Commercial in Confidence

Client Report No. 39406

ECOLOGY AND EPIDEMIOLOGY OF *NECTRIA FUCKELIANA* IN *PINUS RADIATA*: FIRST-YEAR RESULTS

Patricia E. Crane

CLIENT REPORT No: 39406

Commercial in Confidence

Client Report No. 39406

ECOLOGY AND EPIDEMIOLOGY OF *NECTRIA FUCKELIANA* IN *PINUS RADIATA*: FIRST-YEAR RESULTS

Patricia E. Crane

Date: 4 May 2006
Client: Forest Biosecurity Research Council, FRST
Contract NO:

CLIENT REPORT No: 39406

EXECUTIVE SUMMARY

The ecology and epidemiology of a pine canker disease in the south part of the South Island is thought to be caused by the fungus *Nectria fuckeliana*. This northern hemisphere species is the most common fungus isolated from infected wood above pruned branch stubs, but it is not found in all fluted stems or in all parts of the flutes (cankers). Both naturally and artificially inoculated wood show strong activation of tree defensive responses. These responses also occur in wounds without the fungus. In laboratory studies, *N. fuckeliana* grew over a broad temperature range. In nature, spore release and dispersal are correlated with periods of rainfall. Spores are spread by rain and splashing water; therefore spread from a central infection point will be slower than for a wind-dispersed fungus. The ability of *N. fuckeliana* to cause disease likely depends on a complex of host, fungal, and weather factors. It is recommended that pruning be done during dry weather and when trees are actively growing, for maximum defensive response.

Objective

The objectives of this research are (1) to study the microorganisms associated with pine fluting disease; (2) study the ability of the fungus *Nectria fuckeliana* to cause disease in radiata pine; (3) to study the basic biology of *N. fuckeliana* in New Zealand, including conditions for fungal growth, spore production and dispersal.

Key Results

The major results of the study were the following.
(1) *Nectria fuckeliana* is the most common fungus associated with the pine fluting disease, but it may not be present in the entire diseased tissue. Several bacteria are extremely common, and their possible role as pathogens of radiata pine deserves investigation.

(2) Preliminary results of inoculation experiments suggest that the type of wound and the season of wounding are important factors in the ability of *N. fuckeliana* to cause disease in radiata pine. The cambium of the flute of an infected tree is alive, but few new cells are produced and there are strong host defence responses such as traumatic resin ducts.
(3) *N. fuckeliana* can grow over a broad temperature range, but the optimum for growth and spore germination is 18-25°C.

(4) Ascospores are present in fruit bodies in all seasons of the year, but their release and dispersal are correlated with periods of rainfall.

(5) Spores are dispersed by water droplets; therefore spread of the fungus from an infection point will be slower than for a wind-dispersed fungus.

Application of Results

(1) The risk of infection can be minimized by pruning trees in dry weather when risk of spore dispersal is reduced and at times of the year when radiata pine is growing rapidly and can mount effective chemical and physical defensive responses.

(2) Spread of the disease might be minimized by avoiding very close spacing of trees in infected plantations to reduce humidity, and because *N. fuckeliana* spores spread most effectively over short distances.

Further Work

(1) Some of the environmental conditions that favor growth of *N. fuckeliana* have been elucidated, but the role of host physiology and season in tree susceptibility to pine fluting disease should be clarified by further experiments, e.g. inoculation in different seasons.

(2) The effect of cold temperature on ascospore production and viability needs to be clarified, as this has implications for the distribution and potential disease spread in New Zealand.

(3) Spore trapping for at least an entire year will further clarify the effect of weather conditions on spore dispersal.

ensis

CLIENT REPORT No: 39406

ECOLOGY AND EPIDEMIOLOGY OF *NECTRIA FUCKELIANA* IN *PINUS RADIATA*: FIRST-YEAR RESULTS

Patricia E. Crane

May 4, 2006

TABLE OF CONTENTS

Information for Ensis abstracting:

Contract number	
Report ID number	39406
Products investigated	
Wood species worked on	*Pinus radiata*
Other materials used	*Nectria fuckeliana*, cultures of other microorganisms
Location	Otago

INTRODUCTION

Pine fluting disease of radiata pine is thought to be caused by the introduced fungus *Nectria fuckeliana*. The disease is presently confined to the southern half of the South Island. Long flutes or cankers form above branch whorls after pruning operations. The resulting damage is economically important.

Nectria fuckeliana is native to the northern hemisphere (Europe and North America) mainly as a wound invader of conifers, especially spruce and fir.[1] [2] [3] [4] [5] Its association with a stem canker in New Zealand is the first report of natural infection of pines, although five species of pine were shown to be susceptible in inoculation tests.[6] The *Acremonium* stage of the fungus forms on the surface of cut infected wood and in culture and produces clusters of tiny asexual spores (conidia). However, the primary inoculum in nature is thought to be the ascospores[7], produced in red fruit bodies on branch stubs, on bark around wounds, or on the surface of cankers. The climatic conditions in New Zealand are different from those in the native range of the fungus, and the objective of this study is to elucidate some of the host, fungal and environmental factors that have contributed to occurrence of this disease in the South Island. This is a summary of the first-year results from this program. It is hoped that these and future results will help to explain the present distribution, help prevent further disease spread, and contribute to effective management of the disease.

MATERIALS AND METHODS

Microorganisms associated with pine fluting disease

Microorganisms from naturally infected trees

Fungi and bacteria were isolated from disks taken from three naturally infected trees in January 2005. In April 2005, two infected trees near the pruned stub trial (PS5 and PSO, Hetherington Rd., Tokoiti Forest) were chosen for more detailed isolations. Both trees had one or more long flutes extending from one pruned whorl to the pruned whorl above. Successive disks were cut through the entire flutes, and numbered (Table 1). Isolations were made from the cut surface of nine disks from each tree, including infected wood in the flutes, branch stubs, clear wood, and any patches of “whitewood”. Small slivers of wood were removed using sterile technique and placed on 2% malt extract agar. Isolates were later subcultured to produce pure cultures. Location of *N. fuckeliana* was determined, and an attempt was made to identify other microorganisms by microscopic observation.

---

[1] Laing, E.V. 1947. Preliminary note on a disease of Sitka spruce in Cairnhill Plantation, Durris, Kincardineshire (*Picea sitchensis* Carr.). Forestry 21:217-220.

[2] Ouellette, G.B. 1972. *Nectria macrospora* (Wr.) Ouellette sp. nov. (=*N. fuckeliana* var. *macrospora*): Strains, physiology and pathogenicity, and comparison with *N. fuckeliana* var. *fuckeliana*). European Journal of Forest Pathology 2: 172-181.

[3] Roll-Hansen, F.; Roll-Hansen, H. 1979. Microflora of sound-looking wood in *Picea abies* stems. Eur. J. For. Pathol. 9:308-316.

[4] Roll-Hansen, F.; Roll-Hansen, H. 1980. Microorganisms which invade *Picea abies* in seasonal stem wounds. I. General aspects. Hymenomycetes. Eur. J. For. Pathol. 10:321-339.

[5] Roll-Hansen, F.; Roll-Hansen, H. 1980. Microorganisms which invade *Picea abies* in seasonal stem wounds. II. Ascomycetes, Fungi imperfecti, and bacteria. General discussion, Hymenomycetes included. Eur. J. For. Pathol. 10:396-410.

[6] Smerlis, E. 1969. Pathogenicity tests of four pyrenomycetes in Quebec. Plant Disease Reporter 53:979-981.

[7] Vasiliauskas, R.; Stenlid, J. 1997. Population structure and genetic variation in *Nectria fuckeliana*. Can. J. Bot. 75:1707-1713.

