Client Report No. **41261**

Detection of Nectria fuckeliana in wood cores from pruned and un-pruned Pinus radiata. Second year results - February 2007.

Matthew Power and Tod Ramsfield

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Detection of *Nectria fuckeliana* in wood cores from pruned and un-pruned Pinus radiata. Second year results - February 2007.

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Date: **August 2007**
Client: **Forest Health Research Collaborative / Forest Biosecurity Research Council**
Contract No:

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EXECUTIVE SUMMARY

Objective

The objective of this study was to resample pruned and unpruned *Pinus radiata* trees that were first sampled in 2006 and to use a DNA based method, as well as culturing, to detect the presence of *Nectria fuckeliana.* We wanted to confirm our results from 2006 which indicated that there was no significant difference between pruned and un-pruned trees and the presence of *N. fuckeliana*, determine if fungal incidence changed over time, and determine if there was a relationship between presence of the fungus and fluting.

Key Results

Using the molecular identification technique, *N. fuckeliana* was detected in 39 of the 180 cores that were collected and the fungus was isolated from 11 cores by culturing. The fungus was identified in both pruned and un-pruned trees using the molecular identification and culturing techniques. Chi square analysis indicated that there was no significant relationship between the presence of *N. fuckeliana* and whether the tree was pruned or un-pruned. Analysis also showed that there was a significant relationship between the presence of fluting and the presence of *N. fuckeliana* in the tree. The fungus was detected in 41 of the 180 cores collected in 2006; so there was no significant change in the incidence of the fungus from 2006 to 2007.

Fungal growth was recorded from 71 of the 180 surface sterilised cores that were cultured. Of these, *N. fuckeliana* was identified on 11. The identification was confirmed using the DNA identification test, and importantly, it was found that only the 11 isolates that gave positive results by culturing were identified as *N. fuckeliana* by the DNA method. There were no false positives.

Further Work

Destructive sampling of selected trees is planned to take place early 2008 to detect and map the distribution of *N. fuckeliana* within the tree. This will involve further testing using culturing and molecular methods.

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Information for Ensis abstracting:

Contract number	
Client Report No.	**41261**
Products investigated	Nectria fuckeliana
Wood species worked on	Pinus radiata
Other materials used	none
Location	Berwick Forest, Otago Coast Forest, Tokoiti Forest

INTRODUCTION

Nectria fuckeliana is associated with the flute canker disease of *Pinus radiata* in the Otago, Southland and Canterbury regions of New Zealand. The most northerly location record to date is the Banks Peninsula (Turner *et al.*, 2007). The disease is characterised by cambial death, resulting in staining, stem malformation, wood depression (fluting) and growth loss. Fluting has been observed in trees of all ages (Wang & Thode 2004), although is more likely to be seen on trees of 3 years or older.

It has been demonstrated that pruning wounds provide infection courts for *N. fuckeliana*, leading to initiation of the disease (Dick *et al*., 2006). Size of injury (such as branch stub size), depth and height of injury and length of time since injury can affect infection. Winter pruning results in an increase in infection (Dick *et al*., 2006). Research has confirmed that changes in planting and pruning regimes can reduce vulnerability to the disease. It is possible to limit infection by altering the timing of pruning operations (Wang & Thode, 2004; Dick *et al*., 2006). *Nectria fuckeliana* requires an entry point for infection and stem wounds and bark peeling wounds are other possible routes in which the fungus may invade the tree (Roll-Hansen & Roll-Hansen, 1980; Vasiliauskas *et al.*, 1996).

In 2006, a project was initiated to use a DNA based identification system to detect the presence of *N. fuckeliana* within pruned and un-pruned trees and determine if there is a consistent relationship between pruning and the presence of *N. fuckeliana*. Samples were collected using an increment core borer from young (aged from four to nine years) pruned and un-pruned trees in three forests in the Otago region – Tokoiti, Berwick and Otago Coast. The majority of trees sampled did not display symptoms of *N. fuckeliana* infection. DNA was extracted from the wood cores and a previously developed *Nectria fuckeliana* identification method (Langrell 2004) was used to determine if the fungus was present in the wood. It was found that *N. fuckeliana* was present in 41 wood cores: 19 from pruned trees and 22 from un-pruned trees. Positive results were recorded from all three of the forests sampled. Statistical analysis, using the Chi-square technique, indicated that there was no significant difference in the presence of the fungus between pruned and un-pruned trees (Power & Ramsfield, 2006). These results were unexpected as they contradicted the hypothesis which stated *N. fuckeliana* infects the tree primarily through pruning wounds.

