geneaid and 1.50-1.66 for Powerplant DNA). The Geneaid DNA also appeared to have higher molecular weight and less shearing than that produced using the Powerplant kit, as seen by agarose gel electrophoresis. In quantitative PCR, however, both gave amplification efficiences of >1.9 (results not shown). Overall the Geneaid kit (method 3) appears to be the best choice.

2.7.2 Quantitative PCR trialled with DNA samples.

Quantitative PCR was carried out with both pine (CAD) and Trichoderma (LU633) primer sets using a mixture of pine and fungal DNA templates. The aims of this were to:
(a) determine if the pine/fungal reactions can be run as duplex reactions, in which case *CAD* pine gene amplification will be used as an internal normalisation control for *Trichoderma* quantification.
(b) determine if the presence of pine DNA in the sample affects the effciency of fungal amplification with LU633 primers.

A standard curve was prepared with five fold serially diluted LU633 DNA spiked with pine root DNA. The concentration ranged from 50 ng to 3.2 pg of pine and 10 ng to 0.64 pg of fungal DNA. The respective standard curves of the duplex are shown in Figures 3A and 3B.

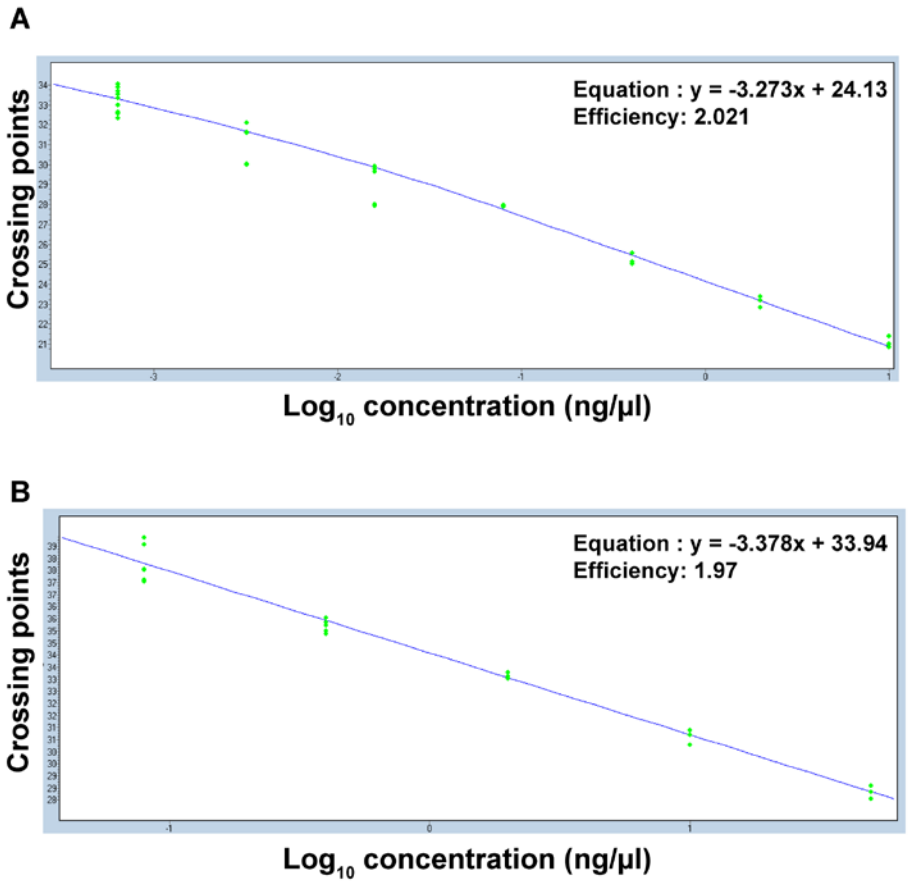

Figure 2: Standard curves prepared to determine if the presence of pine DNA in the sample affects the effciency of amplification with LU633 primers.
The quantification of target DNA (LU633) was done in mixed samples using real-time PCR for (A) *Trichoderma atroviride* strain LU633 and *(B) CAD* gene of pine. Standard curves were obtained with amplification of a 5-fold dilution series of target DNA (LU633) in the presence of pine root DNA at five times the target DNA ratio. The lowest limit of detection of fungal DNA within the linear range was ~0.1 pg.