Table 1: Description of fluted trees used for isolation of microorganisms.

Tree No.	Location	Tree age, yr	No. disks for isolation
G1	Glenledi trial, Tokoiti Forest	8	4
PS20	Nr. pruned stub trial, Poverty Hill Rd., Tokoiti Forest	7	2
M1	McCrosties Block, Tokoiti Forest	5	1
PS5	Nr. pruned stub trial, Poverty Hill Rd., Tokoiti Forest	6	9
PSO	Nr. pruned stub trial, Poverty Hill Rd., Tokoiti Forest	7	9

Interactions of *N. fuckeliana* with other microorganisms

Preliminary laboratory cultural experiments were done to determine the competitive ability of *N. fuckeliana* against other microorganisms commonly isolated from healthy and fluted wood of radiata pine. Three isolates of *N. fuckeliana* (PS20/5-2C, NZFS 990, 1779) were paired on 2% malt agar with *Fusarium* sp., *Stereum sanguinolentum* (NZFS 62A, a decay fungus), slow-growing pink fungus found in "whitewood" (PS5/5-2B), yellow bacteria (PSO/14-1A, common in clear or fluted wood), pink bacteria (PSO/13-6A, from clear wood), orange bacteria (PS5/7-5BB, from clear wood).

Effect of N. fuckeliana on Pinus radiata

Disease progress in artificially inoculated trees

(1) Inoculation to determine effect of spore type and wound type on disease development

An inoculation trial was initiated in April 2005 in the Flagstaff Forest, near Dunedin, to compare infectivity of ascospores from fruit bodies formed in nature and of asexual spores (conidia) from culture, and the effect of three types of wounding. Forty-five 6-year-old unpruned *Pinus radiata* were inoculated using either conidia, ascospores, or water (control). Three kinds of wounds were used (15 trees of each): surface stem wound, deeper wound into sapwood, or a hole drilled into the end of a branch stub. Liquid inoculum was delivered into the wounds using a pipetter. Inoculated trees were examined in early June and in late November 2005. Final assessment of the infections will be done in mid- to late 2006.

(2) Inoculation of trees to study disease progress

Eight healthy young (4 or 5 yr old) radiata pine in the Flagstaff Forest near Dunedin were inoculated with *N. fuckeliana* to observe disease progress. Inoculum was prepared by placing small pieces (about 5 mm long) of wooden dowel on cultures of the fungus (NZFS 1786) for about 2 wk so that they became colonized. Similar pieces of dowel were sterilized by autoclaving, to be used as control inoculum. Each tree was inoculated with *N. fuckeliana* in two places, between two different whorls, by drilling a hole to the cambium and inserting a colonized piece of dowel. On the opposite side of the tree to each inoculation, a similar hole was drilled, and a sterile piece of dowel inserted as a control. Two trees were harvested at 2 months and two at 4 months after inoculation. Stem sections containing the inoculation points were removed. Each stem section was sawn vertically to separate the inoculated and control wounds, then each wound point was sawn through vertically. One vertical face, with the inoculation point, was used for isolation of fungi and bacteria on 2% malt agar, whereas a wedge from the opposite face, including the other half of the wound, was fixed in formalin – acetic acid – ethanol (FAA) for at least 1 week for anatomical studies. Blocks were repeatedly rinsed with distilled

water, then sectioned, both transversely and longitudinally (radial face), at about 30-60 µm using a sliding microtome. Sections were stained with acriflavin (0.1%) and examined by confocal microscopy, or stained with aqueous safranin or lactophenol – cotton blue and examined by light microscopy to elucidate wood cell walls and fungal hyphae.

Anatomy of naturally infected trees

Wood anatomy was examined in two naturally infected trees with long flutes, extending from one pruned whorl to the one above. Small wood blocks were removed from disks of two trees, collected April 2005, including the cambium, bark, and sapwood of infected wood within the fluted areas as well as comparable areas that appeared healthy from within the same disks. Wood pieces were fixed in FAA for at least one week, sectioned, stained, and studied by confocal and light microscopy, as above.

Effect of environmental conditions on N. fuckeliana

Conditions for optimum growth and spore germination of *N. fuckeliana*

Petri plates containing 1.5% malt extract agar were inoculated with a 5-mm plug of a *N. fuckeliana* isolate, either NZFS90, 1786, or 2643. Three plates of each isolate were incubated in the dark at 15, 18, 20, 21, 25, or 28°C. Diameter of each colony was measured in two directions, beginning on the 4th day and every 3rd day thereafter for 16 days. Growth (mm per day) was determined for each isolate and temperature, based on the final mean diameter.

Germination tests were conducted on ascospores obtained from perithecia collected in April 2005 from two locations, Flagstaff Forest near Dunedin and Poverty Hill Rd., Tokoiti Forest. Bark pieces bearing the fruit bodies were stored in a refrigerator at 4°C until used. A small cluster of mature perithecia was removed from the bark with a scalpel, placed in 30% H_2O_2 for 1 min, rinsed twice in distilled water, then soaked in distilled water for 2 h. Softened fruit bodies were then crushed in a small amount of distilled water to release ascospores, and mixed thoroughly. One spore-containing droplet was placed on each microscope slide and five slides were placed in each large glass petri dish lined with moist filter paper. Petri dishes were sealed with plastic wrap and incubated at 5, 10, 15, 18, 20, 23, 25, 28, or 30°C. One slide was removed every 2 h, beginning after 4 h, for the first 12 h, then a final slide was removed after 24 h. To stop germination and facilitate later examination, droplets were immediately covered with a cover slip to prevent clumping of spores, and dried by gentle heating on a hot plate. Percentage spore germination was determined by rehydrating the droplet, and assessing germination of at least 100 spores.

Long-term viability of ascospores

Bark fragments bearing perithecia of *N. fuckeliana*, collected in January 2005 from several locations near Dunedin, were stored in paper bags at room temperature or in plastic bags in a refrigerator at 4°C for 1 yr. At 3, 5, and 7 months and 1 yr, the percentage spore germination was determined, using the method outlined above.

Production of fruit bodies in culture

Initial experiments showed that perithecia could be produced after 3 months when some isolates were paired on sterile pine twigs and grown under natural daylight (Margaret Dick, personal communication). Experiments were done to find a faster, reliable method to produce fruit bodies and to understand the mating system of *N. fuckeliana*. Some isolates readily produced sterile red fruit bodies (without spores), especially on carrot agar, but no ascospores formed under fluorescent light. Therefore, in a second experiment, six isolates of *N. fuckeliana* were paired in all combinations (21 plates) on carrot agar at 20C, 50% relative humidity, under 12 h dark : 12 light (Philips TLD 36W/840 New Generation). The plates were evaluated for the

presence of perithecia containing ascospores after 7 weeks and 12 weeks. In a third experiment, 20 isolates were paired in all combinations (except self-pairing) under the same conditions. They were evaluated after 7 weeks and 10 weeks.

Ascospore release and dispersal and relationship to weather conditions

Bark fragments bearing fruit bodies of *N. fuckeliana* were soaked in distilled water in petri dishes to observe the release of ascospores. They were also suspended above microscope slides in a damp chamber to capture spores that might be forcibly released. Field observations of fruit bodies in dry and wet weather were compared with laboratory observations. Spore release and germination of ascospores produced in culture were studied microscopically.