The same trees were resampled in 2007 to validate the results of the 2006 study. As one more year had passed since pruning was carried out, it was also possible to determine if there was an increase in the number of infections over time.

The presence of the fungus was assessed in 2007 using both the DNA method used in 2006 and culturing from the wood cores. Trees were also visually inspected to compare the relationship between the visual signs (presence of perithecia) and symptoms (presence of fluting) of infection and the DNA and culturing results. Fluting is defined in this report as a stem depression resembling a canker or the early stages of a canker.

MATERIALS AND METHODS

Stands

Table 1: Details of stands sampled

Location	Planting date	Pruning date	Pruned trees sampled	Unpruned trees sampled
Tokoiti, Cpt 35	2002	Oct 2005	25	25
Tokoiti Forest, Cpt 50	2000	Sept 2005	25	25
Otago Coast, Cpt 127	1998	Aug 2004	20	20
Berwick, Cpt 099*	1997	Mar 2005	20	20

*Received first pruning in January 2004.

Sampling

Wood cores were taken from the same 180 trees that were sampled in 2006. Wood cores between 50 and 130 mm long were collected directly above a branch stub on pruned trees, or in the case of the un-pruned trees, from as close to the whorl as possible. Cores were removed from as close as possible to the previous year's sampling point. The core borer was immersed in bleach and then rinsed in water after extracting each core to prevent cross-contamination. Trees were re-labelled with new plastic tags for future assessments, if required.

Trees were visually inspected, with records taken on the presence or absence of fluting and the presence or absence of perithecia. Digital photos were taken of each tree.

Seven trees were felled between the initial core collection in February 2006 and the second core collection in February 2007. It was not possible to visually assess five of the felled trees. Cores were taken from all felled trees.

Surface Sterilisation and Culturing

One small round roughly 3 mm in width and 5 mm in depth, located approximately 10 mm from the bark end of the core were cut from each core. The bark end of the wood core was discarded. Rounds were surface sterilised by immersion in 70% ethanol, followed by 10% bleach and three final rinses in sterile distilled water. Rounds were immersed for 1-2 min at each stage. Sterilised rounds were then placed onto 2% malt extract agar (MEA) plates and incubated at 20°C. Cultures were checked daily and any mycelial growth that emerged from the round was subcultured onto a fresh MEA plate. *Nectria fuckeliana* cultures were identified based on morphology and were retained and added to the NZFS culture collection.

DNA Extraction and Quantification

After removal of rounds for culturing, small rounds were then cut from the remaining core and ground to a fine powder in liquid nitrogen with a mortar and pestle. Rounds were randomly distributed over the remaining core. Enough rounds were cut to produce a volume of 200-500µl of powdered wood tissue after grinding. Approximately 20 rounds were used per core. The mortar and pestle was sterilized between samples by thorough rinsing with ethanol and bleach.

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 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 *N. fuckeliana* specific PCR reactions were carried out. The first round involved amplification with the universal ITS primers ITS-1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The second round reaction involved amplifying the first round PCR products with the *N. fuckeliana* specific primers Cct1 (5'-ACC CCA AAC CCT TAT TTC TG-3') and Cct2 (5'-ACG GCG TGG CCG CGC CGC TT-3'). First round PCR amplification was carried out in a 15µl reaction volume containing 2.5ng of template DNA, 0.05pmol/µl of PCR primers ITS-1F and ITS-4, 0.45 U Taq DNA polymerase (Roche), 1 x reaction buffer (Roche), 1.5mM $MgCl_2$, 0.2mM each of dATP, dGTP, dCTP, dTTP (Roche). The PCR conditions were: 94°C for 3 min followed by 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 45 s, 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 2 min, 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 3 min, then one cycle of 72°C for 7 min, and a 4°C hold.
The PCR reaction contents of the second round reaction were the same as the first round, but the *N. fuckeliana* specific primers were used rather than the universal primers and 1/50 dilutions of first round ITS PCR products were used for the DNA template. Products were amplified using the following profile: 94°C for 3 min followed by 30 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, then one cycle at 72°C for 10 min, and a 4°C hold cycle.

Amplification of the DNA from cultures isolated from wood cores was carried out by scraping a small amount of mycelium directly from the plate using a pipette tip. The tip was placed into the first-round (ITS-1F / ITS-4) reagent cocktail and shaken slightly to dislodge the mycelium which provided the template DNA, then removed. The reaction was carried out as described above. Extra water was added to bring the reaction to 15µl total volume. Second-round reactions were carried out as described above.

