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The survival of ectomycorrhizal fungi (ECM) of Pinus radiata *and* Pseudotsuga menziesii *from the nursery to outplanting – interim report*

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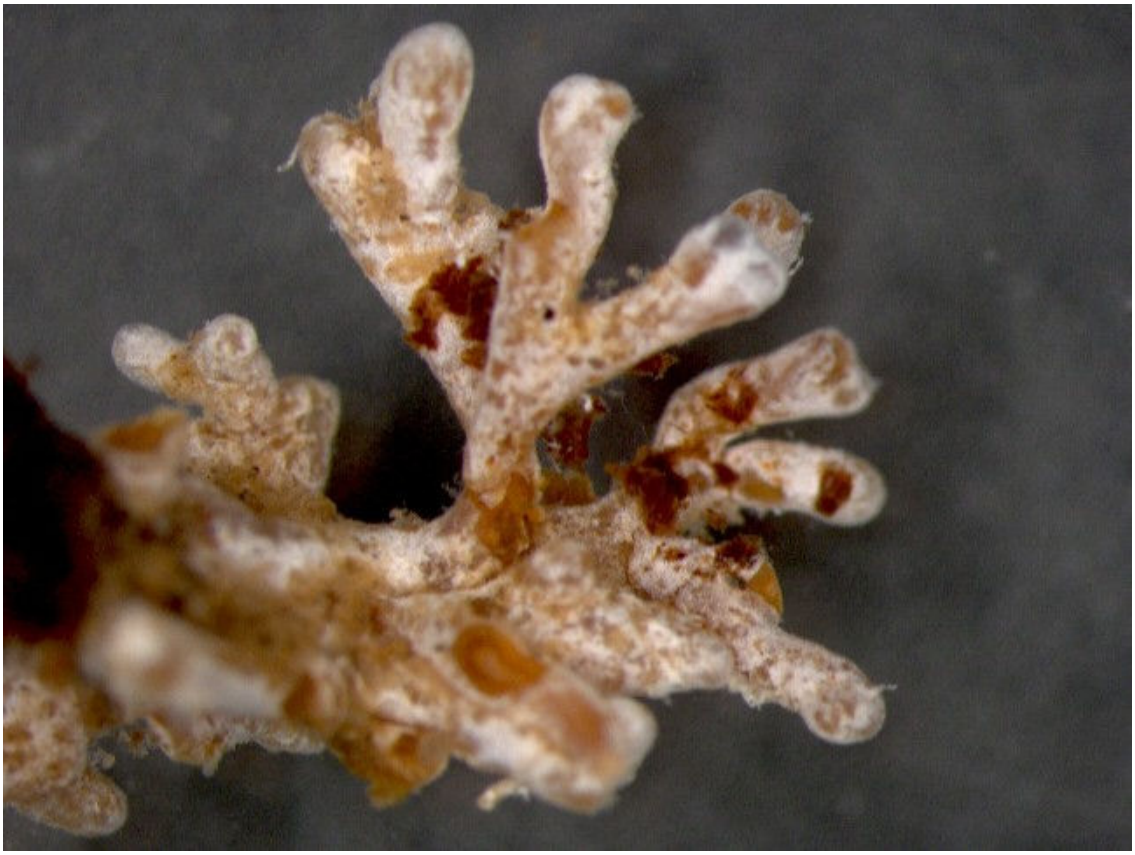
June 2009



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Client Report No.

The survival of ectomycorrhizal fungi (ECM) of *Pinus radiata* and *Pseudotsuga menziesii* from the nursery to outplanting – interim report

K. Walbert

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EXECUTIVE SUMMARY

Objective

To investigate the ectomycorrhizal fungi (ECM) associated with *Pinus radiata* and *Pseudotsuga menziesii* from different nurseries and soil conditions in 2009 and follow up their survival after outplanting in 2009/10 for one year.