Spore trapping was carried out from September 2005 to the present to determine the season(s) of the year or type of weather conditions when ascospores of *N. fuckeliana* are dispersed in Otago. One infected tree was chosen at each of two sites. Each tree had *Nectria* fruit bodies on branch stubs of a whorl fairly low on the tree, so that traps could be located close to them. Hobo weather stations were placed near each tree to record daily temperatures, humidity, dew point, and rainfall. Five wooden stakes were driven into the ground around each tree within 1 m, with the top of each stake just below a cluster of fruit bodies on a stub. Stakes 1 – 5 were around the first tree, and stakes 6 – 10 around the second tree. Spore traps were microscope slides coated with a smooth layer of melted Vaseline, prepared according to a procedure by Ostry and Nicholls[8]. Each week, a coated, numbered slide was attached to the top of a wooden stake with the same number, by means of a bulldog clip (Fig. 1). Slides were replaced each week and stored for later examination. The number of *Nectria* ascospores deposited on each slide was determined after staining with lactophenol – cotton blue and covering the Vaseline layer with a large coverslip (22 x 50 mm). The spores on one-half of the surface were counted by scrolling across the coverslip 10 times at 200x magnification. If necessary, spore identity was verified at 400x.

Fig. 1. Spore trapping method, showing Vaseline-coated microscope slides attached to wooden stakes around an infected tree.

Weather variables (daily rain, rain days, temperature, and relative humidity) were correlated with total number of ascospores deposited each week on all slides around each tree using stepwise regression performed with PROC STEPWISE in the SAS system.

[8] Ostry, M.E.; Nicholls, T.H. 1982. A technique for trapping fungal spores. USDA Forest Service, North Central For. Expt. Stn., St. Paul, MN, Res. Note NC-283.

RESULTS AND DISCUSSION

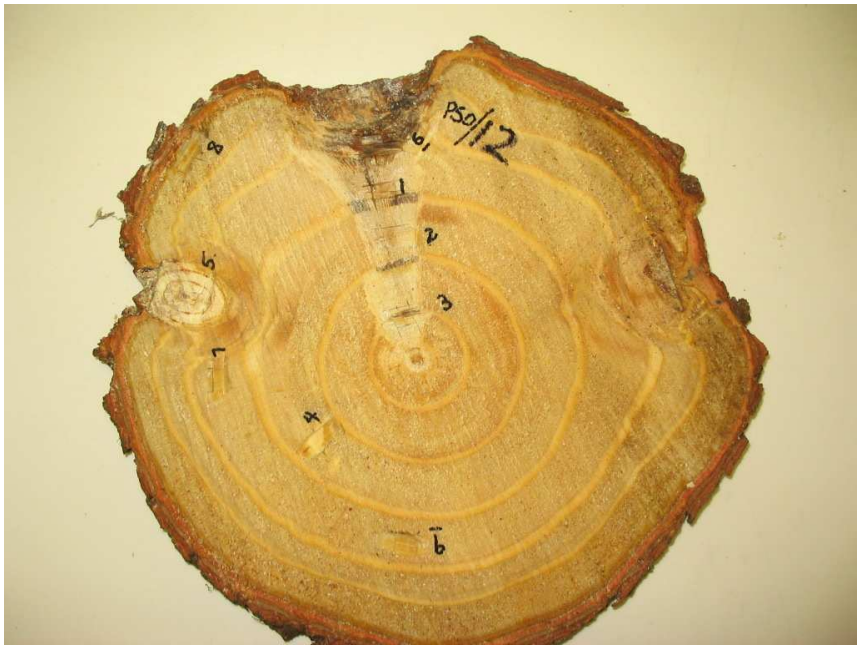
Microorganisms associated with pine fluting disease

Isolates from naturally infected wood

Nectria fuckeliana was the most common fungus isolated from naturally infected, fluted trees examined during this study. It was found in pruned branch stubs and the wood immediately surrounding them, and the cambium and sapwood of the fluted area at and above a pruned whorl. In stem cross section, this area is wedge-shaped, extending from the bark to the pith, and is markedly whiter than surrounding wood; it may have darkened, resinous patches at the ends of growth rings (Fig. 2). In the two trees for which isolations were made through entire flutes, *N. fuckeliana* was found at the whorl and only in the two disks above the whorl in tree PSO; it was found in four disks above the whorl in tree PS5. Isolation of this fungus from clear, healthy-appearing wood of the same trees was rare.

Many other fungi were also isolated from fluted and clear wood. Most were Zygomycetes or Ascomycetes; others did not sporulate and could not be placed in a broad group. Most were isolated infrequently and were not thought to be important in the etiology of the fluting disease. Two slow-growing fungi that did not sporulate ("pink rubbery fungus" and "grey mound-like fungus") were frequent isolates from both healthy and fluted wood. They may be endophytic in radiata pine, living in the tissues, but not causing damage or disease. Most Zygomycetes and Ascomycetes are generally considered to be early colonizers that use easily accessible nutrients before decay fungi begin colonization of wood. Many decay fungi (Basidiomycetes) are capable of degrading cellulose and/or lignin and cause more extensive wood degradation than other groups of fungi. Only one confirmed Basidiomycete was identified from any of the trees examined.

Fig. 2. Cross section of tree PSO, just above a pruned, infected whorl. Note wedge of "whitewood" at branch stub and small patches of whitewood beside numbers 4 and 9. *N. fuckeliana* was isolated from wood beside numbers1, 2, 4, 5, 6, 7, and 9. Note resinous patches at the ends of growth rings near 1, 2, and 3.



Bacteria were very common in both healthy and diseased areas of bark and sapwood. Their role in the pine fluting disease is not known. Bacteria are capable of causing some wood degradation, for example, of resin produced in tree defence, or of the membranes around the

pores or “pits” connecting adjacent tracheids. In this way, they may facilitate access to the wood by fungal hyphae[9]. Some bacteria may also inhibit fungal growth (*see* Interactions of *N. fuckeliana* with other microorganisms).

Interactions of *N. fuckeliana* with other microorganisms

When *N. fuckeliana* was paired in culture with a yellow bacterium commonly isolated from *P. radiata*, the fungus was able to overgrow the bacterial colony, but fungal sporulation was inhibited. Two other bacteria strongly inhibited the growth of *N. fuckeliana* (Fig. 3). When these bacteria were placed on undifferentiated pine callus tissue in another experiment, they rapidly killed the pine cells. The possibility that they are pathogens of pine, or that they could act as biological control agents of *N. fuckeliana*, requires study.

Nectria grew over and around the slow-growing pink rubbery fungus, thought to be a harmless endophyte in radiata pine. Mutual inhibition resulted when *Nectria* was paired with the *Fusarium* spp. and with the decay fungus *Stereum sanguinolentum*. In the latter, an orange line formed across the plate where the colonies met. It is expected that these fungi would not co-exist in the same piece of wood. In fact, a decay fungus such as *S. sanguinolentum* would be expected to colonize a piece of wood at a later stage of wood degradation, after Ascomycete fungi such as *N. fuckeliana* had removed easily obtained nutrients.

Fig. 3. Growth inhibition of *N. fuckeliana* in paired culture with a bacterium (pink) isolated from pine wood.

Effect of Nectria fuckeliana on Pinus radiata

Disease progress in artificially inoculated trees

Isolation of microorganisms from wood that has been infected for several years usually does not give a clear indication of their role in the disease, neither does frequent presence of an organism prove that it causes the disease. For this reason, two experiments were done in which trees were artificially inoculated with *N. fuckeliana* to try to clarify the role of this fungus in the pine fluting disease.

---

[9] Hallaksela A.-M. 1993. Early interactions of *Heterobasidion annosum* and *Stereum sanguinolentum* with non-decay fungi and bacteria following inoculation into stems of *Picea abies*. Eur. J. For. Pathol. 23:416-430.

