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Detection of asymptomatic fungal microorganisms in *Pinus radiata* tissue culture material

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Abstract

Background: Tissue culture has been viewed as a relatively safe method of transporting conifer germplasm as the risk of pathogens associated with tissue cultured material has been assumed to be low. Despite these assumptions, it is unknown whether tissue cultured propagation material contains microorganisms or whether such microorganisms could grow out onto the media used.

Methods: To determine whether asymptomatic tissue culture material contains fungal microorganisms, 30 different *Pinus radiata* D.Don genotypes from four different types of tissue culture material were analysed for the presence of fungal DNA or mycelium. In addition, thirteen fungal and oomycete isolates were cultured on standard tissue propagation media.

Results: Fungal DNA was detected in all samples tested. No fungal or bacterial microorganisms were able to be grown from any of the tissue culture material tested. However, confocal microscopy showed the presence of filaments that could have been fungal mycelium. Growth of thirteen fungal and oomycete isolates on standard tissue propagation media indicated these media can severely limit the growth of some of these microorganisms.

Conclusion: The results from this study show that tissue culture material does contain asymptomatic fungal microorganisms but the exact risk from these microorganisms is unclear.

Keywords: Biosecurity risk; Fungal endophytes; Confocal microscopy; Cryptic infections; *Phytophthora*

Background

The perceived threat that the fungus *Fusarium circinatum*, the causal agent of pitch canker disease, poses to the plantation forest industry has rigorously curtailed the importation of some conifer material into New Zealand. There is currently a complete ban on the importation of any live plant material and restrictions on the importation of seed from *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) or any *Pinus* species (Ministry for Primary Industries 2010). However, new breeding material is required for genetic improvement of existing stocks, to enhance health traits, including resistance to disease, as well as improving productivity and wood quality. Thus, restrictions on the importation of conifer germplasm are of concern to the New Zealand forest industry.

In an effort to find alternative methods to import new *Pinus* spp. or *Pseudotsuga menziesii* material into New Zealand, the importation of live material in tissue culture has been suggested. Tissue culture has been viewed as a relatively safe method of transporting conifer germplasm for a variety of reasons. Firstly, there are very few viruses or bacterial pathogens associated with *Pinus* spp. or *Pseudotsuga menziesii* and those that are known are not considered to be serious pathogens (Sinclair and Lyon 2005; Veliceasa et al. 2006). Secondly, it has been assumed that any fungal or bacterial pathogens present in tissue culture material would grow out onto the growth media. Thirdly, the likelihood that fungi could survive the intensive nature of many currently used tissue culture procedures is thought to be low.

Despite these assumptions, it is unknown whether tissue cultured propagation material contains microorganisms or whether the tissue propagation media would

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contain the required nutrients to support the growth of such microorganisms. Furthermore, the discovery of unculturable microorganisms in plant cultures grown *in vitro* is becoming increasingly common (Thomas 2011). Although these reports have mainly dealt with bacteria that are able to survive antibiotic cleansing treatments, it is possible that similar asymptomatic associations could also occur with fungi in conifer tissue culture. In addition to the possibility of undesirable fungi, *Pinus* spp. are known to contain a wide diversity of fungal endophytes (Deckert and Peterson 2000; Ganley and Newcombe 2006) and total sequencing approaches indicate the number of unculturable fungi present may exceed initial diversity estimates (Fröhlich and Hyde 1999; Hawksworth 2001). The function of fungal endophytes in their hosts is largely unclear (Rodriguez et al. 2009). It is likely that many are involved in beneficial symbiotic associations and it is possible that some may be so intricately linked that they are required for host survival.

The purpose of this study was to determine whether asymptomatic *Pinus radiata* D.Don (radiata pine) tissue culture material contains fungal microorganisms. This species was chosen for study because of its importance as a commercial species in New Zealand. Also, if microorganisms are present, what species are there, what might be their ecological significance, are they viable and do they pose a biosecurity risk? In addition, selected tissue propagation media were tested to see if they could support growth of a selection of fungi and one *Phytophthora* species associated with *Pinus radiata*.

