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Diversity of fungi associated with roots of *Eucalyptus gomphocephala* seedlings grown in soil from healthy and declining sites

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Abstract
We used molecular profiling with 454 pyrosequencing to identify ectomycorrhizal, arbuscular mycorrhizal fungi, and other fungal communities associated with containerized *Eucalyptus gomphocephala* seedling roots. We found a higher proportion of ectomycorrizal fungi associated with seedling roots grown in soil collected from sites with healthy trees, and of arbuscular mycorrhizal fungi from seedling roots grown in soil collected from sites with declining trees. We also found a relatively high proportion of pathogenic fungi present in roots from declining sites compared to healthy sites. This research should be extended to the field to further investigate mycorrhizal and pathogenic fungal populations associated with tree declines.

Introduction

The forests and woodlands of South-Western Australia are vulnerable to climate change (Evans et al. 2013; Laurence et al. 2011; Matusick et al. 2013; Ruthrof et al. 2015) and globally the Mediterranean biome is a high priority for conservation practices (Olson and Dinerstein 2002). *Eucalyptus gomphocephala* dominates a Mediterranean type forest that has experienced severe decline in recent decades (Scott et al. 2013). A range of factors potentially influencing the decline have been explored including soil fertility (Close et al. 2011), drought associated with climate change (Matusick et al. 2012), fire frequency and intensity (Archibald et al. 2010), soil bacteria (Cai et al. 2010), and root pathogenic fungi (Scott et al. 2012), but the primary cause of the decline remains enigmatic.

Ectomycorrizal fungi play an important role in ecosystem function and tree health (Sapsford et al. 2017) and might be related to *E. gomphocephala* health (Ishaq et al. 2013). Our previous bioassay studies found the type of mycorrhiza (arbuscular or ectomycorrhizal) formed in containerized seedlings was related to the canopy condition of *E. gomphocephala* at the sites where the soil cores were taken. Ectomycorrhizal colonization was high in seedlings grown in soil collected from under healthy canopies, and was positively related to the canopy condition of *E. gomphocephala* in the field, suggesting the relative importance of ECM for maintaining the health of *E. gomphocephala* in natural ecosystems (Ishaq et al. 2013). These conclusions, based on morphotyping and microscopic examination only, did not identify fungal taxa associated with seedling roots. In this paper, the roots were re-examined using 454 pyrosequencing to validate the previous findings. This technology has been widely used to analyse fungal diversity including mycorrhizal fungi (Öpik et al. 2009; Blaalid et al. 2012; Kauserud et al. 2012).

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Materials and methods

Root samples

The *E. gomphocephala* roots (96 samples) used for this study were obtained from the previous study (Ishaq et al. 2013). Briefly, *E. gomphocephala* seedlings were grown under

controlled glasshouse conditions for 7 months in soils collected from 12 sites (GPS coordinates are given in Cai et al. 2010), where the health of trees ranged from healthy, to moderately healthy and declining. At each site, four trees were randomly selected, and two intact soil cores each from the north and south were collected 5 m from the trunk of each tree. For this study, a mixture of root fragments and root tips were randomly collected from samples that had been preserved in 100% EtOH at 4 °C for 6 months. The samples were transferred into a 1.5 ml eppendorf tube, allowed to dry in a laminar flow overnight, freeze dried for 3 days (Hetosicc CD 4), and then kept at −80 °C before DNA extraction (Griffin et al. 2002).

Molecular identification

The roots were manually ground by dipping the 1.5 ml eppendorf tube containing root samples into liquid nitrogen until frozen before grinding. The DNA was extracted according to the manufacturer's instruction manual of ZR Fungal/Bacterial DNA MiniPrep™ Catalog No. D6005. The pure DNA, both as full DNA and diluted DNA (1:10 dilution) (Williams et al. 2001), was sent to Australian Genome Research Facility Ltd. (AGRF) Perth-Western Australia (www.agrf.org.au) for 454 sequencing analysis.