Effect of spore type and wound type on disease development

This experiment, begun in autumn 2005, compares the disease-causing ability of conidia, ascospores, and a water control, and the effect of different kinds of wounding. Seven months after inoculation, some trees were showing signs of fluting above and below wounds. Depressions above wounds were longer than below. Trees with deep wounding into the sapwood had the longest depressions and also greatest amount of resinosus (Fig. 4). In the stub treatment, much longer flutes were found with fungal inoculation than with control treatment. Overall, the greatest effect was found with deep wounds (including controls) to the sapwood, than with either inoculation into branch stubs or shallow wounds. The type of wound seemed to be more significant than the type of spore used for inoculation (*see* Appendix A). Assessment after 1 year, planned for mid-year (2006), will give a more complete picture. Felling of trees and reisolation to determine the extent of *Nectria* spread within the trees is planned. Fluting of inoculated trees and consistent reisolation of the fungus are needed to prove conclusively that *N. fuckeliana* is the causal agent of the pine fluting disease.

Fig. 4. Tree inoculated in April 2005 with *Nectria* spores into a deep wound in the sapwood. Note extensive resinosus and the depression developing above the wound after 7 months.

Disease progress in artificially inoculated trees

The second inoculation experiment, initiated in November 2005, is intended to trace the development of the disease at 2-month intervals. After 2 months, the fungus was reisolated from three of the four fungus-inoculated wounds in the two harvested trees: from the cambium and sapwood of the current growth ring, but not more than 0.5 cm above or below the wound. In the fourth case, *N. fuckeliana* was recovered from the control wound but not the inoculated wound. It is likely that an error was made in marking the wounds. Superficially, both control and *Nectria* inoculations appeared similar: the bark was sunken for several millimetres around the wound. Internally, there was evidence of “whitewood” extending up to 1.5 cm into the adjacent sapwood, and a resin-soaked area at the wounded cambium; a gap where inner bark was dead extended above and below the inoculation point. The *Nectria*-inoculated wounds, however, had a more chalky appearance, and adjacent tissues were more crumbly than in the controls. Microscopically, the wounds were surrounded by several complex layers of abnormal cells that sealed off the wound from the surrounding living tissue. These layers were heavily impregnated with resin. In cross section of the sapwood within 1 cm above or below the wound, a row of traumatic resin canals had formed in the sapwood about 3-4 mm from the cambium. Tracheids adjacent to the resin canals were plugged with extractives, likely phenolics and tannins. Resin ducts and the associated antifungal chemicals are part of the defence system of the tree. They form a system of barriers that can wall off invading microorganisms. There was little detectable difference in the responses to *Nectria* and to control wounds at 2 months after inoculation.

Fungal hyphae were seen in some samples, especially along the edge of the dead bark. In one fungal-inoculated wound, they were also common in ray cells and tracheids up to 100 µm from the wound edge.

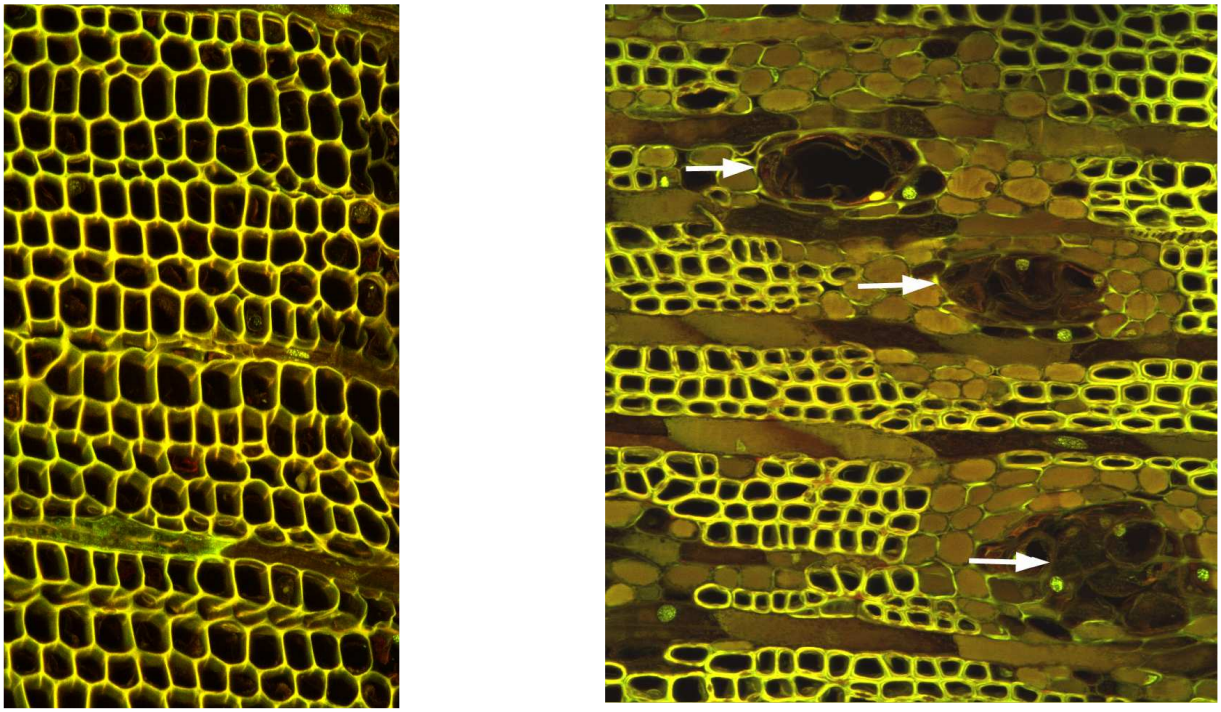
In the two trees harvested 4 months after inoculation, *N. fuckeliana* was recovered from only one of the four points inoculated with the fungus. It was found only in the resinous area inside one wound. Although the sample size was small, this result raises questions about the ability of the fungus to survive and cause disease in actively growing trees (inoculation was done during the spring). On the other hand, it may indicate that shallow wounds, such as those used in this experiment, are easily walled off by a tree's defensive responses. Fungal hyphae were not seen in any samples beyond the dead tissue immediately surrounding the wound. Externally, there was still a slight depression around some wounds, both control and fungal-inoculated, whereas others appeared to have healed completely. In one case, a slight depression had formed 1.5 cm below and 4.5 cm above a fungus-inoculated wound; however, the fungus was not recovered from the latter. Whitewood was seen in the sapwood adjacent to only one wound: the one from which *Nectria* was reisolated. In this case, the whitewood extended 2 cm below the wound, >5 cm above, and 2.5-3 cm radially into the sapwood (Fig. 5). Traumatic resin canals continued to form in new sapwood adjacent to both control and inoculated wounds, but were more scattered than in the 2-month samples. Between the wedge-shaped wound area and the bark of both types of wound, the tracheids were smaller than normal and very dense. The deep staining of their walls with safranin suggests that lignin content was higher than normal. This is another typical resistance response of wood to wounding or infection.

Fig. 5. Vertical section of control wound (left) and *Nectria*-inoculated wound (right) after 4 months. The pencil lines and arrows delineate the area of "whitewood". In the control, the wound area is a well-defined wedge shape, and appears to have healed. *Nectria* was reisolated from the fungal-inoculated wound.

Anatomy of naturally infected trees

Anatomical studies of older infections also showed evidence of defensive responses to wounding or invasion by microorganisms. Unusual proliferation of traumatic resin canals was present within the fluted wood; they were absent in wood near the cambium of the healthy side of the same disk. The cambium, the layer of cells just inside the bark that divides rapidly to produce new wood cells, appeared to be functional, but at a very slow rate, resulting in the indentation (or flute) on one side of the stem. The cambium, ray cells, and cells around resin canals contained nuclei, suggesting they were still alive. The cells of rays and resin canals were occluded with tannins, as shown by $FeCl_3$ staining (Fig. 6). Wood cells were largely intact, with little evidence of wall degradation or decay. However, the resins, tannins, and higher lignin content of affected wood may make the wood unusable for the intended wood products.

