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Density and diversity of fungal endophytes isolated from needles of *Pinus radiata*

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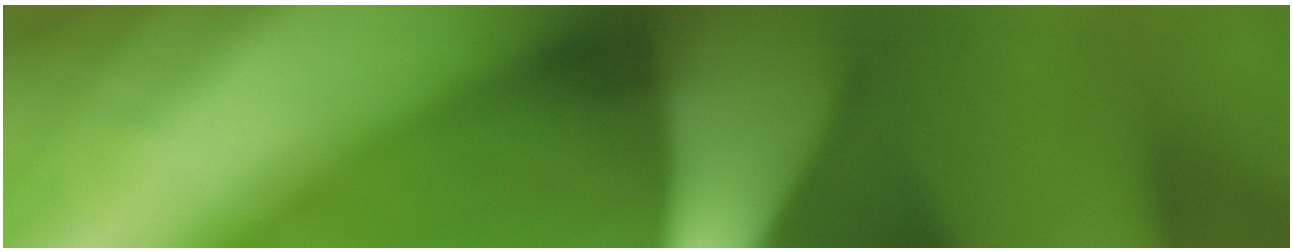

Client Report No. 12925

Density and diversity of fungal endophytes isolated from needles of *Pinus radiata*

Rebecca Ganley

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EXECUTIVE SUMMARY

This research provides an understanding of the fungal communities present in needles of *Pinus radiata* and their associations with diseased trees in comparison to healthy trees. Based on the results of this work, selected endophytes will be identified for future disease resistance and stress tolerance research. Environmentally friendly methods of protection against Cyclaneusma needle-cast will be investigated.

Objective

The objective of this study was to isolate and identify fungal endophytes from foliage of *P. radiata* trees affected by Cyclaneusma needle-cast and Physiological needle blight, and healthy trees in the same stands. Fungal diversity and composition of the diseased and healthy trees were compared, with the aim of identifying any potential beneficial endophytes for future resistance/tolerance research or endophytes that may contribute or be involved in disease expression.

Key Results

A total of 757 colonies of fungal endophytes were isolated from both healthy and Cyclaneusma-affected *P. radiata* trees. The composition and diversity of the fungal endophytes isolated differed between the stands sampled as well as between healthy and Cyclaneusma-affected trees. Identification of 464 of these endophytes showed they represented at least 37 distinct fungal taxa. Nine of these taxa were specific to the Cyclaneusma-resistant trees only and 15 were found only in the susceptible needles sampled.

Application of Results

Further work is required to determine the significance of these fungal endophytes. A selection of the endophytes isolated will be tested, using a variety of *in planta* and laboratory-based methods for their ability to provide beneficial functions in their host plants. The long term goal of this project is to identify fungal endophytes that could help mediate resistance against Cyclaneusma needle-cast and Physiological needle blight in *P. radiata* plantations. In addition, further detailed assessments of the density and diversity of the endophytes between the susceptible and resistant trees will be conducted.

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Information for Scion abstracting:

Contract number	
Client Report No.	12925
Products investigated	
Wood species worked on	*Pinus radiata*
Other materials used	Fungal endophytes
Location	Kaingaroa Forest; Kinleith Forest; Puruki

INTRODUCTION

Fungal endophytes are fungi that live within their host without causing any disease symptoms. Endophytic fungi have been identified in all plant species studied to date, however, the function of these endophytes within their hosts is relatively unknown (Ganley & Newcombe 2006; Kauhanen *et al.* 2006; Stefani & Bérubé 2006). A mutualistic relationship between the endophytes and the plant host has been suggested as the endophytes live within the tissue without causing any apparent disease symptoms (Carroll 1988; Petrini 1991; Carroll 1988). It has been hypothesized that the plant provides nutrients to the endophytes and, in return, the endophytes may confer resistance to the host from attack by pests or perform other beneficial functions (Miller 1986; Hata & Futal 1995; Arnold *et al.* 2003). In some plant species endophytes have been shown to mediate resistance against pests, provide stress tolerance to their hosts or influence community biodiversity (Redman *et al.* 2002; Clay & Holah 1999; Matthews & Clay 2001; Ernst *et al.* 2003).

Only a few studies have looked at the function of fungal endophytes in conifers. In *Pinus monticola*, induced resistance conferred by foliar fungal endophytes, has been demonstrated against the pathogen *Cronartium ribicola*, the casual agent of white pine blister rust. Seedlings colonized with native assemblages of fungal endophytes had greater survival rates than control seedlings and the levels of resistance observed were equivalent to those currently selected in polygenetic breeding programs for white pine species against this disease (Ganley *et al.* 2008) . The resistance demonstrated is thought to be a form of induced systemic resistance (ISR). In some plant species ISR has been shown to be durable and effective against multiple disease problems (Delaney 1997).