Key Results

This report is an interim report on the fate of nursery ECM fungi of *P. radiata* and *Ps. menziesii* in the plantation. *Pinus radiata* and *Ps. menziesii* samples from nurseries over the country varied in the quality of their root system and ectomycorrhizal colonisation. All samples, except for *Ps. menziesii* root trainer stock from Nursery 4, were colonised by ECM fungi. Samples with a good root system had up to 10 times more root tips colonised by ECM fungi. ECM species diversity was overall low, more ECM types were found to be present on *P. radiata* cuttings than on *P. radiata* seedlings. Most ECM types of *P. radiata* were different to those of *Ps. menziesii,* species ID using molecular methods is currently underway.

Further Work

Seedlings investigated in the nurseries in 2009 will be followed up in the plantation for one year. ECM presence and abundance will be assessed as well as survival rate of the ECM types which were present in the nursery.

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INTRODUCTION

ECM fungi facilitate the establishment of seedlings in plantation sites and are important for nutrient and water uptake as well as pathogen protection. In a PhD project under taken in 2005 and 2006, the ectomycorrhizal (ECM) species associated with *P. radiata* at Te Ngae nursery and their fate in the first year of outplanting was investigated (Walbert 2008). This study was continued in 2007 and 2008 with FBRC funding and looked at the second year of outplanting. Contrary to overseas studies, it was found that nursery ECM persisted and dominated in the first year of outplanting. A change to forest ECM only occurred after about 6 yrs of *P. radiata* being in the plantation. This study however was limited to *P. radiata* seedlings grown from seed at Te Ngae Nursery and two stands in Kaingaroa Forest. It is known that ECM species vary depending on soil conditions; it is hence of interest to investigate plants grown and planted under different soil conditions. ECM species also vary depending on the host. Little work on ECM of *Ps. menziesii* has been done in New Zealand to date and none used molecular techniques to determine ECM species colonising root tips, as will be done in this project. Lastly, it is not known if there is a difference in ECM communities between seedlings or cuttings or root trainer stock.

Seedlings and cuttings from *P. radiata* and *P. menziesii* from nurseries around the country were sampled with the aim of identifying the ECM species colonising the respective host. The same seedlings and cuttings will then be followed up in the out planting for a year – we will sample seedlings and cuttings several times from the point of outplanting and identify the ECM colonising the host. ECM colonisation and communities will be assessed using a combined approach of morphological and molecular identification.

MATERIALS AND METHODS

Material used, nursery and plantation sites

In this study, *P. radiata* and *Ps. menziesii* were investigated, both material from seed as well as *P. radiata* cuttings and root trainer stock in the case of *Ps. menziesii*.

Samples were collected and sent by the nursery managers except for one nursery which was sampled by K. Walbert.

Table 1: Host species, sampling location, stock information and seedling numbers sampled

Host	Location/ Supplier	Stock	Number sampled	Notes
Pinus radiata	Nursery 1	Seedlings	10	
	Nursery 1	Cuttings	10	
	Nursery 3	Seedlings	10	
	Nursery 3	Cuttings	10	
	Nursery 5	Cuttings	10	24 months old at time of collection
Pseudotsuga menziesii	Nursery 2	Seedlings	10	20 months old at time of collection (8 months root trainer stock, 12 months in nursery beds)
	Nursery 4	Root trainer stock	10	
	Nursery 5	Seedlings	10	20 months old at time of collection; 5 seedlings with Trichoderma treatment, 5 without Trichoderma treatment

Ectomycorrhizal assessments – sampling and laboratory processing

Nursery sampling

During May and June 2009, ten samples from each host species, location and stock (seedling, cutting, root trainer stock) as listed in Table 1 were extracted from the nurseries.

Laboratory processing

Samples were either processed within the week of arrival or stored in the -20° degree freezer before analysing. The entire root system was examined. Samples were visually assessed for their state of root system development as well as ectomycorrhizal colonisation. ECM colonising root tips were quantified, each ECM tip was counted as a mycorrhiza.