Fig. 6. Cross section of healthy sapwood (left) near the cambium of tree PSO and from the opposite side of the same disk (right) near the cambium of the flute. Arrows indicate traumatic resin ducts. The brown occlusions in the cells around the ducts are tannins, as determined by staining with $FeCl_3$.



Effect of environmental conditions on N. fuckeliana

Conditions for optimum growth and spore germination of *N. fuckeliana*

N. fuckeliana is capable of growing over a broad range of temperatures. Optimum temperature for growth of cultures and for ascospore germination was between 18 and 25°C; within this range, spores produce long, branching germ tubes. Below 18°C and above 25°C, culture growth was slower. Some spore germination did occur, however, at temperatures as low as 5°C. Above 25°C, spore germination was abnormal (Figs. 7-9). In its native habitat, *N. fuckeliana* likely undergoes a dormant period during the winter when temperatures are below freezing. In the milder climate of New Zealand, however, the fungus is likely capable of growing to some extent in all seasons of the year.

Fig. 7. Effect of temperature on growth of cultures of *N. fuckeliana* between 15 and 28°C.

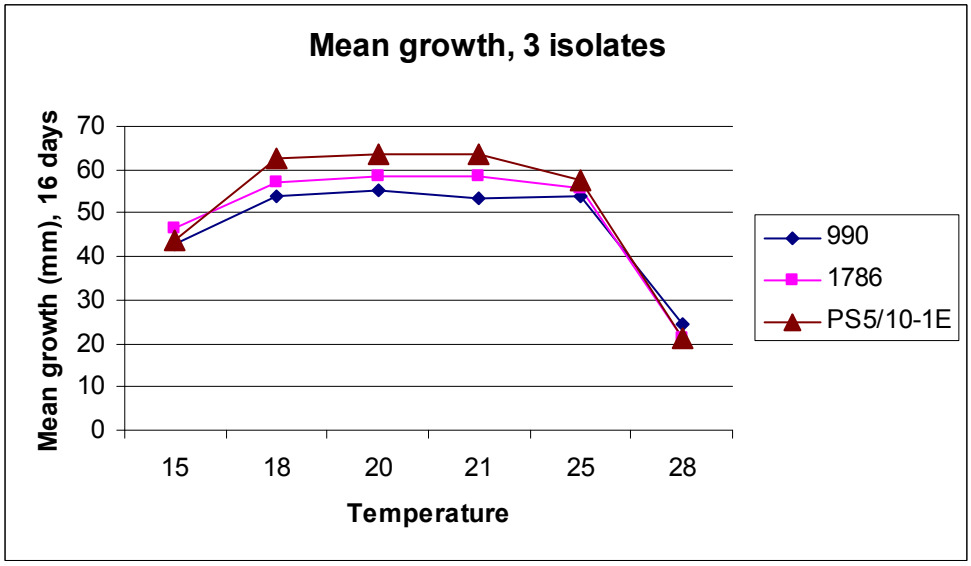
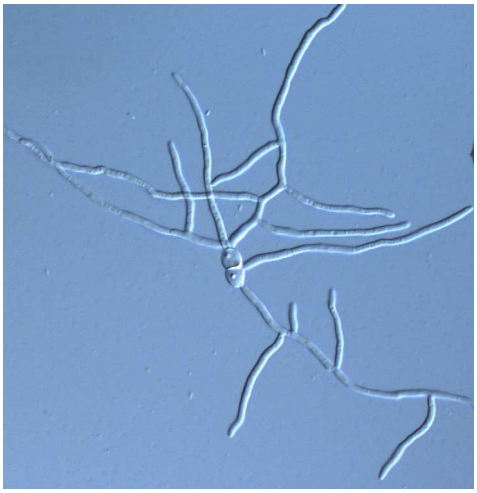


Fig. 8. Left, normal ascospore germination of *N. fuckeliana*, showing branching germ tubes (the two-celled ascospore is in the centre). Right, abnormal ascospore germination at 30°C. Note the knob-like germ tubes.



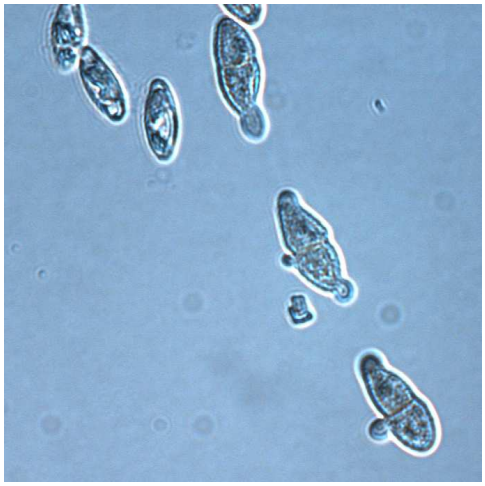
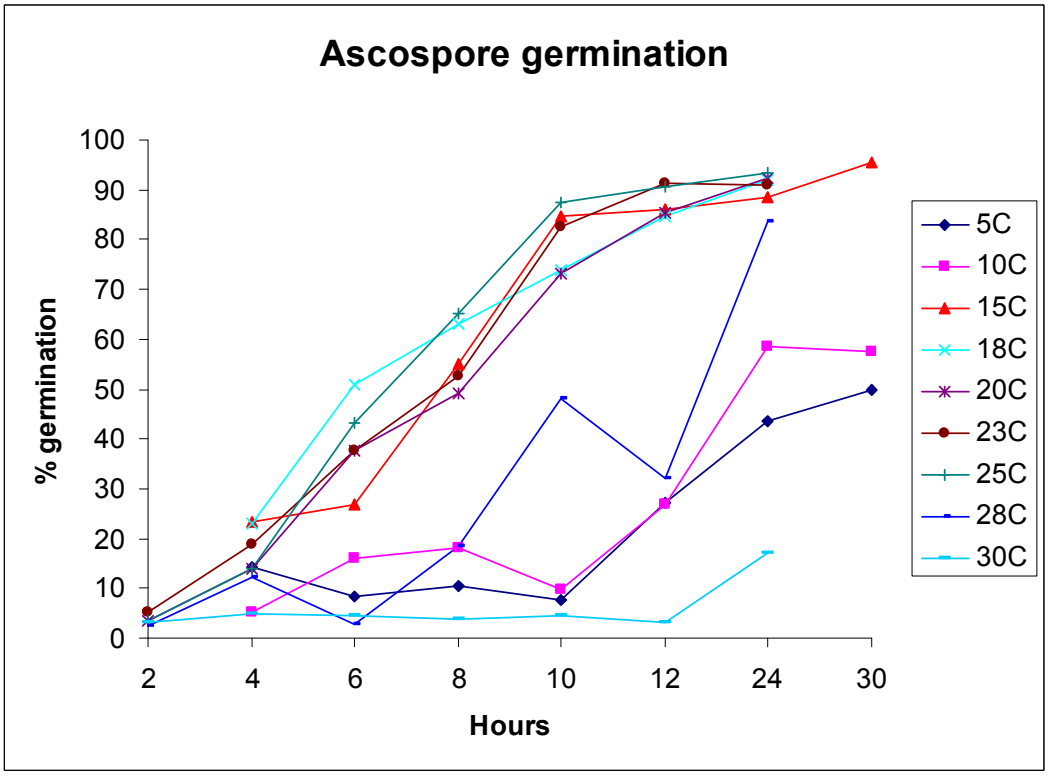


Fig. 9. Effect of temperature on germination of ascospores from perithecia collected at Hetherington Rd., Tokoiti Forest, in April 2005. Ascospores from Flagstaff Forest showed a similar pattern of germination.