Methods

Tissue culture preparation

Four types of tissue culture material were prepared from *Pinus radiata*: embryogenic tissue derived from immature zygotic embryos; adventitious-axillary organogenesis shoots derived from cotyledons dissected from mature zygotic embryos; epicotyl-axillary organogenesis shoots derived from the epicotyls of mature zygotic embryos; and mature organogenesis shoots derived from field grown material and established as *in-vitro* shoot cultures. The same ten genotypes were used for both the adventitious-axillary and epicotyl-axillary organogenesis shoots. Ten different genotypes were used for the embryogenic tissue and another 10 for the mature organogenesis shoots so 30 different genotypes were tested in total. Embryogenic tissue was initiated using techniques described by Walter and Grace (2000) and Walter et al. (2005). The adventitious-axillary organogenesis shoots were prepared from excised cotyledons that were cryopreserved at – 196 °C and then had shoots initiated adventitiously. Non- germinated hypocotyls (including epicotyls) from the embryos that provided the cotyledons for cryopreservation were retained and these provided the non-cryopreserved epicotyl-axillary shoots, thus material was available from two methodology origins using the same genotypes (Hargreaves et al. 2004, 2005). Mature material was established using techniques described by Horgan and Holland (1989).

DNA extractions

Total DNA was extracted from each genotype/cell line for the four different types of *Pinus radiata* tissue culture material prepared. Approximately one gram of embryonic callus material from each cell line was ground to a fine powder in liquid nitrogen using a mortar and pestle. For the adventitious-axillary, epicotyl-axillary and mature organogenesis shoots, one shoot from each cell line was ground to a fine powder in liquid nitrogen using a mortar and pestle. Extraction of DNA was then performed using a DNeasy Plant Mini Kit (Qiagen, California, USA), according to the manufacturer's instructions. For genotypes/cell lines with tissue or shoots that weighed more than 100 mg, the tissue culture powder obtained was separated into samples less than 100 mg and individual DNA extractions were performed on each sample.

PCR amplification

To confirm that the DNA extractions worked, the total extracted DNA was subjected to PCR amplification using plant specific primers ConiferF (5′-GGG AGG TTT TAC TGC AAA TAC TTC-3′) and ConiferR (5′-CAT CCC CAA AGA TCT CGG TCA GAG CAG GC-3′) (Marler et al. 1999). The PCR reaction mixture was prepared in a total volume of 15 μL using 10 mM Tris-hydrochloric acid at pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.2 mM dNTPs, 0.2 mg/mL bovine serum albumin, 5 pmol of each primer, 1.5 U *Taq* DNA polymerase and 1 μL total extracted DNA. All PCR amplifications were performed according to Marler et al. (1999). After PCR amplification, products were run on 1–2 % agarose gels and examined for bands of the expected size to confirm the DNA extractions were successful.

To determine if fungal DNA was present in the tissue culture material, total extracted DNA was tested with fungal-specific nested primers designed to amplify the internal transcribed spacers (ITS) of the fungal ribosomal RNA gene region (rDNA). The fungal-specific primary PCR primers used were NSI1 (5′-GATTGAATGGCT TAGTGAGG) and NLB4 (GGATTCTCACCCTCTAT GAC-3′) (Martin and Rygiewicz 2005), and the nested PCR primers were ITS1-F (5′-CTTGGTCATTTAGAG GAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The PCR reaction mixture was prepared as described above. For the primary PCR, 1 μL total extracted DNA was added to the reaction mixture, whereas 1 μL of purified PCR product was used for the

nested PCR. The purified PCR product was prepared by adding 5 μL of the primary round PCR product to 10 U Exonuclease I (Fermentas) and 2 U Shrimp Alkaline Phosphatase (USB) and incubating on a thermal cycler at 37 °C for 15 mins then 80 °C 15 mins. The primary and nested PCR reactions were incubated in a thermal cycler at 95 °C for 6 min followed by 35 cycles of 95 °C for 30 sec, 60 °C for 40 sec, 72 °C for 40 sec, and finally 72 °C for 5 min. A high annealing temperature was used so that single band products, if present, could be later sequenced. After PCR amplification, products were run on 1–2 % agarose gels and inspected for bands of the expected size.

Each of the ten cell lines/genotypes for the four different tissue culture types were tested for the presence of fungal DNA. In addition, a negative control (water only) was included. For the nested PCR reactions, the negative control from the previous PCR round was PCR amplified as well as a new negative control.