ECM root tips were then categorised into 'ad hoc' morphological groups. The categorisation into morphological groups was based on mantle colour and texture, root branching pattern, root tip shape and the morphology of mycelial strands and emanating hyphae (Ingleby *et al*. 1990; Goodman *et al*. 2003) Representatives from each morphological type from each sampling site were chosen randomly for DNA extraction.

Molecular species identification

DNA was extracted from root tips using the plant DNA extraction kit REDExtract-N-Amp™ Plant PCR kit (Sigma, St. Louis, Missouri, USA). The internal transcribed spacer (ITS) regions of the rDNA was amplified using the fungal specific primer combination of ITS1F and ITS4 (Gardes & Bruns 1996; White *et al*. 1990). PCR was performed using the PCR mix supplied with the REDExtract-N-Amp™ kit, PCR products were purified using the ExoSap (Fermentas, Ontario, Canada). DNA from ECM material was sequenced using the BigDye® Terminator v3.1 & v1.1 Cycle Sequencing Kits (Applied Biosystems). DNA sequences were edited and aligned using Sequencher version 4.9 (GeneCodes Corp. Ann Arbor, MI, USA) and identities were determined by BLASTn searching (Altschul *et al*. 1990) of GenBank and UNITE nucleotide databases.

RESULTS

Root system and colonization – visual assessment

Results of the visual assessments of root system development and ectomycorrhizal colonisation are listed in Table 2.

Pinus radiata cuttings from Nursery 1 had average to good root system development and mycorrhizal colonisation. *Pinus radiata s*eedlings from this nursery however were not well colonised by mycorrhizal fungi. *Pinus radiata* cuttings from the Nursery 5 looked good with well developed root systems and good ECM colonisation. *Pinus radiata* seedlings from the Nursery 3 were of marginal quality. The root system was not well developed neither were roots colonised well. Cuttings from this nursery were of better quality and well colonised. *Pseudotsuga menziesii* seedlings from Nursery 2 had an exceptional well developed root system and a high colonisation rate. *Pseudotsuga menziesii* seedlings from the Nursery 5 also showed a well developed root system. They had a lower colonisation rate but more ECM types present. Surprisingly the *Ps. menziesii* root trainer stock material from Nursery 4 did not show any visible sign of mycorrhizal colonisation yet the root system was well developed. Random samples of the root tips from Nursery 4 were chosen for DNA extraction and PCR/sequence analysis to check whether any mycorrhizal fungi were present or not. This analysis is currently underway.

Table 2: Host species, sampling location, stock information, root system and ectomycorrhizal colonisation assessment (S = seedlings, C = cuttings, R = root trainer stock).

Host	Supplier	Stock	Root system	ECM colonisation
Pinus radiata	Nursery 1	S	Average	Marginal
	Nursery 1	C	Good - average	Good, a lot of dead ECM from previous year
	Nursery 3	S	Marginal	Marginal
	Nursery 3	C	Good - average	Good
	Nursery 5	C	Good	Well colonised
Pseudotsuga menziesii	Nursery 2	S	Good	Well colonised
	Nursery 4	R	Good	Did not appear to have any ECM colonisation
	Nursery 5	S	Good	Well colonised

ECM colonisation – types and abundance

Species ID with molecular methods is currently underway. These results are preliminary and represent a tentative analysis of ECM types and their abundance. Some mycorrhizal species can vary in their morphology depending on age; hence molecular analysis can sometimes determine that two types are one species.

Table 3 summarises the types observed on *P. radiata* and *Ps. menziesii* with tentative species identification, Figures 1 and 2 show examples of ectomycorrhizal types of *Ps. menziesii* and *P. radiata*.