Long-term viability of ascospores

Ascospores from perithecia stored at room temperature gradually lost their viability, whereas those stored at 4°C actually increased in viability to over 90% after 1 year of storage (Table 2). In the cold treatment, the perithecia were stored in plastic bags, which would have retained more moisture in the samples, whereas at room temperature plastic was avoided to prevent moulding. These preliminary findings suggest that in the 4°C treatment, the ascospores matured over time. It may also indicate that a cold period is required to condition maturity of the ascospores. It should be noted that in the ascospore germination studies (Fig. 9, above), all samples had been stored for at least 3 months at 4°C before germination studies were conducted. Further studies, using freshly collected perithecia, are warranted.

Table 2: Long-term viability of ascospores from fruit bodies stored under two different conditions.

Temperature	**Storage meduim**	**Location**	**Germination (%), 24 h**			
			3 mo.	*5 mo.*	*7 mo.*	*12 mo*
Room temp.	Paper bags	Glenledi	67	27	0	-
		Takitoa	42	7	5	-
Fridge (4°C)	Paper bag in plastic	Tokoiti	-	59	81	97
		Glenledi	-	62	82	94

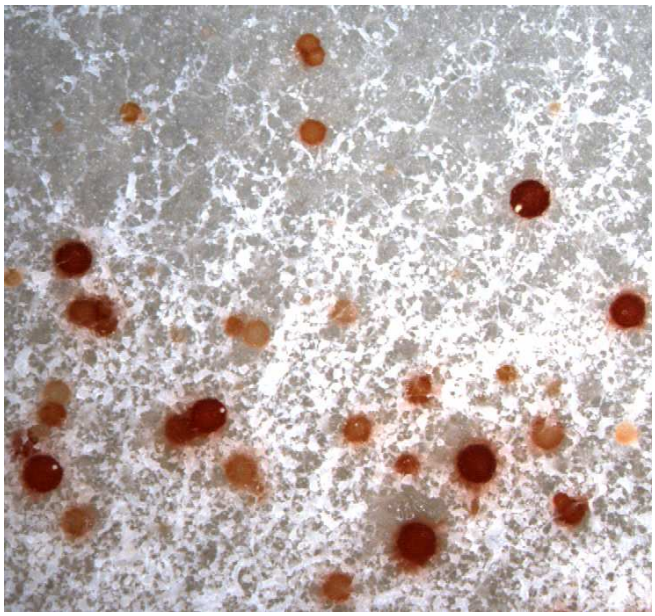
Production of fruit bodies in culture

Ascospores are thought to be the primary means of dispersal of *Nectria fuckeliana* in nature[10]. This stage is seldom produced in culture, but many isolates produce sterile, reddish fruit bodies (protoperithecia) that do not contain spores. Mating of two compatible isolates is required for production of fruit bodies containing ascospores. In addition, a growth medium rich in nutrients, such as carrot agar, and natural, full-spectrum lighting appear to be important requirements for production of fertile fruit bodies.

In the mating experiment with 6 isolates, fertile fruit bodies were produced in 7 plates out of 21 (30%); in the pairing of 20 isolates, 67 of 187 (36%) plates were successful. In culture, fertile fruit bodies were larger than non-fertile ones and often darker red. They were also typically globose, with a long pointed beak, through which mature ascospores are released (Fig. 10). In successful pairings, fruit bodies with this typical morphology could be seen with a dissecting microscope after 7 weeks at 20°C, but mature spores were infrequent. In 3-month-old cultures, ascospores capable of germination were present. Spores were slightly smaller than those found in nature.

It is clear that not all isolates are compatible. The need for two compatible mating types for fruit body production may explain why these structures are produced on some infected trees but not on others. Further work is needed to determine how many mating types are produced by this fungus, and whether there are detectable morphological or genetic differences among them.

Fig. 10. Fertile dark red fruit bodies (perithecia) produced where two compatible isolates of *N. fuckeliana* meet in culture. Small white dots on perithecia are clusters of ascospores. Small, pale orange perithecia are sterile.



[10] Vasiliauskas, R.; Stenlid, J. 1997. Population structure and genetic variation in *Nectria fuckeliana*. Can. J. Bot. 75: 1707-1713.

Relationship of ascospore release and dispersal to weather conditions

Knowing the seasons or weather conditions when spores of *N. fuckeliana* are available to cause infection is important for the timing of tree pruning, which creates a wound through which pathogens can enter the tree. Spore trapping can be done using many different kinds of devices. The traps chosen, microscope slides coated with Vaseline, were simple, inexpensive, and did not require a lot of time to set up each week. The results were also relatively easy to analyze. The two-celled ascospores of *N. fuckeliana* are sufficiently distinct that they could usually be recognized on the spore traps based on shape and size. However, many variables in the field such as wind speed and direction, source of field moisture (melting snow or frost, rain) and maturity of perithecia, as well as interpretation during the laboratory analysis could affect the results. In spite of these limitations, the trapping results are providing important information about the biology of *N. fuckeliana*.

Weekly spore-trap results have been obtained for three seasons of the year: spring, summer, and autumn, and trapping will be continued for at least one complete year. Ascospores of *N. fuckeliana* were found in all three seasons. Spores were also trapped in a preliminary experiment during a wet period in winter (June 2005). The number of spores captured varied greatly from week to week, and the results from tree 1 (Poverty Hill Rd.) and tree 2 (Hetherington Rd.) were not consistent (r^2 = 0.18 when comparing total numbers of spores trapped from each tree over weeks 1-15), although high numbers and low numbers were often obtained on the same weeks. Some traps consistently captured a very low number of spores; therefore after 15 weeks, we decided to continue analysis of the best 3 trap locations around each tree, rather than 5 (*see* Appendix B). These were all 245 mm or less from the tree.

Plotting trapping results with rainfall indicates a strong relationship between total spores each week and the amount of rainfall (Fig. 11.). Stepwise regression confirmed this observation where total weekly rain gave the most significant relationship with weekly spore catches (Table 3). Successive addition of other variables failed to make a significant contribution at the 5% level.

Fig. 11. Total spores trapped each week for tree 2 showing general correlation with weekly rainfall.

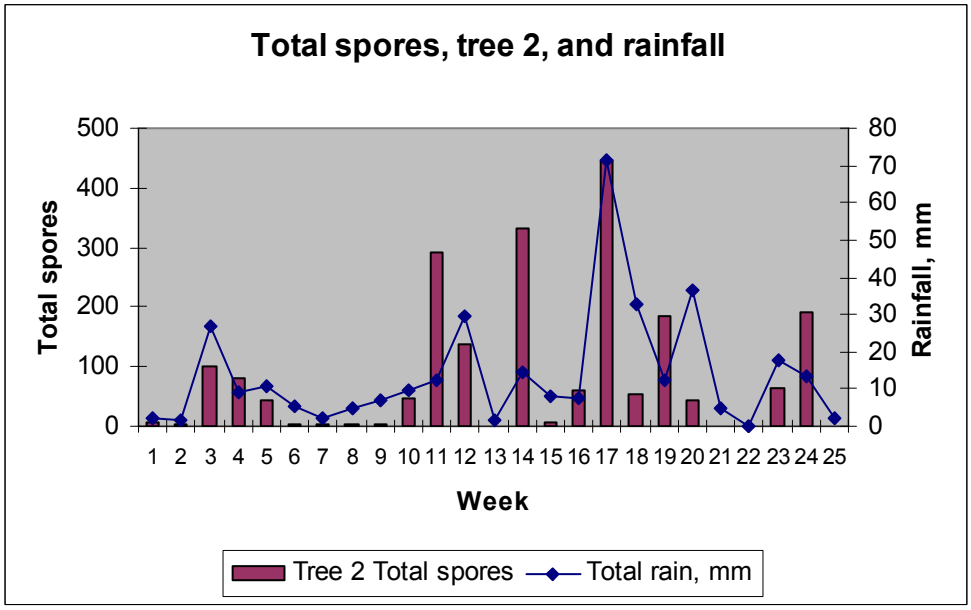


Table 3: ANOVA table and coefficients from PROC STEPWISE for weekly rainfall on weekly spore catches.