In *P. radiata* very little is known about the foliar endophytic assemblages or their functions. Based on findings from studies overseas, it is hypothesised that certain endophytes present within *P. radiata* needles would be able to mediate resistance or provide tolerance against foliar diseases currently affecting New Zealand's forest plantations.

MATERIALS AND METHODS

Needle collection and preparation

Twenty-five needles were collected from one tree with Cyclaneusma needle-cast symptoms and twenty-five needles were collected from one visually healthy tree in the same stand from each of three different plantations, resulting in a total of 75 diseased and 75 healthy needles. The three plantations, all on the volcanic plateau, used were located in: Kaingaroa Forest (Cpt 1078/10; 38°25'.182 S, 176° 30'.928 E; Established 2001), Kinleith Forest (Pokai Road, Tokoroa; 38°13'.198 S, 176 ° 00'.083 E; Established 2000) and Puruki (38°25'.935 S, 176 ° 13'.559 E; Established 1997) (collected by L.S. Bulman). All needles were collected from October - December 2007.

Three field trips were made to a region where it was expected that foliage from trees affected by physiological needle blight (PNB) would be collected. As environmental conditions were not conducive for this disease in 2007 no material was able to be collected. Instead, the number of trees/needles sampled and the subsequent analysis of fungal endophytes in the Cyclaneusma infected stands was increased.

The needles from each tree were surface sterilised in ethanol and bleach solutions according to the methods described by (Ganley & Newcombe 2006). Twenty of these needles were then plated on 2% (w/v) malt extract agar (MEA), sealed with cling film and incubated at 18°C in the

dark to allow any culturable fungal endophytes in the needle tissue to grow out. For the needles collected from Cyclaneusma-infected trees, ten yellow needles and ten green needles were plated on MEA; twenty green needles were used for all of the visually healthy trees. The remaining five surface sterilised needles per tree were used for total DNA extractions.

Fungal endophyte identification

Fungal endophytes that grew out of the *P. radiata* needles onto the MEA were individually subcultured onto new MEA plates. Some isolates were lost during this process as they either did not grow when subcultured, were contaminated or were overgrown by fast growing isolates on the same plate. All isolates that grew out of the needles were recorded regardless of whether they were subcultured. The endophytes were identified using both molecular and morphological techniques. Molecular identification techniques involved sequencing the internal transcribed region (ITS) region of the ribosomal RNA (rDNA) and comparing the sequence against those present in the GenBank database using the BLAST search program (NCBI). Non-sequenced isolates were grouped with sequenced isolates based on similar colony morphology. Traditional morphological identifications were made using colony structures, such as spores, to validate molecular identifications.

DNA extractions and PCR amplifications

Cultures selected for sequencing were grown on cellophane disks overlaid on 1.5% (w/v) MEA. Less than 100 mg of mycelium was scraped from the plates for the extractions and ground in an eppendorf tube using a micropestle. DNA extractions were then performed using a Dneasy Plant Mini Kit (Qiagen, California, USA), according to the manufacturer's instructions.

PCR amplifications were performed using the fungal-specific primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990), which amplify the ITS region. The PCR reaction contained 0.2mM of each deoxynucleotide triphosphate, 0.2 mg/ml bovine serum albumin, 4.5 pmol of each primer, 0.45 U *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany), 1x supplied Roche reaction buffer including Mg, and 1 µl of template DNA. The PCR reactions were incubated in a thermal cycler at 95^{o}C for 6 min followed by 35 cycles of 95^{o}C for 30 sec, 60^{o}C for 40 sec, 72^{o}C for 40 sec, and finally 72^{o}C for 5 min. After PCR amplification, products were run on 1-2% agarose gels and inspected for single band products of the expected size. The PCR products were then purified by adding 5 µl of the PCR product to 10 u Exonuclease I (Fermentas) and 2 u Shrimp Alkaline Phosphatase (USB) and incubating on a thermal cycler at 37^{o}C for 15 mins and then 80^{o}C 15 mins. The resultant purified products were sequenced by Macrogen (Seoul, Korea). The sequences obtained were then compared to those present in the GenBank sequence database using the BLAST search program (NCBI) to determine the closest sequence-based match.

RESULTS AND DISCUSSION

Fungal endophytes were isolated from all 6 trees sampled and from the majority of needles plated (Table 1). Only one of the trees sampled did not have endophytes grow out of all the needles sampled, with 40% of the needles producing no culturable endophytes (Table 1). Interestingly, these needles were green needles taken from a Cyclaneusma susceptible tree. The reason for this result is unclear. The needles could have contained a metabolite that was preventing fungal growth either within the needle and/or out onto the media. Conversely, the needles may have contained no culturable fungal endophytes, although this seems unlikely considering the number that grew from all the other needles tested. Nevertheless, the needles

have been retained and molecular and/or metabolite techniques will be performed on extracts from these needles to help determine the reason behind this unusual result.