To determine if the presence of pine DNA in the sample affects the efficiency of amplification with LU633 primer a comparative analysis was done with the standard curves prepared with or without spiking with pine DNA. The results indicated that the efficiency of LU633 was within the acceptable range (1.85-2.1) in both cases (Table 5). Furthermore no significant difference was observed in Ct values between spiked and non spiked LU633 DNA samples (Table 6) in the same PCR run. However, a significant inter-experimental variation in Ct value at the lower dilutions of 3.2 and 0.64 pg template concentration was observed. This could be explained by the fact that at very low concentrations, a normal distribution of template is not expected. It is suggested that increasing the number of replicates may overcome this problem.

Table 5: Effect of pine root DNA on the efficiency of LU633 quantitative PCR

	Pine	**Fungus**
Without spiking[1]		
mean squared error (E)	0.014	0.020
Slope	-3.42	-3.50
Efficiency	1.96	1.93
With spiking[2]		
mean squared error (E)	0.024	0.068
Slope	-3.38	-3.27
Efficiency	1.97	2.02

[1]Standard curve of pine *CAD* gene as shown in Appendix Figure A3

[2]LU633 DNA was spiked with pine root DNA in a ratio of 1:5; Figure 2B.

Table 6: Effect of pine root DNA on Ct values in LU633 quantitative PCR

Concentration of LU633 DNA (ng per 10 ul reaction)	**S1. Mean Ct**[1] (spiking)	**S2. Mean Ct** (No spiking same expt)	**S3. Mean Ct** (No spiking different expt)	**P value**[2] **S1 vs S2**	**P value S1 vs S3**
10	21.06	20.62	20.58	0.25	0.07
2	23.12	22.98	23.14	0.57	0.94
0.4	25.23	25.23	25.67	0.98	0.15
0.08	27.96	27.59	27.96	0.10	0.98
0.016	29.79	29.75	30.54	0.81	0.05
0.0032	31.78	32.04	32.83	0.64	0.01
0.00064	33.87	33.48	35.27	0.38	0.03

[1] Columns 1, 2 and 3 show mean Ct values of at least two technical replicates of LU633 DNA in a five-fold serially diluted sample. For S1, samples were spiked with 5 times the concentration of pine root DNA compared to LU633 in each tube, whilst in S2 and S3 samples were not spiked so contained no pine DNA. Sample set S3 was an experiment run on different plate and date to S1 and S2.

[2] Probability that Ct values are unaffected by spiking (T-test).

2.7.3 LU633/584 primers tested on forest and controlled environment samples

Details of samples tested are in Table 2. DNA was extracted from root samples using the Geneaid kit method and amplified using the duplex *T. atroviride* LU633 / pine CAD probe-based PCR system. The results are summarised in Table 7.

- Sample set A (PFR) showed significantly higher levels of LU633 amplification in the *Trichoderma*-treated than in the control (untreated) samples. These samples had been collected over a year prior to analysis and stored at -80°C. Although levels of LU633 were not high these results show that the assay can detect low concentrations of LU633 in infected roots.
- Sample set B (Scion/Massey) showed no significant difference between treated and control groups. One reason for this may be that the samples were stored at -20°C rather than at -80°C.
- Sample sets C1-C4 were from forest and nursery sites. Unfortunately they showed no amplification of LU633. Two of these samples (C3, C4) were treated with Trichoderma mix A in 2012. It is possible that other strains within the mix have dominated over LU633, or that the mix has attenuated in concentration over time.
- Sample set D was obtained recently from PFR but no amplification was obtained with LU633 primers. The reason is not known but may be that the infection level is too low for detection with the current assay.
- Sample set E (recent *Trichoderma* treated samples from the tree lab): over 6 times the level of LU633 was detected in the treated than the untreated sample (replicates had to be combined into one sample due to insufficient material for DNA extraction). Unfortunately the pine assay could not be done in time for this report due to both real-time PCR machines being out of action.