Source	DF	SS	MS	F Value	Pr > F
Model	1	147457	147457	17.74	0.0003
Error	23	191175	8311.94492		
Corrected Total	24	338632			

Spore catch = 4.98rain + 15.65 (r^2 = 0.435)

Spore dispersal mechanism

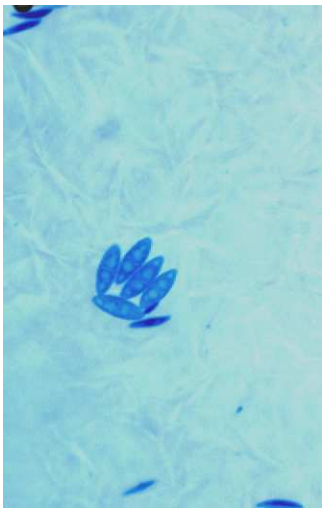
An earlier report[11] suggested that ascospores of *N. fuckeliana* are dispersed mainly by rain and splashing water, rather than by wind. Further evidence supports this hypothesis and is summarized here.

In addition to the correlation of highest spore numbers with weeks of high rainfall (Fig. 11), water dispersal is also supported by the short distance over which spores were trapped. Very few spores were deposited when the traps were greater than one-half metre away from the tree. For the first 15 weeks of trapping, a maximum of 21 spores per week was caught by a trap 678 mm from tree 1, a maximum of 12 spores per week was caught by a trap 611 mm from tree 2. In a preliminary trapping experiment in June 2005, with traps up to 4 m from trees, capture of *Nectria* spores beyond 1 m was rare. One would expect a fairly long-distance spread if spores were moved by wind.

The pattern of deposit of *Nectria* ascospores on the slide traps was also significant. Not only were they often in groups, but they were frequently found within circular areas left by drying of water droplets on the slides (Fig. 12). Often one-half or more of the spores on a trap were found in groups of 2-8 spores, suggesting that they had dispersed together. Interestingly, ascospores are produced in groups of 8 inside the fruit bodies in sac-like structures (asci). Laboratory observations suggest that spores of an individual ascus are squeezed out of the fruit body together, likely due to pressure from water-swollen filaments within the fruit body (Fig. 13). Dispersal in a water droplet would explain why they are found together.

[11] Crane, P.E. June 2005. *Nectria* fluting disease of *Pinus radiata*: ecology and epidemiology. Ensis Client Report No. 38080.

Fig. 12. Left, a group of *Nectria* ascospores on a spore trap (microscope slide coated with Vaseline). Right, *Nectria* ascospores (arrows) within part of a circular area left by drying of a water droplet.



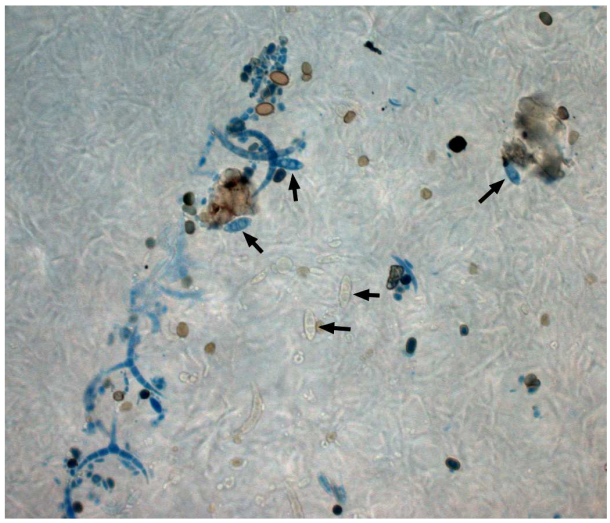
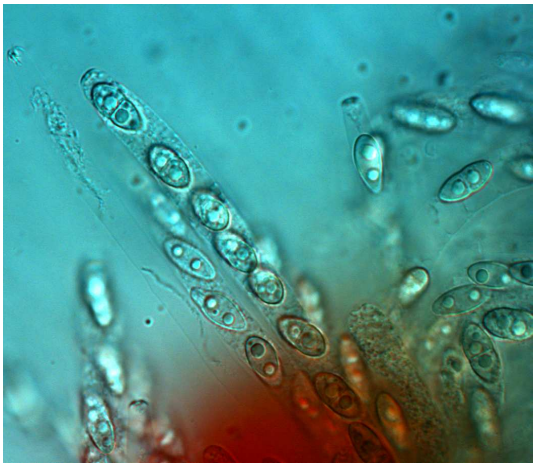
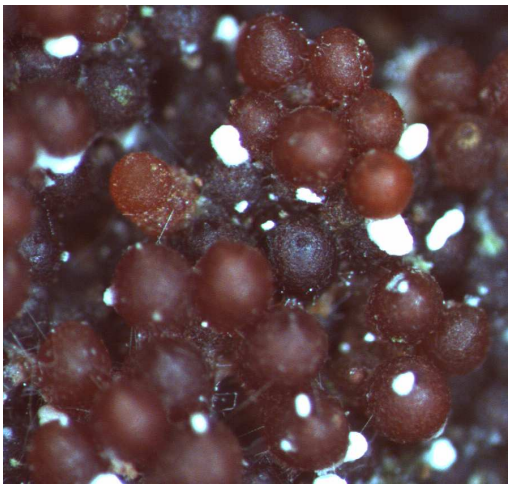


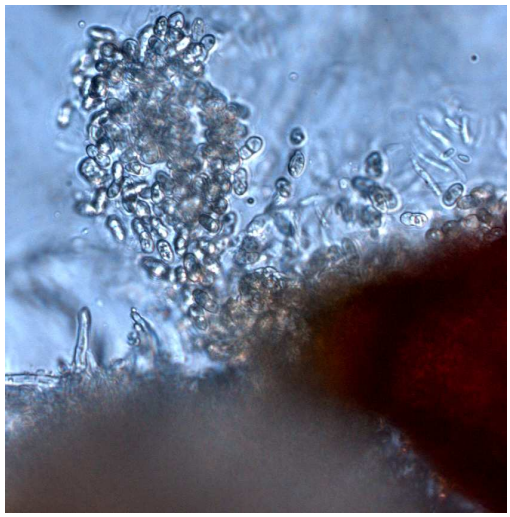
Fig. 13. Left, long, sac-like asci containing ascospores within a fruit body of *N. fuckeliana*. Right, cross section of a fruit body containing asci and filaments (arrow).



Soaking fruit bodies in water in the laboratory causes ascospores to ooze out of the pore of a fruit body. Actual release of ascospores from fruit bodies produced in culture was observed microscopically. When fruit bodies were soaked in water for a few minutes, groups of spores (probably ascus by ascus) were squeezed into the surrounding water droplet (Fig. 14). They are not powdery and are not forcibly ejected in such a way that they would be picked up by air currents under dry conditions. As the air dries, clusters of ascospores dry as a white lump on the surface of the fruit body (Fig. 14). When moistened, the clump readily breaks up and disperses in the water.