The genomic DNA samples were amplified separately in a two step PCR using the fungal primer pair ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3′) to generate PCR ITS rRNA fragments of c. 400 base pairs. In the second round of PCR, the primers were modified to include adaptors for pyrosequencing with ITS1F primer individually barcoded for each sample. The sequence was run on a Genome Sequencer FLX 454 System. After the sequencing run, results were run through Roche's standard amplicon analysis pipeline. This is a very stringent pipeline which results in only the long high quality reads remaining. Demultiplexing of the results based on the barcode assigned to a sample was performed. Each sample underwent the bioinformatic processing for the diversity profiling report, and ITS samples were processed under the UNITE database (http://greengenes.secondgenome.com/). The composition of species diversity in high-throughput amplicon sequencing data was carried out using the Quantitative Insights Into Microbial Ecology (QIIME) software package version (http://qiime.org).

For the calculation of fungal distribution, only identified operational taxonomic units (OTU's) were considered. The calculation was made at phyla and genus levels, except when only mycorrhizal fungi were considered; for these the calculation was at species level and other fungi were not included. Proportional distribution of fungal OTU's of each sample was calculated, and then averaged. At the genus level, all the fungi which might be ecologically important and related to the health of *E. gomphocephala* were included in the calculation. The fungi were grouped into pathogens (leaf, stem, and root pathogens), saprophytes, endophytes, ECM and AMF. Some considerations were taken into account when grouping the fungi. For instance, putative pathogens such as *Botryosphaeria*, *Mycosphaerella* and *Teratosphaeria*, and genera of major fungal plant pathogens such as *Alternaria*, *Cytospora*, and *Phaeosphaeriopsis* were grouped as pathogens. Fungal genera known to cause root rot were also grouped as pathogens. Basidiomycota which were neither mycorrhizal nor root rot fungi were grouped as saprophytes. The endophyte group was considered as neutral endophytic fungi. Pathogenic endophytic fungi, such as the genus *Diaporthe*, were grouped with pathogens.

Statistical analyses

Calculation of proportional distribution of fungal OTU's was performed using Excel Program. To determine the correlation between the richness of ECM/AM fungi OTU's and crown health, Pearson's correlation coefficient was employed. In the calculation, richness is defined as the OTU's at the species level found at the 12 sites. The analyses were conducted using SPSS for Windows version 17.0 (SPSS).

Results

Fungal community

The DNA sequence analysis detected six fungal Phyla associated with the seedling roots, namely Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota, Blastocladiomycota and Zygomycota. The Ascomycota contributed 85.9% of the total DNA sequences detected in the roots from 12 sites, whilst Basidiomycota and Glomeromycota contributed 11 and 2.1%, respectively. The Chytridiomycota contributed 0.9% whereas the Blastocladiomycota and Zygomycota contributed less than 0.1% each.

Related to the canopy condition of *E. gomphocephala* in the field, the highest proportion of the Ascomycota was found for seedling roots grown in soil collected from the moderately healthy sites (90.6%) compared with seedling roots grown in soil collected from the healthy (85.4%) and declining sites (84.4%). The phylum Basidiomycota was mostly detected from seedling roots in soil collected from the healthy sites (13.5%), and a smaller proportion was found for seedling roots grown in soil collected from the moderately healthy (9.23%) and declining sites (8.21%). A higher proportion of the Glomeromycota was identified for roots in soil from the declining sites (5.3%). On the other hand, only 0.65% and

0.11% of this phylum were detected for the seedling roots grown in soil collected from the healthy and moderately healthy sites, respectively.

At the genus level, there were some interesting trends in fungal distribution particularly regarding saprophytic, pathogenic and mycorrhizal fungi between the sites (Supplementary Table 1). The proportion of saprophytes was higher for the seedling roots grown in soil collected from the moderately healthy and the healthy sites, contributing 83.2 and 62.9%, respectively, compared with those seedling roots grown in soil collected from the declining sites (44.8%). On the other hand, the pathogenic fungi were more prevalent with seedlings roots grown in soil collected from the declining sites contributing 36.7% compared to those associated with seedling roots grown in soil collected from the moderately healthy sites (12.7%) and the healthy sites (25.5%). The proportion of ECM to AM fungi was high for seedling roots grown in soil collected from the healthy sites whilst the proportion of AM to ECM fungi was high for seedling roots grown in soil collected from the declining sites. The proportion of endophytes was relatively similar in the seedlings roots grown in soil collected from the healthy and moderately healthy sites, but higher in the seedling roots grown in soil collected from the declining sites (Fig. 1a, b and c).