Table 7. Forest and laboratory samples: amplification results

Sample set	Source[1]	Tricho (T) or Control (C)	Biological (B) and Technical (T) replicates	Mean Cp LU633	Mean +/- SD concentration LU633 (pg/µL)	Mean Cp CAD (pine)	Mean +/- SD concentration pine (ng/µL)	LU633 normalised as ratio to pine
A	PFR	T C	B6 x T4	30.79 29.99	20.39±5.77 31.07±15.29	31.02 30.082	7.22±1.63 14.61±7.72	0.00242** 0.00218
B	Scion/ Massey	T C	B5 x T2	30.14 30.43	25.11±3.36 20.59±3.25	30.19 30.48	12.02±2.83 10.18±2.24	0.00216[ns] 0.00207
C1[2]	PF Olsen nursery	T C	B2 x T4	No amp	-	Not tested	-	-
C2	PF Olsen 2013 Trial	T C	B2 x T4	No amp	-	Not tested	-	-
C3	Wharerata	T C	B2 x T4	No amp	-	Not tested	-	-
C4	Pinnacles	T C	B2 x T4	No amp	-	Not tested	-	-
D	PFR	T C	B2 x T2	No amp	-	32.51 31.17	2.57±0.38 6.82±2.13	-
E[4]	Tree Lab	T C	B2 x T2	29.86 33.31	22.54±13.06 3.65±0.83	Not tested	-	-

[1]Amplification below threshold of standard curve.
[2]No amp (no amplification); nt (not tested as no amp with LU633 or due to non-functioning real-time PCR machine.
**P=0.0046 (2-tailed T-Test with equal variances, testing probability of no difference between control and treated samples.); [ns]no significant difference.

The results suggest that quantification of low levels of LU633 in pine root samples is achieveable with the probe-based PCR assay, but that more optimisation is required to improve sensitivity and reliability. Several approaches will be taken to improve the assay, such as:

- Roots will be freeze-dried prior to DNA extraction to try to obtain higher DNA yields.
- DNA elution volumes will be minimised to increase concentrations of extracted DNA.
- Larger numbers of replicates will be used in the PCR assays for low-concentration samples.
- Different sections of roots (distal, mid, proximal) will be tested to determine if they differ in (a) quality and quantity of DNA that can be extracted and (b) level of *Trichoderma* association.
- Close attention will be paid to sample storage conditions and avoidance of freeze-thaw cycles.
- The concentration of LU584 in the Mix A-treated samples will be determined in case there are differences in abundance of the different strains, using SYBR green primers.
- DNA extracted from soil will be tested from selected samples to determine levels of LU633.

CONCLUSION

Task 2.5 Quantitative PCR methods developed for LU633/584
Task 2.7 LU633/584 primers tested on forest and controlled environment samples

A probe-based quantitative real-time PCR assay method was developed for *Trichoderma atroviride* strain LU633 that will underpin development of a system to test the durability of *Trichoderma* bioinoculants in the forest. The LU633 primer and probe combination was initially tested with purified DNA from the LU633 strain and shown to PCR-amplify the target with good efficiency and sensitivity. When the same assay was done in the presence of pine DNA to better represent the situation in forest samples, there was no loss of efficiency or sensitivity. Furthermore the LU633 primers and probe were shown to be specific for LU633 as they did not detect other *T. atroviride* strains or other fungal species including other forest dwellers.

Attempts to develop an assay for LU584 led to problems in specifity when the new primers/probe combination designed for probe-based real-time PCR were tested with other *T. atroviride* strains. As discussed at the project team meeting on 2 June 2015 (PF Olsen, Rotorua) the LU584 is less of a priority than LU633 at present.