Fungal DNA identification

Products amplified from the nested PCR reactions using the fungal-specific primers were sequenced. Only nested PCR products with single bands were sequenced, and these were purified using Exonuclease I and Shrimp Alkaline Phosphatase as described above. The resultant purified products were sequenced by Macrogen (Seoul, Korea). The sequences obtained were then compared with those present in the National Center for Biotechnology Information (NCBI) GenBank sequence database using the BLAST search program to confirm the products were fungal in origin and to determine closest sequence-based match. Only sequences that showed single peaks were used; sequences that had double peaks, indicating more than one species present, were discarded.

Growth of fungi from tissue culture material

To determine whether any culturable fungi were present, embryogenic tissue and shoots of *Pinus radiata* were plated onto four different general fungal media. The shoot material of these species originated from field collected material that was sterilised and used to establish organogenic cultures. Five genotypes were tested that were different from those used in the DNA analysis. Shoot bases with associated callus from organogenic cultures of *Pinus radiata* were used on the test media. The shoot bases were chosen as test material as they have had the longest contact with the growing shoots. The four types of general fungal media used were 4 % (w/v) potato dextrose agar (PDA), 2 % (w/v) malt extract agar (MEA), carrot agar (CA) and prune agar (PA). The CA was prepared by liquidising 200 g carrots in 1 L distilled water with 15 g agar and the PA was made using 50 mL prune juice, 2.5 g lactose, 0.5 g yeast extract, 10 g agar and 450 mL distilled water, adjusted to pH 5.8–6.0. Embryogenic tissue and shoots were plated onto each type of media, the embryogenic callus was simply placed on the test media surface. The shoot bases were cut length-wise to increase contact of the tissue with the test media. The tissue culture material was incubated in standard growth room conditions (Hargreaves et al. 2005) and monitored for 10 days for any sign of bacterial or fungal growth.

Confocal microscopy

Ten samples of *Pinus radiata* somatic embryogenic tissue growing in sterile culture were examined by a range of light microscopy techniques. Tissue was examined by differential interference contrast (DIC), and by confocal fluorescence microscopy after staining with Alexa labelled-wheat germ agglutinin (WGA), a lectin which binds to chitin in some types of fungal hyphae, or by Acriflavin, a general purpose fluorescent stain. Cotton blue and toluidine blue stains were also used on some samples.

Growth of microorganisms on standard tissue propagation media

All isolates were obtained from the Forest Research Culture Collection at Scion. Twelve fungal (*Amylostereum aerolatum* NZFS 2534, *Armillaria novae-zelandiae* NZFS 1973, *Colletotrichum acutatum* NZFS 7 T/3, *Cyclaneusma minus* NZFS 682, *Cylindrocladium scoparium* NZFS 1828, *Dothistroma septosporum* NZFS 2728, *Fusarium oxysporum* NZFS 2952, *Junghuhnia vincta* NZFS 2473, *Leptographium procerum* NZFS 626, *Lophodermium conigenum* NZFS 709, *Pestalotiopsis* sp. NZFS 3259, *Strasseria geniculata* NZFS 2998) and one oomycete (*Phytophthora cinnamomi* NZFS 2968) were selected to encompass the wide array of microorganisms known to be associated with *Pinus radiata*.

All isolates were initially grown on MEA or CA for *Phytophthora cinnamomi*. For each isolate, a 5 mm plug of mycelium obtained from these plates was placed in the centre of a 90 mm diameter Petri dish containing one of the five nutrient media. The four standard tissue culture media were: shoot initiation medium ½LP5 (Hargreaves and Menzies 2007), shoot multiplication medium LPch (Hargreaves and Menzies 2007), embryogenesis medium EDM6 (Hargreaves et al. 2009) and embryogenesis medium Glitz (Hargreaves et al. 2009). The Petri dishes were sealed and placed in a culture cabinet at 20 °C in the dark. Three replicates were completed for each of the five nutrient media. The diameter of each colony was measured in two directions at 7, 14, 21, 28 and 35 days.

