



Massey University

Report Ref: project 2008-03

Project Title: Herbarium specimens: opening the treasure trove.

Staff: Dr Rosie Bradshaw, Dr Arne Schwelm and Mr Tim Owen

Research Provider:

Centre for Advanced Bio-Protection Technologies
Institute of Molecular BioSciences
Massey University
Palmerston North

Collaborator:

Dr Tod Ramsfield
Scion Forest Biosecurity and Protection
49 Sala Street
Rotorua

Final Report: 31 March 2009

EXECUTIVE SUMMARY

An improved method was developed for extracting DNA from pine needles. The best method involved using some components from a commercial DNA extraction kit, along with enzymatic lysis and a simplified purification step. A significant (> 4-fold) increase in yield was obtained using the new method compared to using either of two popular commercial DNA extraction kits. All DNA extracted was tested for suitability for PCR amplification using pine-specific PCR primers.

The new extraction method was tested with needles infected with *Dothistroma septosporum*. It was possible to extract and PCR-amplify both fungal and plant DNA from single lesions. The method was then successfully used to extract DNA from single infected lesions of dried herbarium specimens and will underpin a future PhD project in which these samples will be investigated further.



Report Ref: project 2008-03

TABLE OF CONTENTS

Page

1	EXECUTIVE SUMMARY
3	TABLE OF CONTENTS
4	INTRODUCTION
5	MATERIALS AND METHODS
8	RESULTS AND DISCUSSION
12	FUTURE WORK
12	ACKNOWLEDGEMENTS
12	REFERENCES

1. INTRODUCTION

DNA identification of pathogens is an increasingly important tool in forest pathology. Diagnostic tests can be specific for a particular species or strain, and can be either quantitative or qualitative. Furthermore it is possible to examine material where the pathogen is no longer viable, but has left sufficient DNA for analysis.

Although sensitive polymerase chain reaction (PCR)-based assays form the basis for many diagnostic assays, obtaining sufficient purified DNA for analysis can be problematic. Pine needles are rich in resins and other complex metabolites that can hinder the extraction of DNA and inhibit PCR. Dried herbarium specimens are a potentially valuable source of historical information about pathogens but the limited samples are precious. Hence an efficient method to extract high-quality DNA from small samples is required.

This project follows on from FHRC project 2007-04 (DNA-based identification of needle pathogens). The 2007 project involved developing identification tools for *Dothistroma* spp. and is being continued as part of the Better Border Security programme (in collaboration with Tod Ramsfield, Scion). During the 2007 project some preliminary work was carried out with herbarium specimens of needles infected with *Dothistroma* spp. Extraction of DNA from dried herbarium needles was achieved but was inefficient and required sacrificing several lesion samples in order to obtain sufficient DNA for PCR analysis.

The main aim of this work was to optimize the recovery of PCR-amplifiable DNA from small samples (e.g. single *Dothistroma* lesions) and herbarium specimens. Physical grinding of the plant tissue is often a limitation so improving this step was an important component of this work. Use of a "Whole Genome Amplification" method that can increase the amount of DNA in a sample by several orders of magnitude (Foster & Monahan, 2005) was also considered.

A secondary aim of this work was to use *Dothistroma*-specific PCR primers developed in FHRC 2007-04 to answer the following questions:

- Is there any evidence that *Dothistroma septosporum* is present as an endophyte or latent pathogen in uninfected needles?
- Can DNA of sufficient quality be obtained from herbarium specimens containing *D. septosporum* to enable comparative sequence analysis with modern-day isolates?

Although *Dothistroma septosporum* is the target organism in this study, the methods will be generally applicable to all pathogen and endophyte species from forest needle samples.

Agreed Description of Work:

- A reliable and sensitive method to obtain PCR-quality DNA from fungi in pine needles, where limited starting material is available.
- DNA amplified from *Dothistroma*-infected herbarium needle samples and some initial characterisation achieved.
- FHRC report

NOTE: A 3 month extension was granted for this work following the departure of Dr Arne Schwelm to Europe. The work was continued by MSc student Tim Owen.