In total, 757 colonies grew out from the needles plated. The majority of these were subcultured to fresh media. For all three locations, more endophytes grew from the susceptible yellow needles than the green needles (Kaingaroa Forest: yellow = 8.7 endophytes per needle, green = 3.8; Kinleith Forest: yellow = 13.5, green = 10.1; Puruki: yellow = 8.3, green = 0.2) (Table 1). More fungal endophytes per needle also grew from the susceptible trees than the resistant trees from both Kaingaroa Forest and Kinleith Forest (Table 1). Interestingly, approximately the same number of endophytes grew from the susceptible and resistant trees from Puruki. However, if the unusual green needles that grew very few endophytes are removed, then more endophytes per needle grew from the susceptible than the resistant needles. The trend of more endophytes in the susceptible needles is not surprising as many fungal endophytes in conifers have been purported to be involved in early decomposition of needle tissue (Carroll 1992; Müller *et al.* 2001). Needle senescence is likely to have triggered the fungal endophytes into a growth/decomposition phase versus the slow growing state that has been documented in healthy needle tissue (Suske & Acker 1986; Deckert *et al.* 2001). The average number of endophytes per needle is likely to be an over estimation of the number of individual culturable endophytes within the needles. Some of the individual endophytes could have grown out over more than one region of the needle producing multiple colonies that were genotypically identical.

The fungal endophytes that were successfully subcultured were identified using a combination of molecular and morphological techniques. In total 464 endophytic cultures were identified (Table 2). There were problems with identification of 35 of the isolates and these isolates are still undergoing molecular and morphological analysis. From the 464 cultures, 37 individual taxa were identified based on unique ITS sequences obtained (Table 2). Of these, 9 taxa were only identified from the resistant needles and 15 were only found in the susceptible needles sampled. The closest GenBank match gives an indication of the type of fungi these 37 taxa represent. All taxa isolated were ascomycetes although a number were in the anamorphic form. Some of the cultures had ITS sequences that were identical to those on GenBank whereas others were quite divergent. Several fungi commonly present on *Pinus radiata* in New Zealand were isolated: for example, *Dothistroma septosporum*, *Cyclaneusma minus* “simile” and *Strasseria geniculata* (Table 2). *Cyclaneusma minus* “simile” is one of the strains of *Cyclaneusma* which has been found in New Zealand (Bulman & Gadgil 2001). In addition, numerous isolates of *Lophodermium conigenum* were isolated (Table 2). *Lophodermium* spp. have previously been isolated from Cyclaneusma-susceptible *P. radiata* needles in New Zealand. Patterns of colonisation between *C. minus* and *Lophodermium* spp. in *P. radiata* needles have shown that the numbers of *C. minus* isolates per needle increase from August to October and then remain at a high level and colonisation by *Lophodermium* spp. Lags behind that by *C. minus* by approximately 1-2 months but shows a similar trend (Gadgil 1984).

Surprisingly, several of the species in the *Xylariaceae* had identical sequences to cultures obtained from New Zealand native trees such as *Kunzea ericoides*, *Darydium cupressinum* and *Podocarpus totara*. This would suggest that native New Zealand fungal endophytes have developed the ability to colonise *P. radiata* needles. It is unlikely that the endophytes have transferred from *P. radiata* into the native tree species as the number of *P. radiata*-specific endophytes that would have established in New Zealand would be expected to be low as the frequency of endophytes in seed and nursery stock has been found to be low in *P. monticola* (Ganley & Newcombe 2006). It is also unlikely that they would be able to out-compete the co-evolved native fungal endophytes within their hosts. It is possible that these endophytes may also be functional in *P. radiata* and could be potential candidates for induced resistance/tolerance studies.

Some fungal endophytes from the resistant trees have been selected to be tested for potential resistance/tolerance against diseases of *P. radiata*. These consist of fungal endophytes found only in the resistant trees, some of the putative New Zealand host-derived *Xylariaceae* spp. And endophytes found more frequently in resistant trees than in their susceptible counterparts. Further selection and testing of these fungal endophytes will be performed using a variety of *in planta* and laboratory-based tests. Further evaluation of the endophyte isolates will be performed once the final 35 fungal endophytes have been processed (Table 1). This will include more detailed assessments of the density and diversity of the endophytes between the susceptible and resistant trees.

Total DNA was extracted from a sample of needles taken from each tree. All extractions were successful and all were found to contain fungal DNA. Further work using DGGE or metagenomic assessment techniques are required to evaluate the fungal DNA content. This work is outside of the scope of this study.