The average colony diameter ± standard deviation was calculated from the three replicates on each of the five nutrient media using SAS software. Analyses of variance were also performed to determine any significant difference in

colony growth on the five nutrient media for each of the 13 microorganisms at 7, 14, 21, 28 and 35 days. Fisher's Least Significant Difference (LSD) ($\alpha = 0.05$) was used to determine significant differences between the five nutrient media when sample sizes were equal ($n = 30$). When sample sizes were unequal ($n < 30$), due to contamination, the Student-Newman-Keuls procedure ($\alpha = 0.05$) was used.

Results

Fungal DNA in tissue culture

Total DNA was successfully extracted from all the tissue culture samples. Amplification of PCR using the general nested fungal primers NSL1/NLB4 and ITS1-F/ITS4 detected DNA in all the tissue culture material tested and sequencing of single band PCR products confirmed the DNA was fungal (Table 1). The number and intensity of the bands amplified varied among the samples (Fig. 1). All four tissue culture types produced multiple and single band PCR products, including the cryopreserved (adventitious-axillary) shoot cultures. Analysis of DNA sequences obtained from single band PCR products showed a variety of different fungal microorganisms present (Table 1).

Growth on general fungal media

No fungal or bacterial growth was observed from any of the tissue culture material plated onto the four general fungal media (PDA, MEA, CA and PA) tested (data not shown).

Confocal microscopy

There was no clear unambiguous evidence of fungal mycelium in any of the tissue culture samples analysed. None of the stains used were useful in highlighting any low level fungal infection and the embryogenic tissue reacted with the WGA reagent reducing the potential for specific labelling of hyphae amongst the mass of tissue. However, filaments were observed in four out of the 10 samples examined. It is possible that these filaments were fungal hyphae. The cytoplasmic appearance of these filaments would suggest they were alive. There were two types of structures observed that might represent fungal mycelium. The first were bundles of filaments 1–5 μm wide with granular cytoplasmic contents that appeared relatively stiff compared with suspensors (Fig. 2). These structures were non-pigmented, and WGA negative. No evidence of clamp connections could be seen. The second were minute filaments attached to the outer surface of suspensor cells (Fig. 3). These filaments were definitely outside of the cell wall of the suspensor and had a cytoplasmic appearance. The cell wall was very thin so was difficult to assess but appeared to be WGA positive.

Growth on standard tissue propagation media

Growth of the microorganisms on standard tissue propagation and general fungal/oomycete media differed for each of the 13 isolates tested (Table 2, data only shown for six of the isolates tested). There were significant differences in growth on the five different media for all of the isolates except one, *Junghuhnia vincta*. There was no difference in growth for *J. vincta* on any of the media tested and by 21 days this isolate had grown to the edge on all Petri dishes. For two of the isolates, *Cylindrocladium scoparium* and *Dothistroma septosporum*, there was no significant difference in growth at 7 days, but after 21 days growth differences were significant (Table 2). Conversely, *Colletotrichum acutatum* showed significant differences in growth until 35 days when the isolate grew to the edge of the Petri dish on all plates. The remainder of the isolates showed significant differences in growth over the entire 35 days.

The majority of isolates grew better on the five nutrient media in the following order: MEA (CA for *Phytophthora cinnamomi*), Glitz, EDM6, ½LP5, LPch. The only two isolates that did not grow best on the general fungal medium (MEA) were *Colletotrichum acutatum* and *Lophodermium conigenum*, which both grew best on Glitz. Conversely, the isolates tended to grow slowest on LPch, with 11 of the isolates growing significantly slower on LPch than on MEA (CA for *Phytophthora cinnamomi*) and two of these isolates (*Cyclaneusma minus* and *L. conigenum*) did not grow on the LPch medium beyond the original plug. The only isolates with no significant difference in growth between these two media were *Cylindrocladium scoparium* and *J. vincta. Colletotrichum acutatum* grew significantly faster on MEA than LPch from 7–21 days, although there was no difference in growth after 28 days.