2. MATERIALS AND METHODS

2.1 Sources of uninfected and infected needles

Uninfected and *Dothistroma*-infected *Pinus radiata* needles were obtained from Gordon Kear forest, Manawatu, in 2008. Uninfected needles were used for development of improved DNA extraction methods, initially with approximately 10 mg fresh weight (~ 3 cm length) needle tissue in each DNA extraction. This amount of tissue was reduced in subsequent trials. Herbarium specimens were obtained from the Scion herbarium, courtesy of Margaret Dick.

2.2 DNA extraction: needle grinding methods

One of the main limiting factors to obtaining sufficient good-quality DNA from pine needles is the difficulty in breaking open needle tissue sufficiently well to release the DNA. Freeze-dried samples can be ground thoroughly with a mortar and pestle but this is impractical for very small amounts of tissue, such as single needle lesions. Parameters investigated were:

- Freeze-dried vs non freeze-dried samples (for fresh needles).
- Grinding tissue with a plastic Eppendorf pestle and/or using a Fastprep machine (Bio101 ThermoSavant Fastprep FP120) set at maximum speed (6.5 m/s) for 45 seconds. In both cases glass beads (~ 30 mg of diam 650 μm) were used.
- Ground needles incubated with 10 mg/mL Glucanex enzyme (Chemcolour Industries, Auckland, NZ) at 37°C for 30 minutes after initial lysis.

2.3 DNA extraction: extraction procedures and kits

To maintain a standard baseline, optimization was carried out using uninfected needles (to extract pine DNA), rather than infected needles that could contain variable quantities of fungal DNA. DNA was initially extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or a Phytopure DNA extraction kit (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire), both of which are also routinely used for fungal DNA extraction. Subsequent developments involved using a combination of these kits:

(1) Qiagen lysis components, up to step 10 of the mini-protocol in the manufacturer's instructions, using modified volumes (see below) and a single 50 μL elution, and (2) Phytopure purification components, from step 10 of the small sample protocol in the manufacturer's instructions. Ultimately it was possible to replace components of these kits such that only the Qiagen lysis buffers are required (see page 5).

After extraction the DNA was quantified using Nanodrop spectrophotometry (Thermo Scientific NanoDrop 1000). The 260/280 nm absorbance ratio was also measured. DNA has an absorption maximum at 260 nm and protein at 280 nm, thus the 260:280 ratio indicates the purity of the DNA, with pure DNA having a ratio of 1.8-2.0.

2.4 Whole genome amplification

Whole genome amplification was carried out using the MDA (multiple displacement amplification) method of Foster and Monahan (2005). The method involves use of short (random hexamer) primers and a polymerase enzyme from bacteriophage phi29 that can displace previously copied DNA strands. The reaction is carried out at 30°C for 18 hours. Modifications included use of PCR buffer (Roche: 2X final concentration) instead of TE buffer (10 mM Tris, 1mM EDTA) during the initial MDA denaturation step (Gadkar & Rillig, 2005).

Optimised method for extraction of DNA from pine needles.

Volumes and amounts are given for extraction from large (3 cm) needle sections, followed [in parenthesis] by those used for small (3 mm) samples.

- 1) Cut pine needle sample into sections 1 cm or smaller and place pieces in a 1.5 mL microcentrifuge tube. Determine weights before and after freeze-drying.
- 2) Place closed tubes containing samples in liquid nitrogen until frozen, then remove tubes and grind samples with a sterile microcentrifuge pestle until no large sections are present. Add approximately 30 mg of 650 μm diameter glass beads and grind again with pestle until samples are roughly homogenized.
- 3) Add 300 μL [100 μL] of Qiagen lysis buffer AP1 (heated to 65°C), then grind until homogenization is complete.
- 4) Place tube in 65°C water bath for 10 minutes, mixing several times.
- 5) Remove tube from water bath and add 100 μL [35 μL] of 10 mg/mL Glucanex enzyme in CTAB buffer (see recipe below).
- 6) Add 3 μL [1.5 μL] RNase (Invitrogen 46-7604, Carlsbad, California, USA) and incubate at 37°C for 30 minutes.
- 7) Remove from water bath and add 130 μL [45 μL] Qiagen AP2 precipitation buffer then incubate on ice for 5 minutes.
- 8) Centrifuge at 8,000 g for 10 minutes, and transfer supernatant to a new tube.
- 9) Run a chloroform extraction: add 500 μL ice-cold chloroform along with 100 μL of 5 x TE buffer. Centrifuge at 1300 g for 1 minute then transfer top layer to a fresh tube.
- 10) Precipitate the DNA. Add an equal volume of isopropanol, mix gently, with inversion. Centrifuge at 8,000 g for 5 minutes. Carefully remove supernatant from pellet. Wash pellet with 70% ethanol (30% TE). Air dry pellet, then resuspend in 20 μL of Qiagen AE buffer (or TE buffer).