Fig. 14. Left, white clumps of ascospores that were extruded during rainy weather, and have later dried on the surface of fruit bodies. Right, a fertile fruit body from culture that is releasing spores after being placed in water on a glass slide for a few minutes. Ascospores are squeezed out in small groups (probably one ascus at a time) from the opening in the neck (red conical structure at lower right) of the fruit body.





The morphology of *Nectria* ascospores is also consistent with water dispersal: they sink in water rather than float, they are elongated and thin-walled, and the walls are fairly smooth[12]. The mode of spore dispersal has important management implications for spread of a fungal disease. Fungi that are spread by rain and splashing water may build up to high infection levels in a localized area, but the long-distance spread will be slow. Anecdotal evidence suggests that the pine fluting disease occurs in "pockets", consistent with this mode of dispersal. Avoiding overcrowding of trees in infected plantations may help to reduce spread from tree to tree.

[12] Fitt, B.D.L.; McCartney, H.A.; Walklate, P.J. 1989. The role of rain in dispersal of pathogen inoculum. Annu. Rev. Phytopathol. 27:241-270.

RECOMMENDATIONS AND CONCLUSIONS

The ecology and epidemiology of a pine canker disease in the south part of the South Island is thought to be caused by the fungus *Nectria fuckeliana*. This northern hemisphere species is the most common fungus isolated from infected wood above pruned branch stubs, but it is not found in all fluted stems or in all parts of the flutes (depressions). Both naturally and artificially inoculated wood show strong activation of tree defensive responses (unusual number of resin canals, tannins, phenolics and other extractive chemicals). These responses also occur in wounds without the fungus. In laboratory studies, *N. fuckeliana* grew best at 18-25°C, but it could grow to some extent even at 5°C. Spores were trapped near infected trees in all seasons of the year, but spore release is greatest in weeks of high rainfall. Field and laboratory evidence suggests dispersal of the fungus by rain and splashing water. The ability of *N. fuckeliana* to cause disease likely depends on a complex of host, fungal, and weather factors. It is recommended that pruning be done during dry weather and when the trees are actively growing. Avoiding crowding of trees will minimize humidity and ability of the fungus to spread in infected plantations.

ACKNOWLEDGEMENTS

I am grateful to Margaret Dick for her collaboration on this project and technical help in the laboratory and field; to Lindsay Bulman for his support and advice on many aspects of the study, especially statistical analysis; and to Tod Ramsfield, Ian Hood, and Peter Gadgil for helpful discussions. Lloyd Donaldson and Jacqueline Bond willingly shared their expertise on wood sectioning and confocal microscopy. I thank the following people for their cheerful assistance in field or laboratory: Judy Gardner, Pam Smith, Rita Tetenburg, Pam Taylor, Matt Power, Chrystal Kelly, and Rachel Hood. Many aspects of the field studies would not have been possible without the excellent support and cooperation of City Forests, Dunedin, especially Peter Oliver, Ross Chambers, and Ross Edgar.

APPENDICES

Appendix A – Seven-month results of inoculation experiment to assess effect of spore type and wound type

Flagstaff inoculations, done Apr. 05, assessed 28.11.05

Treatment		Tree No.	Height Above (mm)	Height Below (mm)	Width (mm)	Widest point*	Resin**
1	Stub	10	100	0	10	Above	x
	ascospores	14	0	0	0		x
		16	0	0	0		x
		18	115	0	11	Above	x
		40	0	0	0		x
	Mean		***43***	***0***	***4.2***		
2	Stub	3	115	95	26	Below	x
	conidia	6	80	0	8	Above	x
		15	0	0	0		x
		21	165	0	14	Above	x
		28	0	0	0		x
	Mean		***72***	***19***	***9.6***		
3	Stub	2	0	0	0		x
	water	5	0	0	0		x
		13	50	0	8	Below	x
		41	0	0	0		x
		91	40	40	6	Above	x
	Mean		***18***	***8***	***4.7***		
4	Shallow	4	20	35	10	Above	x
	ascospores	8	70	35	12	Middle	R
		11	0	0	0		x
		24	0	0	0		x
		30	35	22	5	Middle	x
	Mean		***25***	***18.4***	***5.4***		
5	Shallow	1	50	40	20	Below	R
	conidia	* 7	113	35	20	Above	R
		17	25	40	18	Below	x
		34	0	0	0		x
		90	50	60	13	Middle	R
	Mean		***47.6***	***35***	***14.2***		
6	Shallow	12	60	50	12	Below	x
	water	27	0	0	0		x
		33	0	0	0		x
		38	60	50	10	Below	R
		99	0	0	0		x
	Mean		***24***	***20***	***4.4***		
7	Deep	9	46	80	25	Middle	R
	ascospores	19	130	52	12	Middle	FR
		20	120	110	12	Middle	R

Flagstaff inoculations, done Apr. 05, assessed 28.11.05

Treatment		Tree No.	Height Above (mm)	Height Below (mm)	Width (mm)	Widest point*	Resin**
		31	100	65	20	Above	RT
		92	115	77	15	Above	x
	Mean		***102.2***	***76.8***	***16.8***		
8	Deep	22	65	50	17	Middle	R
	conidia	23	155	60	15	Below	R
		25	55	55	12	Middle	R
		36	0	0	0		R
		37	85	130	25	Middle	FR
	Mean		***72***	***59***	***13.8***		
9	Deep	29	56	55	15	Middle	x
	water	32	62	85	17	Middle	FR
		35	45	35	18	Above	x
		39?	95	42	12	Middle	R
		42	63	45	12	Middle/sunken	R
	Mean		***64.2***	***52.4***	***14.8***		

*Above = widest point is above wound; below = widest point is below wound.

**R=resin, RT = large amount of resin, FR = fresh resin, x = no resin.

Appendix B—Numbers of spores trapped on 3 traps adjacent to tree 1 (Poverty Hill Rd.) and tree 2 (Hetherington Rd.), Tokoiti Forest, begun August 31, 2005

Tree 1 Dist., mm**	114	195	227		**Tree 2 Dist., mm**	245	252	238	
	Trap No.					**Trap No.**			
Week	**3**	**4**	**5**	***Total/wk***		**6**	**7**	**10**	***Total/wk***
1	20	18	53	*91*		2	3	1	*6*
2	25	12	15	*52*		0	3	2	*5*
3	130	14	9	*153*		40	2	60	*102*
4	23	7	36	*66*		6	18	56	*80*
5	39	31	31	*101*		13	2	29	*44*
6	8	4	4	*16*		1	3	0*	*4*
7	0	6	12	*18*		2	3	0*	*5*
8	0*	0*	0*	*0**		1	0*	2	*3*
9	134	12	6	*152*		0	2	0*	*2*
10	152	4	20	*176*		46	0	0	*46*
11	65	23	14	*102*		39	24	230	*293*
12	1	0	3	*4*		27	5	104	*136*
13	3	0	0	*3*		0	0	1	*1*
14	110	22	47	*179*		98	124	110	*332*
15	39	12	22	*73*		4	1	2	*7*
16						25	17	19	*61*
17						104	49	292	*445*
18						12	8	35	*55*
19						9	28	148	*185*
20						7	1	34	*42*
21						1	0	0	*1*
22						0	0	0	*0*
23						22	7	35	*64*
24						78	40	72	*190*
25						0	0	0	*0*
26						14	0	70	*84*
27						27	34	179	*240*
28						7	2	199	*208*

*Data unavailable because of damage to trapping surface.

**For tree 1, analysis of data beyond 15 weeks is in progress.

Note: Traps were installed directly below fruit bodies. Distance refers to distance of the trap from the tree.