Table 3: ECM morphotypes observed on *P. radiata* and *Ps. menziesii* and tentative species identification

Pinus radiata	**Description**	**Tentative identification**
	White + hyphal net, irregular - dichotomous branching	*Hebeloma* sp.
	White, thick mantle,dichotomous branching, rhizomorphs	*Rhizopogon rubescens*
	Clear-skin colour, dichotomous branched, smooth	*Thelephora terrestris*
	Brown, strong dichotomous branching, smooth	*Tuber* sp.
	Brown, thick tips with swollen tips, dichotomous - irregular branching, smooth	*Tuber* sp.
	Brown, not to irregular branched, smooth	*Wilcoxinia mikolae*
	Black + black hyphal net, unbranched	Unknown
	Brown + white, fluffy hyphae	Unknown
	Skin colour with white patches, long, dichotomous branching, hyphae	Unknown
	Green, not branched	Unknown
Pseudotsuga menziesii	**Description**	**Tentative identification**
	White, thick mycelium, thick rhizomorphs, monopodial pinnate branching	*Rhizopogon parksii*
	White and black, thick mycelium, thick rhizomorphs, monopodial pinnate branching	Unknown, variety of *Rhizopogon parksii?*
	White + hyphal net, not to irregular branched	*Hebeloma* sp.
	Black + black hyphal net, not to irregular branching	Unknown
	Black - iridescent, smooth, monopodial-pinnate	Unknown
	Honey-brown, monopodial-pinnate branching, smooth	Unknown
	Purple - iridescent, straight, non branched	Unknown
	White and thick feltlike hyphal cover, thick rhizomorphs, monopodial pinnate branching	Unknown

Figure 1: Examples of ECM types found on *Pinus radiata* during this study.

Figure 2: Examples of ECM types found on *Pseudotsuga menziesii* during this study.

Numbers of root tips colonized by ECM fungi are listed in Table 4 and Table 5. These numbers are absolute numbers of ECM root tips, we did not count root tips that were not colonised. As expected, *P. radiata* seedlings had low species diversity and about three different ECM types were present on the root system. Eight different ECM types were found on the *P. radiata* cuttings from Nursery 3, six ECM types on *P. radiata* cuttings from the Nursery 5. More ECM types were found on the cuttings than on the seedlings, except for the material from Nursery 1. As noted earlier, this material was of poorer quality. The ECM count was higher for cuttings than for seedlings – 29,050 vs. 3,569 ECM tips for Nursery 3 material and 13,010 vs. 2.913 ECM tips for the Nursery 1 material. ECM count for Nursery 5 was 8,518. In all cases the *Rhizopogon rubescens* type dominated on the samples.

As for the *Ps. menziesii* samples, three ECM types were found on seedlings from Nursery 2 and five types on the seedlings from Nursery 5. No ECM colonisation was apparent on the root trainer stock from the Nursery 4. The latter is surprising as a preliminary analysis of material from this nursery found five different ECM types. This matter is currently being followed up. Ectomycorrhizal types found on *Ps. menziesii* showed high branching patterns (Figure 2), which is reflected in the high count of mycorrhizal root tips from Nursery 2 seedlings. As with *P. radiata,* more ECM tips were counted on cuttings (40,952) than on seedlings (9,828). Again, the *Rhizopogon* type (*R. parksii*) dominated the colonisation of the seedling roots.