Notes:

CTAB buffer: 2% (w/v) hexadecyl-trimethyl-ammonium bromide (CTAB), 1% (w/v) PVP40, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH 8.0).

5 x TE buffer: 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA

Qiagen AP1 and AP2 buffers can be purchased separately from the DNeasy Plant Mini Kit as product numbers 1014630 and 1014717 respectively.

2.5 PCR amplification

To assess whether the modified DNA extraction methods yielded PCR-quality DNA, plant DNA was targeted using primers designed to the single-copy cinnamyl alcohol dehydrogenase (CAD) gene. This provided a standard internal reference.

To assess the suitability of the new method for extraction of fungal DNA from lesions on infected needles, fungal DNA was amplified using primers designed to amplify either the ribosomal ITS (multicopy target) region or the polyketide synthase (*pksA*) dothistromin biosynthetic gene (single-copy target) (Bradshaw *et al* 2006). Nested primers were used to increase sensitivity and specificity at both of these loci.

Table 1: Primers and primer combinations used in this work.

ITS1 and ITS4 primers are from White *et al* (1990). The *Dothistroma* spp. *pksA* gene primers were designed in the FHRC 2007-04 work and *Pinus radiata* CAD (Moyle *et al* 1998) primers designed by Dr Rebecca McDougal at Massey University.

Gene/ Locus	Primer name	Sequence (5' to 3')	Product size (bp)	Specificity
ITS	ITS1	TCCGTAGGTGAACCTGCGG	~500	Fungal
	ITS4	TCCTCCGCTTATTGATATGC		
ITS (nested)	DPS ITS spec fwd	CTGAGTGAGGGCGAAAG	406	<i>Dothistroma</i>
	DPS ITS spec rev2	CTCTTCAGCGAAATATATG		
<i>pksA</i>	DPS <i>pksA</i> fwd2	GCCTCTGGGAAGCG	219	<i>Dothistroma</i>
	DPS <i>pksA</i> rev2	GACTGAGCTCCCAAGG		
<i>pksA</i> (nested)	DPS <i>pksA</i> fwd3	GCGAAAGATTCGTTGATTGT	142	<i>Dothistroma</i>
	DPS <i>pksA</i> rev3	CCAAGGCTCCTCAAGTC		
CAD	CAD 793	GGGATTGTAACAGAGATTGGTAG	200	<i>P. radiata</i>
	CAD 973	ATACTGCTTGCAAATCCTCC		
CAD	CAD 920	GCAAGAGGATTTGGACCTAC	478	<i>P. radiata</i>
	CAD 1378	TGCTAACAAAGATAAGCATCG		

DNA was PCR-amplified using 1 μ L each of 10 μ M P_{for} and P_{rev} , 1 μ L of 1.25 mM dNTPs, 0.75 μ L of 50 mM $MgCl_2$, 2.5 μ L of 10 x PCR buffer (Roche), 0.2 μ L (1 unit) of Taq polymerase, 2 μ L of sample and water up to 25 μ L (for each reaction).

After initial incubation at 94°C for 4 min, 36 cycles of PCR were run with 30 sec denaturation at 94°C, 30 sec annealing at 58°C and 30-40 sec extension at 72°C, followed by a 4 min additional extension period at 72°C to finish.

10 μ L of PCR products from each reaction were run on a 2% agarose gel, stained for 10 minutes in ethidium bromide solution, and visualised using a Bio-Rad gel documentation system.