ECM/AM communities

The DNA sequence analysis identified six ECM families associated with seedlings roots, namely Cortinariaceae, Rhizopogonaceae, Sclerodermataceae, Sebacinaceae, Thelephoraceae, and Tuberaceae. All six families were found

for the seedlings roots in soil collected from the healthy sites, whereas only two families (Cortinariaceae and Tuberaceae) were detected from roots in soil from the moderately healthy sites, and four families (Sclerodermataceae, Sebacinaceae, Thelephoraceae, and Tuberaceae) from declining sites. At the species level, not many ECM fungal communities associated with seedling roots were detected by molecular analysis, and only fourteen ECM fungi OTU's in total were found (Table 1). Pearson's correlation analysis revealed that the number of ECM fungi OTU's present at the species level was not significantly correlated with canopy condition of *E. gomphocephala* in the field; however, there was a trend for more ECM fungi OTU's for seedlings roots grown in soil collected from the healthy sites than in the declining sites. Twelve of the 14 ECM fungi OTU's were associated with seedling roots grown in soil collected from the healthy sites, whilst only two and eight ECM fungi OTU's were found for seedling roots grown in soil collected from the moderately healthy and declining sites, respectively.

There were four AM families associated with the roots. These were Diversisporaceae, Claroideoglomeraceae, Glomeraceae and unidentified Glomeraceae. All four AM families were found for seedling roots grown in soil collected from the declining sites, whereas only two AM families (Diversisporaceae and Glomeraceae) were identified from seedlings grown in soil collected from the healthy and one family (Diversisporaceae) from moderately healthy sites. At the species level, a total of 13 AM fungi OTU's were found in the roots grown in soil collected from the 12 sites (Table 1). *Glomus* was the dominant AM genus present in roots followed by *Rhizophagus*. Pearson's correlation analysis revealed that

Fig. 1 Proportional distribution of fungal groups at the genus level colonizing the seedling roots grown in soil collected from; **a** healthy, **b** moderately healthy, and **c** declining sites
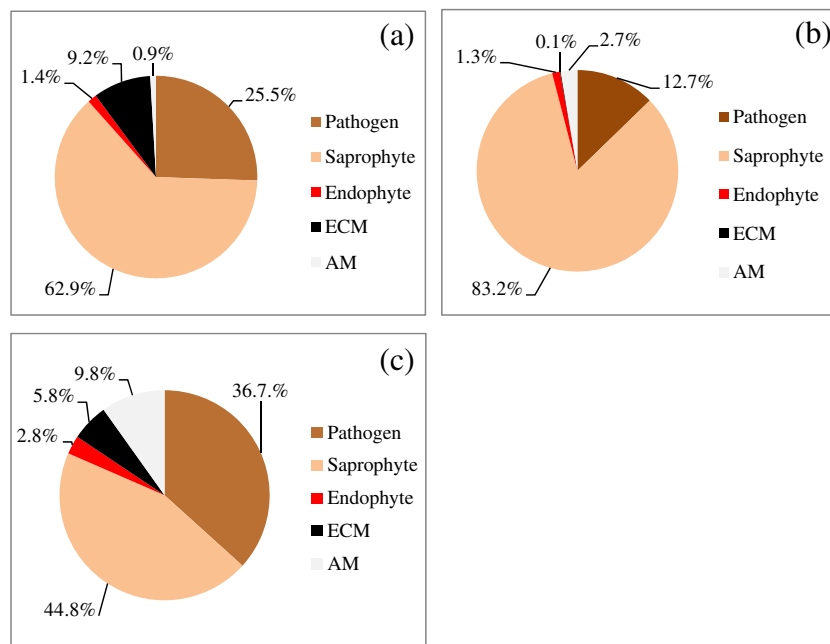
Table 1 Richness of ECM/AM fungi associated with seedling roots grown in soil collected from healthy (H), moderately healthy (M) and declining (D) sites at the species level