Statistical Analysis

Chi square analysis was carried out to compare pruned and unpruned trees and the presence of the fungus as determined by PCR as well as culturing. In addition, Chi square analysis was carried out to compare the presence of fluting and the presence of *Nectria fuckeliana*. The Chi square test was conducted using two by two contingency tables. This allowed comparison of DNA results and pruning status, DNA results and presence or absence of fluting, and culturing results and presence or absence of fluting. Analysis was conducted using an online Chi square calculator (http://www.graphpad.com/quickcalcs/contingency1.cfm). In addition to analysing the 2007 data separately, the 2006 and 2007 positive results were pooled and analysed using the Chi square technique.

RESULTS

Field Assessment

Fluting was observed on both pruned and un-pruned trees. *Nectria fuckeliana* perithecia were observed only on one tree which was dead. The majority of wounds created by sampling in 2006 were well healed. Flutes were recorded on 56% of the pruned trees assessed and on 38% of the unpruned trees assessed. Most flutes were small and not well developed.

Table 2: Incidence of fluting

Fluting Present	Pruned	Unpruned
No	44%	62%
Yes	56%	38%

Culture Results

Fungal growth was recorded from 71 of the 180 surface sterilised cores that were plated out. Of the 71, the morphology of 11 matched *N. fuckeliana*, six of the matches coming from pruned trees, and five coming from un-pruned trees. The identification of these 11 isolates was confirmed using the DNA identification test. There was one tree where *N. fuckeliana* was confirmed by culturing but was not confirmed by the DNA method.

DNA Results

Nectria fuckeliana DNA was identified from 39 of the 180 wood cores using the *N. fuckeliana* specific nested PCR method. Positive results were recorded from pruned and un-pruned trees displaying both symptomatic and asymptomatic traits. All three of the forests sampled produced at least one positive result.

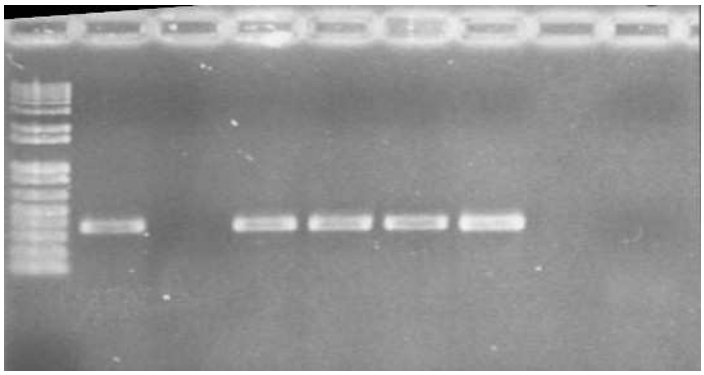

Fig 1: *N. fuckeliana* specific PCR reaction.
Band indicates positive identification for *N. fuckeliana*.

Chi square analysis was carried out to compare the presence of *Nectria* between pruned and un-pruned trees (Table 4). A Chi square statistic of 0.818 was generated, which indicated that there was no significant difference between pruned or un-pruned trees and the presence of *N. fuckeliana*.

Table 3: 2007 DNA identification results compared to pruning status

Pruning Status	Nectria present	Nectria absent	% Present
Pruned	17	73	18.9
Un-Pruned	22	68	24.4

Relationship between fluting and the presence of *N. fuckeliana*

Nectria fuckeliana was detected by culturing and by the DNA method in both pruned and unpruned trees with and without evidence of fluting (Table 5). There were pruned trees which were fluted in which the fungus was present (Fig 2) as well as pruned trees which were fluted but the fungus was absent (Fig 3). When a Chi-square analysis was conducted to compare the presence of flutes with the presence of *N. fuckeliana,* as determined by DNA, a significant relationship was observed (Chi square = 8.082) (Table 6); trees with *N. fuckeliana* were more likely to be fluted. When the analysis was conducted on trees that were identified as *N. fuckeliana* infected by culturing, there was no significant relationship (Chi square = 1.665).

