Commercial in Confidence

Client Report No. 39801

Detection of *Nectria fuckeliana* in wood cores from pruned and un-pruned *Pinus radiata* – Interim Report

Matthew Power and Tod Ramsfield

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Matthew Power and Tod Ramsfield

Date: **June 2006**
Client: Forest Health Research Collaborative / Forest Biosecurity Research Council
Contract No:

ensis

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EXECUTIVE SUMMARY

Objective

The objective of this study was to use a DNA based method to detect the presence of *Nectria fuckeliana* in both pruned and un-pruned *Pinus radiata* trees and to determine if the presence of the fungus was related to pruning.

Key Results

A previously developed polymerase chain reaction (PCR) protocol was used to detect *N. fuckeliana* in wood cores collected from four different stands of *Pinus radiata* in Southland. Using this protocol, *N. fuckeliana* infection was detected in 41 of 180 trees.

Positive *N. fuckeliana* results were identified in trees from all three of the forests from which samples were gathered – Tokoiti, Berwick and Otago Coast – and in both pruned and un-pruned trees. Chi square analysis indicated that there was no significant difference in the presence of *N. fuckeliana* between pruned and un-pruned trees.

Further Work

Further investigation of the trees sampled in this study will take place February / March of 2007. This will include a visual assessment of each tree to record any symptoms of the flute canker disease and will include further sampling for DNA analysis.

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Information for Ensis abstracting:

Contract number	
Client Report No.	39801
Products investigated	*Nectria fuckeliana*
Wood species worked on	*Pinus radiata*
Other materials used	none
Location	Tokoiti Forest, Berwick Forest, Otago Coast Forest

INTRODUCTION

Nectria fuckeliana is widely distributed throughout the Otago/Southland region and it is hypothesized that the fungus is the causal agent of the flute canker disease of *P. radiata.* It is believed that the disease is initiated by the entrance of *N. fuckeliana* spores through pruning wounds. The infection then spreads upwards and downwards from the entry point, killing cambium cells and resulting in the flute canker symptom (Wang and Thode, 2004).

A specific test to detect the presence of *N. fuckeliana* within woody tissue has been developed previously (Langrell, 2004) and was optimized for New Zealand on material gathered from the Otago/Southland region (Power and Ramsfield, 2005).

The objective of the research conducted within this project was to use the DNA based identification system to detect the presence of *N. fuckeliana* within pruned and un-pruned trees to determine if there is a relationship between pruning and the presence of *N. fuckeliana*.

MATERIALS AND METHODS

Collection

Wood cores were collected from forests, one core per tree, located around the Otago region, from Tokoiti, Otago Coast and Berwick Forests (Appendix A), using an increment core borer. From each site half of the cores collected were from young trees which had recently received their first pruning, and the other half from trees in the same stand which had not been pruned (followers).

One core was removed from every tree sampled, resulting in a total of 180 increment cores from 180 trees. Of these, 90 cores were taken from trees which had been pruned in the previous year, and 90 cores were taken from trees which had not been pruned. One hundred cores were collected from two different stands in Tokoiti Forest and forty cores were taken from both Otago Coast and Berwick Forests.

Wood cores between 5 and 13 cm long were collected from the stem of the tree, directly above a pruning wound on pruned trees, or in the case of the un-pruned trees, from as close to the whorl as possible. The core borer was immersed in bleach and then rinsed in water after extracting each core to prevent cross-contamination.

Every tree that was sampled was numbered and marked with yellow paint so that follow-up assessments can be made.

DNA Extraction and Quantification

The bark end of the wood core was removed and discarded. Small rounds were then cut from the remaining core and ground to a fine powder in liquid nitrogen with a mortar and pestle. Enough rounds were cut to produce a volume of 200-500µl of powdered wood tissue after grinding.