Discussion

The results from this study show that *Pinus radiata* tissue culture material is able to carry cryptic infections of asymptomatic fungal microorganisms. All of the tissue culture material tested was found to carry fungal DNA and multiple bands were found in many of the DNA samples tested, indicating more than one species present. Overall, this suggests that fungal DNA is present in the embryo and is propagated throughout the tissue culture process, also indicating that the fungal microorganisms present are viable. If the fungal microorganisms present were non-viable then fungal DNA would be expected to be found only in the embryonic tissue and not be propagated through to the adventitious- or epicotyl-axillary organogenesis shoot material. Hence the presence of fungal DNA, and the implied viability of the microorganisms, in the adventitious-axillary shoots indicates that cryopreservation has no effect on the ability of this material to harbour cryptic fungal microorganisms and cannot

Table 1 Fungal DNA present in *Pinus radiata* tissue culture material

Tissue culture type and genotype number	PCR product amplified[a]	Closest GenBank match of sequenced single-band PCR products		
		Fungal microorganism	Accession number	Sequence identity (%)
Embryogenic tissue				
1	yes	-	-	-
2	yes	-	-	-
3	yes	-	-	-
4	yes	Uncultured ectomycorrhiza	EF218819	98
5	yes	-	-	-
6	yes	*Mycena rubromarginata*	EF530939	91
6	yes	*Botryosphaeria parva*	AM410965	99
7	yes	-	-	-
8	yes	-	-	-
9	yes	-	-	-
10	yes	*Resincium bicolor*	AF518763	99
Adventitious-axillary organogenesis shoots				
11	yes	*Epicoccum nigrum*	AF455403	99
12	yes	*Trametes* sp.	AY968077	75
13	yes	-	-	-
14	yes	-	-	-
15	yes	*Oligoporus placentus*	AJ249267	79
16	yes	*Drechslera erythrospila*	AY004782	99
17	yes	*Cladosporium* sp.	EF432298	99
18	yes	*Oligoporus placentus*	AJ249267	79
19	yes	Basidiomycete	AF519892	79
20	yes	-	-	-
Epicotyl-axillary organogenesis shoots				
11	yes	Fungal sp.	AM231388	98
12	yes	-	-	-
13	yes	-	-	-
14	yes	*Armillaria hinnulea*	AF394918	97
15	yes	-	-	-
16	yes	*Coriolaceae*	AY840564	93
16	yes	*Botryobasidium subcoronatum*	AJ389785	95
17	yes	*Cyclaneusma minus*	KM216324	100
18	yes	-	-	-
19	yes	*Phellinus* sp. or *Trametes* sp.	AF251437/ AY840588	99
20	yes	*Rhodotorula yarrowii*	AF444628	98
Mature organogenesis shoots				
21	yes	*Trametes versicolor*	AF13996	95
22	yes	-	-	-
23	yes	-	-	-
24	yes	*Petriella sordida*	AY882360	98
25	yes	-	-	-
26	yes	-	-	-
27	yes	-	-	-

Table 1 Fungal DNA present in *Pinus radiata* tissue culture material *(Continued)*

28	yes	*Resinicium bicolour*	AF518763	99
29	yes	-	-	-
30	yes	-	-	-

[a]PCR products generated from nested PCR amplification using primers NSL1/NLB4 and ITS1-F/ITS4 (see Methods and materials)

be relied on as a sanitising method for tissue culture material.

Mature organogenesis shoot material can have a high level of fungal contamination, which grows out onto the tissue culture media used during the first stages of the tissue propagation process and is discarded as contaminated. The majority of the contaminants are likely to be fungal endophytes, which are known to reside within mature needle tissue (Ganley and Newcombe 2006; Johnson and Whitney 1989). The remainder of the fungal microorganisms retained in the asymptomatic mature shoot material, as identified in this study, are likely to represent non-culturable fungal assemblages present in mature needle tissue. A high annealing temperature was used in this study so single-band products could be obtained. If a lower annealing temperature was used it is expected more species would have been amplified, if present in the tissue culture material. To get a better understanding of the fungal biodiversity present, the use of next-generation technologies would be recommended, and the use of quantitative PCR methods could be informative in understanding the extent of colonisation for specific species.

A BLAST search analysis of the single-band PCR products generated revealed a variety of fungal species present. The majority of the GenBank taxa with close sequence identity matches to the tissue culture sequences were fungi known to have conifer associations, had been isolated as fungal endophytes from other plant species or had been obtained from environmental samples from forest settings, according to their GenBank accession details. There was a wide variation in the sequence identity to the closest GenBank match, ranging from 79 to 100 % identity. The *Cyclanuesma minus* sequence that was 100 % identical to sequences on GenBank matches shows known fungal associates of *Pinus radiata* are able to survive the tissue culture process. *Cyclaneusma minus* is a pathogen of *Pinus radiata* that causes a disease known as cyclaneusma needle cast (Gadgil 1984). However, it can be readily isolated from healthy *P. radiata* needles suggesting it also has an endophytic phase (unpublished data). Molecular analysis has indicated that *C. minus sensu lato* contains multiple species (Prihatini et al. 2014) and potentially the species within this complex may encompass a range of pathogenic, saprophytic or endophytic behaviours.