Table 4: Evidence of infection compared to molecular and culturing results

Pruning Status	Fluting Present?	DNA Result	Culturing Result	No. of Trees
Pruned	Yes	Positive	Positive	2
Pruned	Yes	Positive	Negative	11
Pruned	Yes	Negative	Positive	2
Pruned	Yes	Negative	Negative	35
Pruned	No	Positive	Positive	1
Pruned	No	Positive	Negative	2
Pruned	No	Negative	Positive	1
Pruned	No	Negative	Negative	35
Un-pruned	Yes	Positive	Positive	4
Un-pruned	Yes	Positive	Negative	8
Un-pruned	Yes	Negative	Positive	1
Un-pruned	Yes	Negative	Negative	19
Un-pruned	No	Positive	Positive	0
Un-pruned	No	Positive	Negative	9
Un-pruned	No	Negative	Positive	0
Un-pruned	No	Negative	Negative	45

- These results do not include those trees which were felled before 2007 sampling.

Fig 2: Tree 22 – Pruned, Fluting present, No perithecia, Positive DNA result, Positive culturing result

Fig 3: Tree 15 – Pruned, Fluting present, Negative DNA result, Negative culturing result

Fig 4: Tree 131 – Un-pruned, Fluting present, Positive DNA result, Positive culturing result

Table 5: DNA results compared to presence or absence of fluting

	N. fuckeliana **Positive**	***N. fuckeliana*** **Negative**	**Positive (%)**
Fluting	25	57	30
No Fluting	12	81	13

- Results for this table omit the 5 trees which were cut down before sampling in 2007 and were not assessed for fluting.

PCR specificity

As fungi were isolated from a total of 71 trees, and only 11 of the isolates matched the morphology of *N. fuckeliana,* the PCR method was tested on all 71 of the isolates to ensure that the technique was specific to *N. fuckeliana.* It was found that only the 11 isolates that gave positive results by culturing were identified as *N. fuckeliana* by PCR. The PCR method did not amplify DNA from non-*Nectria* cultures.

Relationships between the 2006 and 2007 DNA results

In 2006, 41 *N. fuckeliana* positive trees were recorded using the DNA technique, while in 2007, 39 positive trees were recorded.

There were some inconsistencies in the results between 2006 and 2007:

- of the 39 *N. fuckeliana* positive trees in 2007, only 19 were *N. fuckeliana* positive in 2006, i.e. 19 trees that were that were found to be *N. fuckeliana* positive in 2006 were confirmed as positive in 2007.

- of the 39 *N. fuckeliana* positive trees in 2007, 17 were negative in 2006, i.e. 17 trees that were not infected in 2006 became infected by 2007.

- of the 41 *N. fuckeliana* positive results for 2006, 25 were negative in 2007.

- Seven cores produced inconsistent results (both positive and negative results recorded in multiple PCR tests) in 2006. Three of the inconsistent results recorded in 2006 produced positive results in 2007. This suggests that the infection by *N. fuckeliana* has become more widespread in the tree, thus increasing the probability of successfully detecting the fungus. Four of the trees which produced inconsistent results in 2006 were negative in 2007, suggesting that the increment core borer may have penetrated a small pocket of infection in 2006 and that sampling in 2007 missed the infected material.

Pooling of 2006 and 2007 results

In 2006, 41 trees were found to be positive, while in 2007, 39 trees were found to be positive for the presence of *N. fuckeliana*. As there were 19 trees that were found to be positive in both 2006 and 2007, pooling the data from both years suggests that a total of 61 of 180 trees were found to contain the fungus. When the 7 inconsistent results (i.e. both positive and negative results were recorded in multiple PCR tests) from 2006 and the one culture positive that was not backed up by a DNA positive are included, there were a total of 69 trees in this experiment that were found to contain *N. fuckeliana*. This analysis assumes that the trees which were found to be positive in 2006 but negative in 2007 were the result of the increment core borer sampling *N. fuckeliana* infected wood in 2006 but missing the infected wood in 2007. Of the 69 trees that were found to be positive for *N. fuckeliana*, 32 had been pruned while 37 had not. Calculation of the Chi square statistic (Chi square = 0.588) indicated that there was no significant relationship between pruning and the presence of *N. fuckeliana*.

DISCUSSION

To study the effect of pruning on the presence of *N. fuckeliana*, the fungus associated with the flute canker disease of *P. radiata*, samples were collected from pruned and unpruned trees in Otago. DNA analysis and culturing have both shown that there was no significant relationship between the presence of *N. fuckeliana* in the sapwood and whether the tree was pruned or un-pruned.