DNA was isolated from the powdered tissue using the FastDNA® kit, with DNA extraction buffer CLS-VF, and the FastPrep® instrument (Qbiogene, Inc., CA) following the manufacturer's protocol with the following modifications: Homogenisation was carried out with lysing matrix A and an additional ceramic sphere at speed 5 for 20 seconds and repeated three times. All incubation steps were carried out at room temperature.

Following DNA extraction, the concentration of DNA in every sample was determined using a FLUOstar Galaxy fluorometer (BMG Lab Technologies) and the DNA was then diluted to a concentration of 1 ng/µl with sterile water for the PCR analysis.

PCR Amplification

Nested PCR reactions were carried out as described in Power and Ramsfield (2005). The first round PCR reaction was performed on DNA that was diluted to 1 ng/µl and used the conserved PCR primers ITS-1F and ITS-4 to amplify any fungal DNA present in the wood. The PCR products from this reaction were then diluted 1/50 with ultra-pure water and re-amplified with the *N. fuckeliana* specific primers Cct1 and Cct2. Generation of a band of 360 base pairs following the *N. fuckeliana* specific PCR reaction was considered to be a positive diagnostic result. The PCR test was duplicated on every sample to ensure that the results were consistent.

PCR products were electrophoresed on 1% agarose gels, stained using ethidium bromide and then visualised and photographed under UV light.

Samples which failed to produce PCR products for both first and second round reactions were re-amplified using 1/200 dilutions of extracted wood core DNA.

Statistical Analysis

To determine if the presence of the fungus was related to pruning, the Chi square test was conducted on a two by two contingency table. This allowed comparison of the following categories: Pruned and *N. fuckeliana* present, pruned and *N. fuckeliana* absent, un-pruned and *N. fuckeliana* present, and un-pruned and *N. fuckeliana* absent. Only samples that gave consistent results after two replications of the PCR test were included in the analysis (173 out of 180).

RESULTS AND DISCUSSION

All nested PCR reactions were repeated to confirm the results of the previous test. Following the initial round of amplification, 122 of the 180 cores gave consistent results. DNA from thirty-two wood cores failed to amplify PCR products with the ITS primer pair. DNA from a further twenty-six wood cores failed to produce consistent amplification when reactions were repeated.

The DNA that did not amplify was re-tested using 1/200 dilutions of DNA stock rather than a 1ng/µl dilution of the stock and amplification proved successful with this template concentration. All samples that were tested at this concentration produced PCR products with the ITS primers. All but two of these samples produced consistent results for the Nectria-specific reaction.

DNA from cores which gave inconsistent results was re-amplified using the original nested procedure. These reactions were also duplicated. Five of these cores continued to give inconsistent results. These problems may be due to the wood sample themselves, rather than the procedure and the trees that gave inconsistent results will be resampled in 2007.

The seven cores that did not give consistent results for the Nectria-specific reaction were distributed amongst all of the forests tested, and came from both pruned and un-pruned trees (Table 1). These samples were not included in the statistical analysis.

Table 1: Wood cores giving inconsistent results

Wood Core	Forest	Pruning Status
82	Tokoiti	Un-Pruned
108	Otago Coast	Pruned
123	Otago Coast	Un-Pruned
126	Otago Coast	Un-Pruned
134	Otago Coast	Un-Pruned
143	Berwick	Pruned
159	Berwick	Pruned

Nectria fuckeliana was detected in trees from all forests and in both pruned and un-pruned trees (Tables 2 and 3).

Table 2: Comparison of positive results for Pruned and Un-pruned trees

Location	Number of positive results	
	Pruned	Un-pruned
Tokoiti Cpt 35	1	1
Tokoiti Cpt 50	0	4
Berwick Cpt 99	8	7
Otago Coast Cpt 127	10	10

Table 3: *N. fuckeliana* positive results per forest

Location	Number of Trees tested	Number of Inconsistent Results	Number of Positive Results	% of Positive Results*
Tokoiti Cpt 35	50	0	2	4
Tokoiti Cpt 50	50	1	4	8.2
Berwick Cpt 99	40	2	15	39.5
Otago Coast Cpt 127	40	4	20	55.6

* = inconsistent samples are not included in the percentage calculation.