CONCLUSIONS

This work represents a comprehensive study of the culturable fungal endophyte communities present within *Pinus radiata* needles in a small selection of trees in the central North Island. Knowledge of endophyte composition and diversity in *P. radiata* will contribute to enhanced understanding of the role of these fungi. However, further work is required to elucidate the function of the endophytes isolated and to assess their ability to contribute towards increased resistance against diseases within New Zealand's forests.

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Table 1. Fungal endophytes isolated from needles of Cyclaneusma needle-cast susceptible and resistant *Pinus radiata*.

		Number of fungal endophytes isolated						
		Cyclaneusma susceptible trees[a]				Cyclaneusma resistant trees[b]		
	Needle	Kaingaroa Forest	Kinleith Forest	Puruki	Needle	Kaingaroa Forest	Kinleith Forest	Puruki
Yellow needles	**1**	9	18	8	**1**	2	9	7
	2	6	14	8	**2**	3	5	4
	3	8	10	14	**3**	5	5	1
	4	14	11	3	**4**	3	5	6
	5	6	12	12	**5**	4	10	5
	6	7	12	9	**6**	6	6	6
	7	8	17	11	**7**	4	6	5
	8	13	14	7	**8**	7	8	4
	9	9	13	5	**9**	2	8	3
	10	7	14	6	**10**	2	8	7
Green needles	**11**	4	7	0	**11**	7	11	4
	12	3	8	1	**12**	3	7	2
	13	2	6	0	**13**	1	10	1
	14	7	7	0	**14**	6	8	3
	15	3	2	0	**15**	5	5	6
	16	3	7	0	**16**	2	10	4
	17	4	19	0	**17**	2	8	5
	18	2	25	1	**18**	1	12	4
	19	4	7	0	**19**	4	6	6
	20	6	13	0	**20**	3	8	1
Total number of endophytes isolated		125	236	85		72	155	84
Total number of needles with endophytes		20	20	12		20	20	20
Needles colonised (%)		100	100	60		100	100	100
Average number of endophytes per needle		6.25	11.80	4.25		3.60	7.75	4.20

[a]Needles 1-10 were yellow and needles 11-20 were green, see Methods and materials.
[b]All needles used were green

Table 2. Diversity of endophytes isolated from needles of Cyclaneusma needle-cast susceptible and resistant *Pinus radiata*.

		Number of fungal endophytes identified						
		Cyclaneusma susceptible tree			Cyclaneusma resistant tree			
Distinct endophyte taxa	**Closest GenBank taxa**	**Kaingaroa Forest**	**Kinleith Forest**	**Puruki**	**Kaingaroa Forest**	**Kinleith Forest**	**Puruki**	
E1	Bionectria sp.	1						
E2	Coniothyrium sp.			4		1		
E3	Coniothyrium sporulosum				1			
E4	Cryptosporiopsis sp.			1				
E5	Cyclaneusma “simile”	9	35	12	4	7	3	
E6	Dothistroma septosporum			1				
E7	Epicoccum nigrum			1	1			
E8	Fusicladium sp.			1				
E9	Helotiales sp.	1						
E10	Hormonema sp.						1	
E11	Lophodermium conigenum	39	29	21				
E12	Lophodermium molitoris	1						
E13	Mycosphaerella sp.			1		1		
E14	Pestalotiopsis sp.			2		1		
E15	Pezicula sp.	1						
E16	Phaeomoniella sp.				1			
E17	Sporomiaceae sp.	1						
E18	Strasseria geniculata			6			1	
E19	Unknown 1	1						
E20	Unknown 2	26	15	1	42	83	4	
E21	Unknown 3						1	
E22	Xylariaceae sp.1	3	10	1	2	5	17	
E23	Xylariaceae sp.2		2					
E24	Xylariaceae sp.3						2	
E25	Xylariaceae sp.4	1	2		6	2	8	
E26	Xylariaceae sp.5						2	
E27	Xylariaceae sp.6	1						

(contd)

Distinct endophyte taxa	Closest GenBank taxa	Kaingaroa Forest	Kinleith Forest	Puruki	Kaingaroa Forest	Kinleith Forest	Puruki	
E28	Xylariaceae sp.7		1					
E29	Xylariaceae sp.9	3	8	2	1	2	7	
E30	Xylariaceae sp.10						2	
E31	Xylariaceae sp.11					1	1	
E32	Xylariaceae sp.12		2			2		
E33	Xylariaceae sp.13		1					
E34	Xylariaceae sp.14	1						
E35	Xylariaceae sp.15						1	
E36	Xylariaceae sp.16		1					
E37	Xylariaceae sp.17		2					
Total endophytes identified		89	108	54	58	105	50	464
Lost cultures		29	120	27	10	43	29	258
Under process		7	8	4	4	7	5	35
Total endophytes isolated		125	236	85	72	155	84	757