2.6 Statistical analysis

An unequal variance T-test (Ruxton 2006) was used to compare DNA yields obtained using different extraction methods, based on a null hypothesis of no significant difference in each case.

3. RESULTS AND DISCUSSION

3.1 Optimisation of DNA extraction from pine needles.

A step-wise approach was taken to optimize DNA extraction. When a procedure showed significant improvements, that procedure was used as the baseline for further improvements. The yield of DNA extracted from pine needles using different methods is shown in Figure 1. The 'standard' method shown in lane 3 involves manual grinding of pine tissue followed by extraction using a DNeasy Plant Mini Kit (Qiagen). Both the Qiagen and the alternative Phytopure kits involve steps to lyse the tissue followed by steps to purify the DNA.

A **hybrid combination of Qiagen and Phytopure kits** (see methods and lane 1 in Figure 1) gave a higher DNA yield than using either Qiagen (lane 3, $P_{3=1} = 0.011$) or Phytopure (lane 5, $P_{5=1} = 0.005$) kits alone. The combination shown in lane 1 involved use of the Qiagen lysis components followed by use of the Phytopure purification resin (removes polysaccharide contaminants) and chloroform extraction (instead of the Qiagen purification column).

Freeze dried needles (lane 1) yielded significantly more DNA than fresh needles (lane 2; $P_{2=1} = 0.006$), thus all further extractions used freeze-dried needles.

Using the Qiagen/Phytopure combination, pre-digestion with **glucanex enzyme** (a mixture of β -glucanase, chitinase and cellulase) increased DNA yield (compare lanes 7 and 1; $P_{7=1} = 0.001$). Lane 8 shows a 'mock digestion' without Glucanex ($P_{8=7} = 0.003$).

Use of a commercial **Fastprep bead beater** machine *instead of* manual grinding with an Eppendorf pestle resulted in a very low yield of DNA (compare lane 10, beating, with lane 7, manual grinding; $P_{10=7} = 1.42 \times 10^{-8}$). Use of the fastprep bead beater *in addition to* manual grinding gave a small but non-significant increase in yield (compare lanes 9 and 7; $P_{9=7} = 0.068$). However the DNA extracted using the Fastprep bead beater was not consistently PCR-amplifiable, possibly due to degradation of the DNA by strong shearing forces. Moreover, use of the Fastprep machine increased the complexity and time taken to extract DNA, hence only manual grinding was used in future work.

Due to the high cost of using two commercial DNA extraction kits, efforts were made to replace the kit components with laboratory-prepared alternatives. Extensive testing with various buffer formulations did not yield replacements for Qiagen buffers AP1 and AP2 (results not shown), but these can be purchased separately from the remainder of the kit. **Deletion of the Phytopure resin** step prior to chloroform extraction caused a severe decline in yield (compare lanes 7 & 11; $P_{11=7} = 1.72 \times 10^{-8}$) but addition of 100 μ L TE buffer in place of the resin gave a yield comparable to the Qiagen/Phytopure hybrid method (compare lanes 7 and 12; $P_{12=7} = 0.65$). TE buffer includes EDTA that chelates Mg^{2+} ions required by nuclease enzymes, suggesting that the extracted DNA is very vulnerable to attack by nuclease enzymes at this time.