In order to apply the PCR assay to root samples, four DNA extraction methods were compared and a Geneaid kit method determined to be the best in terms of DNA quality, ease of use and cost. The assay was tested with root samples taken from the forest, nurseries or controlled environment laboratories. Whilst many of the samples showed poor, or no, amplification of LU633, two of the sample sets showed the presence of LU633, including plants treated in 2013 and harvested in April 2014. Improvements will be made to the assay to further increase sensitivity and reliability.

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Appendix: Additional quantitative PCR data

Figure A1. Primer specificity in SYBR Green qPCR using *Trichoderma atroviride*-, LU584- and LU633-specific primers.

A-C show qPCR results from three experiments where blue, green and red font colour (at the base of arrow) indicate the sets of *Trichoderma atroviride*-specific, LU584-specific and LU633-specific primers respectively. The *T. atroviride* template DNA (LU584 or LU633) is indicated before the primer set. Poor amplification of LU633 with LU633-specific primers RM5/RM6 (higher Ct values) suggested a problem with these primers.

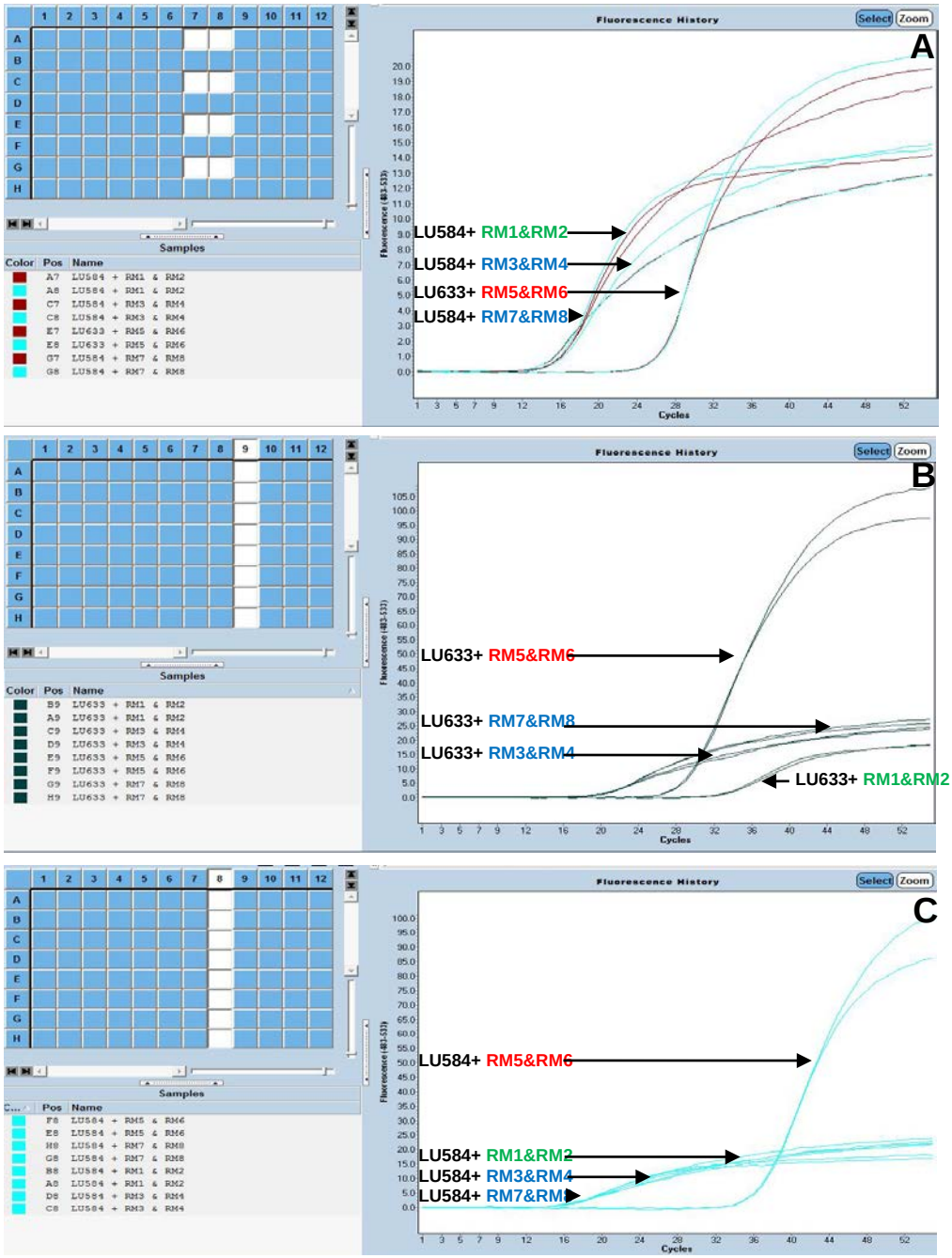

Figure A2: Determination of primer specificity using fluorescent probe/primers.

QPCR quantification of (A) LU633 with LU584-specific probe/primers and (B) LU584 with LU633-specific probe/primers. No fluorescence was obtained until 55 cycles. (c) No template control.

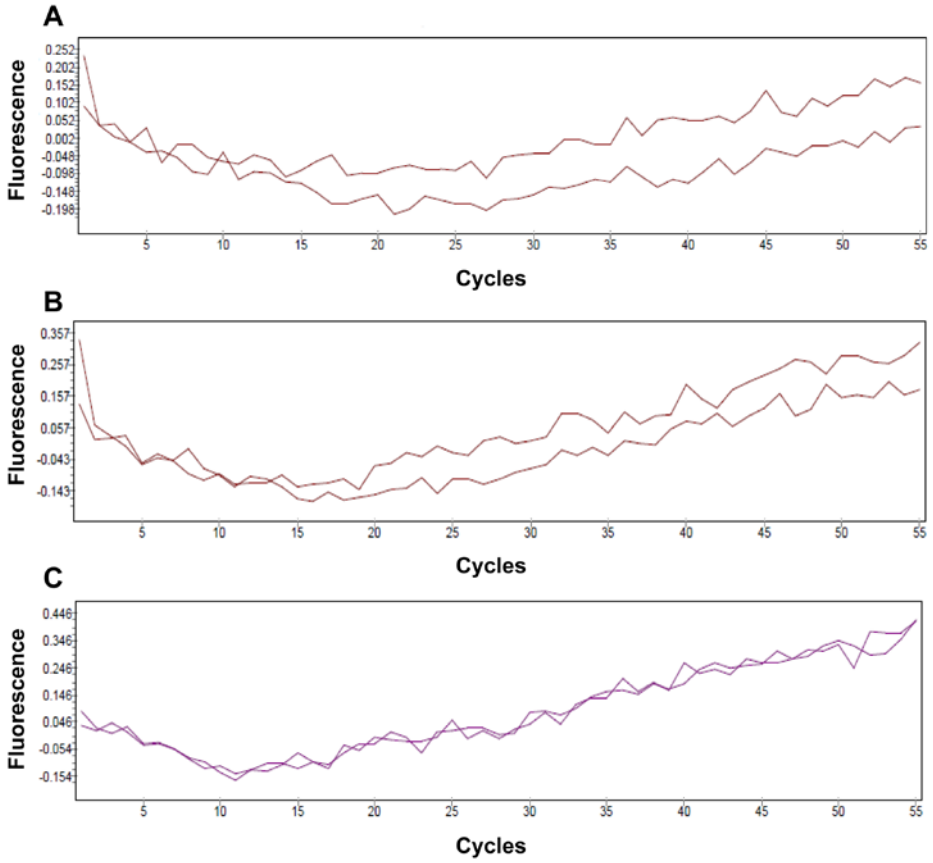

Figure A3: Quantification of serially diluted pine root genomic DNA.

Standard curve obtained by plotting the concentration of DNA versus the cycle number required to elevate the fluorescence signal above the threshold. The lowest limit of detection of pine DNA within the linear range was ~32 pg. The equation shown is that of a straight line y=mx+c where y is the ct value on y axis, m is the slope, x is the log of DNA concentration and c is the y intercept.