Table 4: Numbers of ECM morphotypes assessed on *P. radiata* during this study

Pinus radiata			
Nursery 3 *P radiata* seedling	**Description**	**Count**	**%**
	White, thick mantle, dichotomous branching, rhizomorphs	2,962	83.0
	Clear-skincolour, dichotomous branched	384	10.8
	White + hyphal net, irregular - dichotomous branching	223	6.3
	Total	**3,569**	
Nursery 3 *P radiata* cuttings	**Description**	**Count**	**%**
	White, thick mantle, dichotomous branching, rhizomorphs	15,206	52.3
	Brown, not to irregular branched, smooth	7,537	25.9
	Brown, strong dichotomous branching, smooth	4,863	16.7
	Skincolour with white patches, long, dichotomous branching	697	2.4
	Brown + hyphal fluff, not branched	529	1.8
	White + hyphal net, irregular - dichotomous branching	128	0.4
	Clear-skincolour, dichotomous branched	64	0.2
	Black + black hyphal net, not to irregular branching	26	0.1
	Total	**29,050**	
Nursery 1 *P radiata* seedlings	**Description**	**Count**	**%**
	White, thick mantle, dichotomous branching, rhizomorphs	2,053	70.5
	Brown, thick tips with swollen tips, dichotomous - irregular branching, smooth	832	28.6
	Clear-skincolour, dichotomous branched	28	1.0
	Total	**2,913**	
Nursery 1 *P radiata* cuttings	**Description**	**Count**	**%**
	White, thick mantle, dichotomous branching, rhizomorphs	7,415	57.0
	Brown, not to irregular branched, smooth	4,827	37.1
	White + hyphal net, irregular - dichotomous branching	768	5.9
	Total	**13,010**	
Nursery 5 *P radiata* cuttings	**Description**	**Count**	**%**
	White, thick mantle, dichotomous branching, rhizomorphs	5,705	67.0
	Clear-skincolour, dichotomous branched, smooth	1,499	17.6
	Brown, not to irregular branched, smooth	925	10.9
	White + hyphal net, not to irregular branched	220	2.6
	Brown + white, fluffy hyphae	134	1.6
	Green, not branched	35	0.4
	Total	**8,518**	

Table 5: Numbers of ECM morphotypes assessed on *Ps. menziesii* during this study

Pseudotsuga menziesii			
Nursery 2 *Ps menziesii* seedlings	**Description**	**Count**	**%**
	White and black, thick mycelium, thick rhizomorphs, monopodial pinnate branching	38,312	93.6
	Purple - iridescent, straight, non branched	1,476	3.6
	White and thick feltlike hyphal cover, thick rhizomorphs, monopodial pinnate branching	1,164	2.8
	Total	**40,952**	
Nursery 5 *Ps menziesii* seedlings	**Description**	**Count**	**%**
	White, thick mycelium, thick rhizomorphs, monopodial pinnate branching	6,237	63.4
	Honey-brown, monopodial-pinnate branching, smooth	2,225	22.6
	White + hyphal net, not to irregular branched	1,346	13.7
	White and thick feltlike hyphal cover, thick rhizomorphs, monopodial pinnate branching	23	0.2
	Black - iridescent, smooth, monopodial-pinnate	7	0.1
	Total	**9,838**	
Nursery 4 *Ps menziesii* root trainer stock	**Description**	**Count**	**%**
	Total root tips, no ECM colonisation visible	**5217**	

DISCUSSION

This report presents interim results on a project which followed ectomycorrhizal species from the nursery into the out planting. At that stage, *P. radiata* and *Ps. menziesii* samples were processed, ECM fungi grouped into morphotypes based on morphology and abundance was measured. Species identification with molecular methods is currently underway.

At this point of the project we can see a variety of quality in seedlings from different nurseries. Seedlings with a poorly developed root system did not have good mycorrhizal colonisation. Seedlings with good root systems and colonisation had up to ten times higher root tip counts compared with samples having poor root systems and colonisation. Higher root tip counts and ECM colonisation translates into a bigger root surface area of the plant for nutrient and water uptake, which in turn will lead to a healthier and more resistant seedling which is less dependent on additional fertilisers. Especially certain ectomycorrhizal types, like the *Rhizopogon* type of *Ps. menziesii* or the *Tuber* type found on *P. radiata* show a very high branching pattern (Figures 1 and 2), which increases the root system surface area of the host plant greatly (up to 38, 312 ECM root tips were counted for the

As expected, ECM fungal diversity was low in the nurseries and abundance was generally dominated by *Rhizopogon* types. Cuttings showed a higher fungal diversity. This could be explained by the fact that these plants are physiologically older and have spent more time in nursery beds than seedlings.

Some material from Nursery 1 and Nursery 3 did not have a well developed root system and were not well colonised by mycorrhizal fungi. Abundance of mycorrhizal fungi varies depending on soil conditions and is reduced by high applications of nitrogen fertiliser. These factors could have reduced the presence of mycorrhizal fungi, which will be further investigated. It will be interesting to see how these seedlings establish in the plantation and how their mycorrhizal communities will develop over the first year of out planting.

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