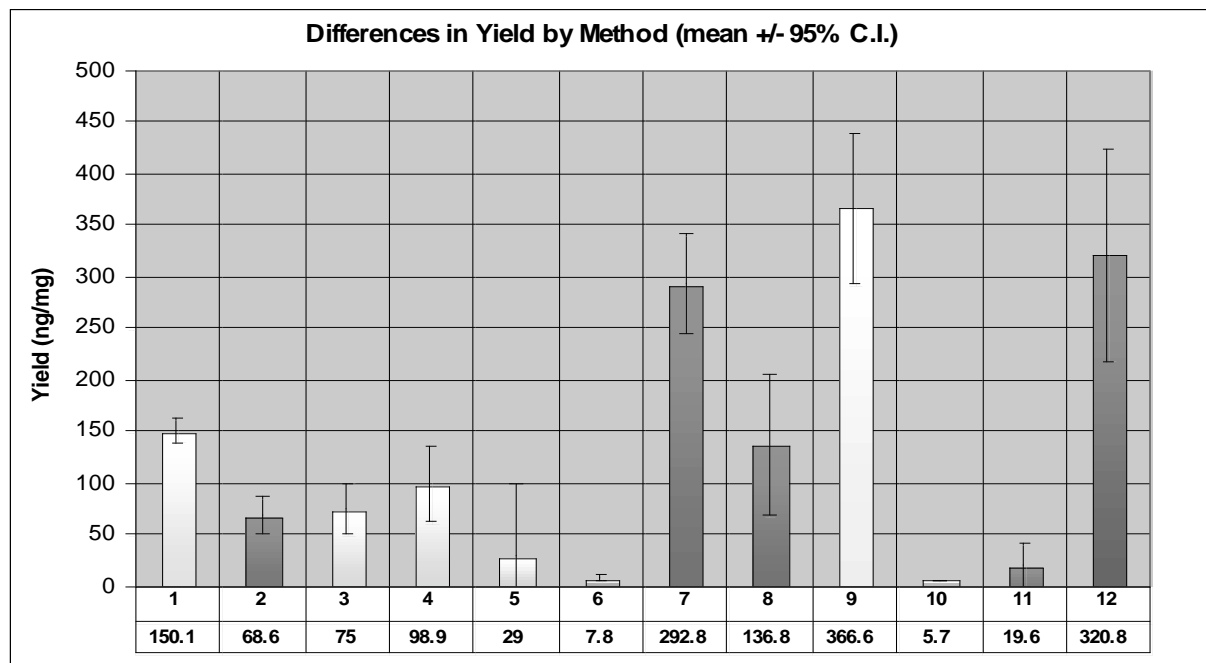
The final, modified method shown in lane 12 does not require any components from the Phytopure kit, consistently gives DNA with a 260:280 nm absorbance ratio of >1.8 that is PCR-amplifiable, and is the 'final method' shown on page 6.

This method shows a > 4-fold increase in yield over Qiagen and Phytopure kits. It is possible that yield from the Qiagen kit is compromised by retention of high molecular

weight DNA on the column that is normally used for purification. Conversely the Phytopure kit may be less efficient during the initial lysis stages.

Figure 1: Summary of DNA extraction optimization experiments

'Standard' methods are shown in lanes 3 (Qiagen) and 5 (Phytopure) and the final modified method in lane 12.



Mod.	1	2	3	4	5	6	7	8	9	10	11	12
Fresh	-	+	-	-	-	-	-	-	-	-	-	-
Glnx.	-	-	-	+	-	+	+	N	+	+	+	+
Grnd.	+	+	+	+	+	+	+	+	+	-	+	+
Btr.	-	-	-	-	-	-	-	-	+	+	-	-
Lysis	Q	Q	Q	Q	P	P	Q	Q	Q	Q	Q	Q
Purif.	P	P	Q	Q	P	P	P	P	P	P	C	M
n	18	3	3	3	3	3	18	6	3	3	3	6
PCR	++	++	++	++	+	-	++	++	-	-	++	++
A260/280	1.74	1.87	1.71	1.94	1.22	1.51	1.77	1.71	1.84	1.13	1.44	1.83

Yield is given as μg DNA/mg fresh weight of green needle tissue.

Key to modifications made to method

Fresh: +, Needles not freeze-dried before extraction.

Glnx.: +, Ground needles incubated 30 minutes with Glucanex enzyme.

N: Ground needles incubated with CTAB buffer (negative control for Glucanex).

Btr.: Needles ground using a Fastprep bead beater.

Grnd.: +, Some needles were not manually ground (to test if Fastprep is sufficient by itself).

Lysis: Kit used for lysis of ground pine needle sections, Qiagen (Q) or Phytopure (P).

Purif.: Kit or method used for purification of DNA, Qiagen (Q), Phytopure (P), chloroform (C), or modified chloroform (M).

n: Number of samples tested.