Comparison of culturing and DNA results have indicated that the DNA method may be a far more sensitive than culturing, due to the greater number of *N. fuckeliana* positive results obtained (39 positive DNA results in 2007 compared to 11 positive culturing results). However, in must be noted that the higher number of positive results with the DNA method might have been a result of the fact that DNA was extracted from a larger percentage of the core than the single disk that was cut from the core for culturing. The DNA test effectively sampled much more of the core because DNA from the different regions of the tree was pooled. In future, the increment core will be sampled so that isolations are made from the vascular cambium to the pith of the tree, to allow for the possiblity that the fungus is not distributed uniformly. Further support for uneven fungal distribution in the tree is made by the finding that one tree was found positive from culturing and negative from DNA analysis. The lower number of positive results from culturing may have been a result of competition between fungi as they grew from the sample; *N. fuckeliana* may have been out-competed by faster growing fungi present in the wood cores. Dick & Tetenberg (2007) found that two fungi commonly isolated from *Pinus radiata* stems affected by the pine fluting disease, *Stereum sanguinolentum* and *Sphaeropsis sapinea*, out-competed *Nectria fuckeliana* when growing on wood blocks.

When the samples were collected, the trees were visually inspected for the presence of flutes and *N. fuckeliana* perithecia. Flutes were present on both pruned and unpruned trees but statistically, the presence of fluting was significantly associated with the presence of the fungus. There were instances however of trees without flutes being positive for the presence of *N. fuckeliana*. The fungus has been isolated previously from non-wounded, healthy looking wood (Vasiliauskas and Stenlid, 1998; Roll-Hansen and Roll-Hansen, 1979; Huse, 1981). This

suggests that *N. fuckeliana* may be capable of living asymptomatically within healthy looking tissue, or that infection occurred through previous stem wounds, which have been overgrown, hidden or overlooked (Huse, 1981). It must be pointed out that most of the flutes observed were very small and that any stem depression was considered to be a flute. With time, it may be possible to distinguish between flutes caused by *N. fuckeliana* and other causes. Perithecia are a clear visual sign of the fungus and these were only observed on one tree that had died in the period between the 2006 and 2007 samplings. As perithecia were only observed on one dead tree and the flutes were very small, the current impact of the disease in the stands that were sampled is very small.

When the 2006 results were compared with the 2007 results, it was found that there were inconsistencies between years. Trees that were positive in 2006 became infected prior to sampling in 2006, while trees that were negative in 2006 and positive in 2007 either became infected after sampling in 2006, or infection was present in 2006 but not detected. The number of trees which were positive in 2006 and negative in 2007 highlight the difficulty in detecting the fungus in wood samples. *Nectria fuckeliana* may have been not present in the wood core that was collected, or may have been present in the wood core, but not the part of the wood core used for the DNA extraction or culturing. Sampling method, along with detection method, is extremely important when trying to determine the presence of the fungus in a tree.

Pooling the results from the 2006 and 2007 assessments resulted in positive samples from 69 of 180 trees. When these results were examined statistically, the trend was the same as was observed when the 2006 and 2007 data were examined independently; there was no significant relationship between the presence of *N. fuckeliana* and pruned trees. This suggests that pruning wounds are not the only route by which the fungus can infect the tree.

The total number of *N. fuckeliana* positive trees detected in 2007 was similar to the number detected in 2006, despite the increased time available for infection. Pruning wounds may be susceptible for at least three months after pruning (Dick *et al*., 2006); therefore it is unlikely that infection took place through the pruning wounds in the year between samplings. Infection may have occurred through the wounds created by the increment core in 2006; however these wounds were very small and are not believed to have a major impact on the 2007 totals. The fact that the total number of positive trees did not increase dramatically between 2006 and 2007 indicates that *N. fuckeliana* is not aggressively infecting trees in the sampled forests. However, comparison between years is made difficult with sampling method being a confounding factor.

Further work is intended for this project. The trees will be resampled in 2008 and when cultured, samples from the increment core representing the different regions of the tree from the vascular cambium to the pith will be plated out so culturing and DNA analysis will be conducted using similar material. Three consecutive years of sampling will allow us to observe any trends in infection levels and disease expression. Selected trees will also be sampled destructively in order to map the presence of *N. fuckeliana* in the tree so that we can deduce the infection route and elucidate the influence of sampling on results to date.

The results from 2007 confirm those observed in 2006 and it is hoped that as we continue to monitor this experiment in 2008 we will learn more about the interaction between *N. fuckeliana* and the flute canker disease. A thorough understanding of the role of this fungus in the disease process will lead on to management strategies that can reduce the impact of the disease.

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