For the remainder of the putative fungal isolates identified, whether the tissue culture sequences represent fungal microorganisms that are the same species or genus as their closest GenBank match is unclear. Likewise, the possible function of these microorganisms within *Pinus radiata* tissue is unknown. Fungal

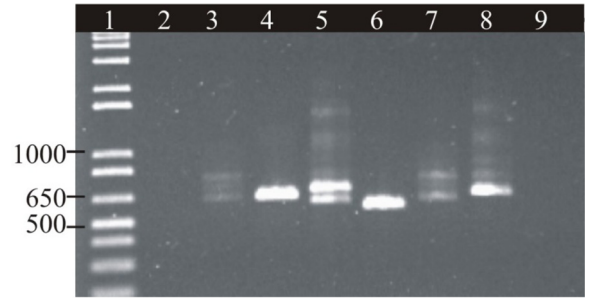

Fig. 1 PCR products generated from nested PCR amplification using primary primers NSL1/NLB4 followed by the nested primers ITS1-F/ITS4, visualised on a 1 % agarose gel. Lane 1: 1 kb ladder, band sizes (bp) are indicated on the left. Lane 2: primary negative control, NSL1/NLB4 negative control re-amplified with nested primers ITS1-F/ITS4. Lane 3–8: adventitious-axillary shoot PCR products, Lane 3: genotype number 19; Lane 4: 16; Lane 5: 13; Lane 6: 11; Lane 7: 15; and Lane 8: 18. Lane 9: secondary negative control, amplified with primers ITS1-F/ITS4 only

Fig. 2 Potential strands of 2–3 mycelia amongst the embryo tissue (*arrow*). Field of view 1000 × 750 μm

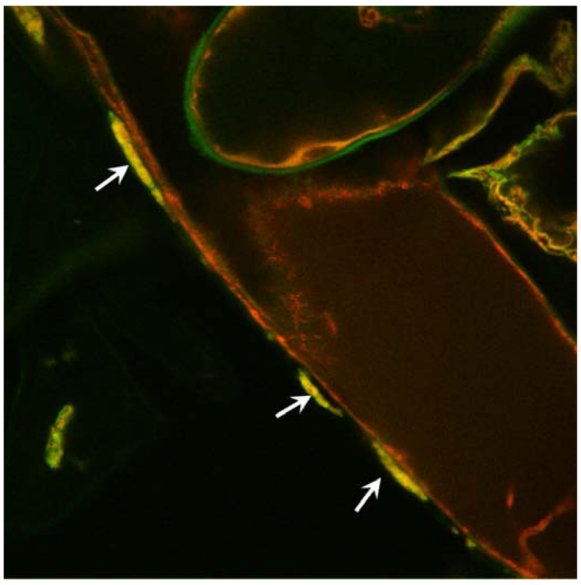

Fig. 3 Potential mycelium (*arrows*) adhering to the outside surface of a suspensor cell. Confocal fluorescence image of WGA stained tissue. Field of view 159 × 159 μm

organisms that live entirely endophytic lifestyles are unlikely to pose a biosecurity risk, in contrast to fungi such as *F. circinatum* that are pathogenic but can live endophytically within their hosts (Storer et al. 1998). Although host switching or the discovery of unrelated host species of pathogens does occur, for example the recent discovery that corn is a hosts of the pine pathogen *F. circinatum* (Swett and Gordon 2015), the frequency of such occurrences are low.

Fungal DNA was found in all tissue culture samples tested but no fungal microorganisms grew out of the tissue culture material plated on the general fungal media tested. The lack of fungal outgrowth was not surprising as most of the closely related GenBank taxa were listed as uncultured or were from DNA-based assessments of environmental samples. Others which are known to be readily culturable would have the potential to grow out on general fungal media. However, it is not known if these fungal microorganisms are capable of outgrowth onto general fungal media during the endophytic or latent phase of their lifecycle. The abundance of these fungal microorganisms in the tissue culture material is also likely to be very low based on the low number of filaments observed microscopically and the lack of amplification of fungal DNA using single primer sets. It is possible that the sample of tissue culture material tested did not contain culturable fungal microorganisms.