PCR: Amplification from purified product; ++ consistently good, + poor/variable, - no amplification

3.3 Whole genome amplification

Whole genome amplification was achieved with purified fungal genomic DNA template as well as with DNA isolated from herbarium samples. Amplification was more efficient with TE buffer than with 2 x PCR buffer during the denaturation step, in contrast to published reports (Gadkar & Rillig, 2005). However it was decided not to continue with whole genome amplification as the amplification process was unreliable, with some samples in most batches not being amplified. Furthermore, technical improvements in the DNA extraction process, followed by nested PCR, gave a high level of sensitivity specific for *Dothistroma* (as opposed to amplification of plant and fungal material) and therefore WGA was not considered worth pursuing further.

3.4 Extraction from small samples

The method shown on page 6 was tested with smaller samples, with a view to being able to extract DNA from single infected needle lesions. In this trial uninfected needles and pine CAD PCR primers were used as before, but the extraction procedure was scaled down such that only a 1 cm or 3 mm length of needle was included in each extraction tube (instead of 3 cm). Although there was some variation in yield of DNA (see Table 2 below) it was possible to extract DNA from single lesions and to amplify the DNA with pine CAD primers.

Table 2: Yields of DNA from small samples of needles.

Sample size (mm)	DNA conc. (ng/ μ L)	Total DNA (ng)	Mean fresh weight (mg)	Yield (ng/mg FW) Mean \pm SD	Mean dry weight (mg)	Yield (ng/mg DW) Mean \pm SD	<i>n</i>	PCR
30	167.8	3356.8	10.6	321 \pm 124.9	4.5	772 \pm 356.3	6	++
10	24.2	484.7	3.2	149 \pm 98.8	1.7	300 \pm 227.5	3	++
3	17.9	358	1.3	272 \pm 47.1	0.8	470 \pm 58.1	3	++

3.5 Extraction from infected needles

DNA was extracted from 3 mm length sections of pine needle lesions infected with *Dothistroma septosporum*. These samples were expected to yield a mixture of pine and fungal DNA, including DNA from resident endophytes as well as from *D. septosporum*. Extraction from *Dothistroma*-infected lesions on needles gave more variable amounts of DNA than extraction from 3 mm sections of uninfected needles: 150 – 1000 ng compared to 330 – 370 ng (total DNA) from uninfected needles.

Because of tissue dehydration in the infected needle tissue, the 'wet weights' are not comparable with those from uninfected pine needle samples. The weight of the lesions was extremely low, meaning that they had to be weighed together and an average weight taken. Dry weights were too low to be accurately measured for these samples. However the yield of DNA is still higher than the yield per dry weight from uninfected needles. This could reflect increased accessibility of DNA from the degraded plant cells, and/or increased abundance of fungal biomass in the lesions compared to uninfected needles.

Table 3: Yields of DNA from infected needles.

Surface sterilized	DNA conc. (ng/ μ L)	Total DNA (ng)	Abs 260/280 nm	Mean weight (mg)	Yield (ng/mg) Mean \pm SD	<i>n</i>	PCR	Nested PCR
-	13.4	267.3	1.45	0.3	827.8 \pm 520	3	++	++
+	29.0	579.5	1.34	0.5	1091 \pm 418	8	-	+

DNA extracted from surface-sterilised needles (sterilization as per Ganley and Newcombe 2006) was a weak template for PCR amplification with fungal ITS and *pksA* primers, requiring nesting to give a product visible on a gel. However it is likely that this could be improved with optimization of the surface sterilisation method.

3.6 PCR from uninfected needles

The nested PCR approach provides a very high level of sensitivity and specificity as it involves two rounds of PCR and two sets of primers. It was hoped that we could determine if *D. septosporum* can be identified in needles that do not show needle blight symptoms, even at a low level. If so, this would indicate that *D. septosporum* can be present as a latent pathogen or endophyte in the needle. PCR with universal ITS1 and ITS4 primers will amplify DNA from any fungi present in or on the needle, hence serves as a control. However the nested ITS primers are specific and only amplify from *D. septosporum* and *D. pini*. The ITS region is a multicopy target in the fungal genomes, thus providing a sensitive test.