A Chi square analysis (Chi square = 0.327) was carried out comparing positive Nectria results between pruned and un-pruned trees (Table 4). The analysis indicated that there is no significant difference between the pruned and un-pruned trees and the presence of *N. fuckeliana*.

Table 4: Results utilized for the Chi Square analysis

Pruning Status	Nectria present	Nectria absent
Pruned	19	68
Un-Pruned	22	64

RECOMMENDATIONS AND CONCLUSIONS

Nectria fuckeliana was detected in both pruned and un-pruned trees from all forests that were surveyed. Statistical analysis of the results suggest that the presence of the fungus was not dependent on entrance through pruning wounds as there was no significant difference in the presence of the fungus between pruned and un-pruned trees.

The results obtained from this study were unexpected. The majority of the trees sampled appeared healthy at the time of core collection; therefore, a low number of positive results were expected. Also, the great majority of un-pruned, trees were not expected to be positive for the presence *of N. fuckeliana* based on the current hypothesized mechanism of host infection by *N. fuckeliana*

It is believed that *N. fuckeliana* enters the tree through pruning wounds; however, the fungus has been identified in wood cores from un-pruned trees. This indicates that the fungus has entered though another wound of some kind, or infection occurs via a different mechanism. Our current understanding of the infection process of this fungus is not complete and other studies of the epidemiology of the pathogen are being conducted. As the results of these other studies are analysed, it is hoped that we will be able to piece together the mode of infection of this fungus.

The identification of *N. fuckeliana* in the wood cores does not necessarily constitute a disease outbreak in the affected forests. The majority of the trees sampled showed none of the symptomatic traits associated with *N. fuckeliana* infection (i.e. fluting or fruiting bodies). It should be emphasised that these results are preliminary and future investigation is planned.

The field sites will be revisited in 2007 and all trees will be visually inspected to observe and record any symptoms of the fungus. Additional samples from the trees which had inconsistent results, and trees that appear to have early symptoms of infection by *N. fuckeliana,* will be collected and analysed in 2007.

ACKNOWLEDGEMENTS

Peter Oliver of City Forests Ltd and Paul Greaves of Wenita Forest Products Ltd identified sites and allowed access to the forests. Funding for this research was provided by the Forest Health Research Collaborative and Forest Biosecurity Research Council.

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Power, M., Ramsfield, T. 2005: Investigation of the relationship between *Sphaeropsis sapinea* and *Nectria fuckeliana*. Ensis Client Report No. 38904. 18p.

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APPENDIX

Appendix A – Collection site details

Site 1
Tokoiti forest, Compartment 35. Follow Hetherington Rd. After going through second gate, the road forks, take fork to right up hill, past gravel pit. Pruned samples 1-25, unpruned samples 26-50. Sampled trees are scattered on the downhill side of the site.

Site 2
Tokoiti forest, Compartment 50. Follow Heatherington Rd down, go through gate to main road, turn right and drive to gravel pit. The site can also be accessed by driving on HWY 1 to fork. Fork near where the train track parallels the road (Road possibly called Lakeside Rd). Take L fork and follow road to just before Kaitangata and turning L on private road. Pruned samples 51-75, unpruned samples 76-100. The trees are on both sides of the track and are not far off the track.

Site 3
Otago Coast Forest, Compartment 127. Trees located on N side of Centre Road. Plot starts 0.9 km from the intersection of Longway Rd and Centre Rd. Pruned samples 101-120, unpruned 121-140. Access via Glenledi Road and then turn L on Big Bush Road. Trees are all on the side of the road.

Site 4
Berwick Forest, Compartment 099. Trees located on N side of Prentice road. Plot starts 200m from intersection of Prentice Road and Longspur Road. Pruned trees 141-160, unpruned 161-180. Most trees close to the side of the road although not all.