The results from this study show that standard tissue culture propagation media can severely limit the growth of some fungal and oomycete microorganisms. In view of these results, it cannot be assumed that all pathogens present in tissue culture material would be able grow out and be detectable. For the isolates that had severely restricted growth on some of the tissue propagation media, especially LPch, it is likely that the original agar plug of mycelium was able to support limited growth of the isolates. It is unknown whether these isolates would have been able to grow at all on these media in the absence of the original agar plug, such as would occur under normal tissue propagation conditions.

In general, the length of time that tissue culture material is grown on EDM6, Glitz or ½LP5 should be sufficient for any culturable fungi present to grow. Callus tissue derived from zygotic embryonic tissue is usually grown on EDM6 or Glitz for 10–14 days before it is sub-cultured and can be grown on these media for up to 12 months until plants are formed. For most of the fungi tested, and *Phytophthora cinnamomi*, a detectable colony should have grown within this timeframe as growth tended not to be as restricted on these media. Likewise for ½LP5, a medium used for between 7–21 days for shoot initiation, colonies should be detectable if left on this medium for longer than seven days. As a shoot multiplication medium which contains charcoal, LPch is generally used for between 28–42 days; growth on this medium would be dependent on the microorganisms present.

Conclusions

The exact risk of importation of asymptomatic fungal microorganisms in tissue culture material is unclear. This study indicates that fungi do occur on *Pinus radiata* tissue culture material and that these microorganisms are alive. However, there is no indication of long-term survival of these microorganisms, whether they would be functional (either positively or negatively) in the host plant or whether they could become established outside of their host in a new environment. For example, current scientific understanding of the symbiotic relationship between endophytic microorganisms and their hosts is poor so it is possible these microorganisms could be intricately involved in plant survival, and without some of them the plant may not survive. Likewise, the transmission of endophytes is poorly understood; fungal endophytes frequently do not sporulate in culture and observations of fruiting bodies under natural conditions are uncommon. Finally, the risk of these microorganisms becoming pathogenic in their host or host switching under new environmental conditions is expected to be low. Further research is required to confirm whether these microorganisms are alive in tissue culture material and to elucidate whether they pose a biosecurity risk.

Table 2 Average colony diameter for fungal and oomycete isolates grown on standard tissue propagation and general fungal or oomycete media