Unfortunately no conclusive results were obtained in the time available, since most of the time in this project was spent optimizing the DNA extraction method. However our current hypothesis for the growth of *D. septosporum in planta* is that there is a latent pre-symptomatic period after needle infection during which very little growth of the fungus occurs (Schwelm 2009). A PhD student who will commence work in my laboratory later in 2009 (Mr Kabir, recipient of a Massey Doctoral Scholarship) will investigate this hypothesis. The optimised DNA extraction method outlined in this report will be of considerable benefit for this new project.

3.7 Extraction from herbarium samples

One lesion was taken from each of twelve dried herbarium specimens prepared in 2006 and the DNA extracted. The concentration of DNA obtained (ng/ μ L) was similar to that of the small samples and single lesion extractions, although the mean Abs 260/280 ratio was low. As with the single lesion extractions, DNA yield per mg plant tissue cannot be reliably compared with previous extractions. The samples were very dry and light and could not be weighed accurately.

Table 4: Yield of DNA from herbarium samples (needle lesions)

Surface sterilized	DNA conc. (ng/ μ L)	Total DNA (ng)	Abs 260/280 nm	Mean weight (mg)	Yield (ng/mg) Mean \pm SD	<i>n</i>
+	33.0	660	1.32	0.5	1438 \pm 1072	12

Initial experiments suggest that this DNA can be amplified both with *Dothistroma*-specific ITS and with nested *pksA* primers, leading to the possibility of cloning and sequencing these and other regions of the genome, but further work is required.

4. FUTURE WORK

The optimized DNA extraction method outlined here will greatly facilitate further work. It will be published along with a new quantitative real-time PCR method that is currently being developed by Dr Rebecca McDougal at Massey University.

As outlined in section 3.6, PhD student Mr Kabir will use the new DNA extraction method to examine whether *D. septosporum* has a latent period *in planta* prior to symptom development. This is part of a larger project aimed at determining whether dothistromin toxin has a role in competition against other microorganisms *in planta*.

The work with herbarium samples will be continued partly by Mr Tim Owen as part of his MSc. He will attempt to obtain PCR-quality DNA from older herbarium samples of *Dothistroma*-infected needles using the new DNA extraction method. Following on from that, a Bio-Protection CoRE-funded PhD student (Mr Chettri) commencing August 2009, will attempt to clone and sequence the *pkxA* and other variable regions from herbarium isolates and compare sequences to those of modern day isolates, including some from epidemic areas in Canada and Europe.

ACKNOWLEDGEMENTS

We would like to thank the New Zealand Forest Health Research Collaborative for funding this project, Margaret Dick (Ensis) for the provision of herbarium specimens and Dr Rebecca McDougal for provision of CAD primers and for helpful comments on the report.

REFERENCES

- Bradshaw, R.E., Jin, H.-P., Morgan, B.S., Schwelm, A., Teddy, O.R., Young, C.A. and Zhang, S.G. (2006) A polyketide synthase gene required for biosynthesis of the aflatoxin-like toxin, dothistromin. *Mycopathologia* **161**, 283-294.
- Foster, S.J. & Monahan, B.J. (2005) Whole genome amplification from filamentous fungi using Phi29-mediated multiple displacement amplification. *Fungal Genetics and Biology* **42**, 367-375
- Gadkar, V. & Rillig, M.C. (2005) Suitability of genomic DNA synthesised by strand displacement amplification (SDA) for AFLP analysis: genotyping single spores of arbuscular mycorrhizal (AM) fungi. *Journal of Microbiological Methods* **63**, 157-164
- Ganley, R. J. and G. Newcombe (2006) Fungal endophytes in seeds and needles of *Pinus monticola*. *Mycological Research* **110**, 318-327.
- Moyle, R. (1998) Nucleotide sequence of a cinnamyl alcohol dehydrogenase gene (Accession No. AF060491) from *Pinus radiata* (PGR98-118). *Plant Physiology* **117**, 1125
- Ruxton, G.D. (2006) The unequal variance *t*-test is an underused alternative to Student's *t*-test and the Mann–Whitney *U* test. *Behavioral Ecology* **17**, 688-690
- Schwelm, A., Barron, N.J., Baker, J., Dick, M., Long, P.G., Zhang, S. and Bradshaw, R.E. (2009) Dothistromin toxin is not required for Dothistroma needle blight in *Pinus radiata*. *Plant Pathology* **58**, 293-304.