		Average colony diameter ± standard deviation on nutrient media (mm) [significant difference][a]							
Taxa	Day	MEA	Glitz	EDM6	½LP5	LPch	n	F	P
Amylostereum aerolatum	7	26.7 ± 2.2 a	21.0 ± 3.1 b	10.8 ± 1.0 c	10.0 ± 2.8 c	12.3 ± 1.0 c	26	54.7	<0.0001
	14	76.3 ± 2.0 a	67.3 ± 3.3 b	52.5 ± 2.9 c	33.3 ± 8.4 d	16.0 ± 0.8 e	26	142.4	<0.0001
	21	85.0 ± 0.0 a	83.5 ± 1.5 a	75.5 ± 2.6 b	52.2 ± 3.3 c	17.3 ± 1.0 d	26	874.3	<0.0001
	28	85.0 ± 0.0 a	85.0 ± 0.0 a	78.5 ± 3.7 b	54.3 ± 2.3 c	21.0 ± 2.9 d	26	787.1	<0.0001
	35	85.0 ± 0.0 a	85.0 ± 0.0 a	82.8 ± 1.0 a	54.8 ± 2.3 b	24.0 ± 2.6 c	26	1389.2	<0.0001
Colletotrichum acutatum	7	34.3 ± 1.6 c	42.5 ± 1.4 a	38.5 ± 2.7 b	40.3 ± 2.5 ab	17.2 ± 1.8 d	30	143.3	<0.0001
	14	68.0 ± 5.4 a	69.8 ± 3.8 a	64. 8 ± 4.3 a	69.8 ± 4.2 a	44.5 ± 3.1 b	30	38.8	<0.0001
	21	84.2 ± 2.0 a	84.2 ± 1.3 a	75.2 ± 6.7 b	74.5 ± 2.4 b	71.5 ± 6.1 b	30	11.0	<0.0001
	28	85.0 ± 0.0 a	85.0 ± 0.0 a	81.0 ± 3.1 b	85.0 ± 0.0 a	83.7 ± 1.2 a	30	8.2	0.0002
	35	85.0 ± 0.0	85.0 ± 0.0	84.0 ± 1.5	85.0 ± 0.0	85.0 ± 0.0	30	2.5	0.0681
Cyclaneusma minus	7	16.8 ± 0.8 a	9.3 ± 1.5 c	10.5 + 0.5 b	6.2 ± 0.4 d	6.3 ± 0.5 d	30	158.5	<0.0001
	14	32.5 ± 2.0 a	15.7 ± 2.7 c	18.7 ± 1.2 b	9.0 ± 1.3 d	6.2 ± 0.4 e	30	224.1	<0.0001
	21	49.5 ± 1.4 a	26.8 ± 3.8 b	25.3 ± 1.6 b	14.7 ± 2.1 c	6.3 ± 0.5 d	30	341.0	<0.0001
	28	59.8 ± 3.5 a	32.8 ± 3.4 b	32.3 ± 1.8 b	18.7 ± 2.4 c	6.5 ± 0.5 d	30	354.9	<0.0001
	35	65.7 ± 3.7 a	37.3 ± 3.6 b	37.7 ± 2.6 b	23.0 ± 2.6 c	6.8 ± 0.8 d	30	349.0	<0.0001
Dothistroma septosporum	7	8.0 ± 2.4	7.5 ± 1.5	9.2 ± 2.9	6.2 ± 0.8	6.5 ± 0.5	30	2.5	0.0674
	14	13.7 ± 1.8 a	10.3 ± 1.2 b	10.8 ± 1.2 b	8.3 ± 1.2 c	8.0 ± 0.9 c	30	19.1	<0.0001
	21	19.3 ± 2.1 a	13.5 ± 1.5 b	15.0 ± 2.4 b	13.0 ± 1.3 b	8.8 ± 1.0 c	28	24.6	<0.0001
	28	25.8 ± 0.5 a	15.5 ± 1.2 c	19.2 ± 2.0 b	15.5 ± 2.1 c	12.0 ± 1.4 d	28	48.5	<0.0001
	35	32.5 ± 1.7 a	18.8 ± 1.2 c	23.5 ± 2.3 b	19.8 ± 2.4 c	15.2 ± 1.3 d	28	57.2	<0.0001
Junghuhnia vincta	7	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	83.0 ± 3.6	81.8 ± 3.8	30	2.4	0.0787
	14	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	83.8 ± 2.9	30	1	0.4261
	21	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	85.0 ± 0.0	30	-	-
Phytophthora cinnamomi	7	82.0 ± 0.0 a	71.0 ± 6.9 b	74.3 ± 2.9 b	58.3 ± 2.7 c	16.5 ± 1.9 d	30	303.5	<0.0001
	14	85.0 ± 0.0 a	83.3 ± 2.0 ab	82.0 ± 1.9 b	85.0 ± 0.0 a	18.8 ± 2.5 c	30	1829.6	<0.0001
	21	85.0 ± 0.0 a	85.0 ± 0.0 a	84.5 ± 0.9 a	85.0 ± 0.0 a	19.8 ± 2.0 b	30	5215.9	<0.0001
	28	85.0 ± 0.0 a	85.0 ± 0.0 a	85.0 ± 0.0 a	85.0 ± 0.0 a	23.2 ± 3.9 b	30	1532.8	<0.0001
	35	85.0 ± 0.0 a	85.0 ± 0.0 a	85.0 ± 0.0 a	85.0 ± 0.0 a	40.0 ± 24.3 b	30	20.6	<0.0001

[a]Average colony diameters with the same letter were not significantly different; those with different letters were significantly different

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors have contributed substantially to this manuscript and were involved in planning, data analyses and manuscript writing. RG carried out the DNA and growth media experiments; CH prepared all tissue culture material; and LD carried out the microscopy experiments. All authors read and approved the final manuscript.

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