	Canopy condition			Note
	H	M	D	
Putative ECM fungal species				
Astraeus sp.	√	–	–	Known to form ectomycorrizas on *Eucalyptus* species in Australia
Rhizopogon luteolus	√	–	–	Commonly associated with *Pinus* spp. in Western Australia, and has been reported to form only surface colonization with a loose mantle in associations with eucalypts
Scleroderma areolatum	–	–	√	Common in *Eucalyptus* forest in Australia
Sebacina sp. AA39	√	–	√	*Sebacinaceae* known to form mycorrhiza with Australian orchids, but some species could form ECM on *E. marginata* (Glen et al. 2002)
Sebacina vermifera	√	√	√	
Tuber indicum	√	–	–	Not reported for Australia but has been imported as food
Uncultured *Hebeloma*	√	–	–	*Hebeloma* spp. are common in *Eucalyptus* forests in Australia. *H. lacteocoffeatum* has wide distribution from Western Australia to New South Wales (Rees et al. 2013).
Uncultured *Sebacina* 1	√	–	√	
Uncultured *Sebacina* 2	√	–	–	
Uncultured *Sebacina* 3	√	–	√	
Uncultured *Sebacina* 4	–	–	√	
Uncultured Thelephoraceae	√	–	√	Thelephoraceae family known to form ECM in *Eucalypt* forest in Australia
Uncultured *Tomentella*	√	–	–	*Tomentella* spp. are common in *Eucalyptus* forests in Australia
Uncultured *Tuber*	√	√	√	
Richness	12	2	8	
Putative AM fungal species				
Claroideoglomus sp.	–	–	√	
Diversispora celata	√	–	–	
Diversispora sp. W5257	–	–	√	
Funneliformis mosseae	–	–	√	
Glomus dimorphicum	–	–	√	*Glomus* genera are common in Australian native plant communities, also known to form association with *Eucalyptus*.
Glomus macrocarpum	√	–	–	
Glomus sp. 8 SUN 2011	√	–	–	
Rhizophagus intraradices	–	√	√	
Rhizophagus irregularis	√	√	√	Can form association with *E. marginata* seedlings (Kariman et al. 2012).
Rhizophagus sp.	–	–	√	
Uncultured Glomeraceae	–	–	√	
Uncultured *Glomus*	√	√	√	
Uncultured Glomeromycota	–	–	√	
Richness	5	3	10	

the number of AM fungal OTU's was significantly (r = −0.41) negatively correlated to crown health of *E. gomphocephala*. Ten AM fungi OTU's out of 13 were found for seedling roots grown in soil collected from the declining sites compared with three OTU's for roots in soil collected from moderately healthy and five OTU's from healthy sites.

Proportion of ECM/AM fungi within the roots

For the calculation of the proportional distribution of mycorrhizal fungi associated with the seedling roots, only the ECM and AM fungi were included. Ectomycorrhizal fungi contributed 75% of the total proportion of mycorrhizal fungi

colonizing the seedling roots grown in soil collected from the healthy sites, whilst the AM fungi only contributed 25%. On the other hand, seedling roots grown in soil collected from the declining sites were highly colonized by AM fungi. The AM fungi contributed 52.1% of the total proportion, whilst the ECM fungi were 47.9%. A high proportion of ECM fungi (66.7%) was found for mycorrhizal fungi colonizing the seedling roots grown in soil collected from the moderately healthy sites, whilst the AM proportion was low (33.3%) (Fig. 2).

Discussion

Our molecular findings indicate the dominance of ECM fungi for the seedling roots grown in soil collected from the healthy sites, and vice versa for the AM fungi for the seedling roots grown in soil collected from the declining sites. This finding validated our previous study where the mycorrhizal assessment was based on microscopic examination (Ishaq et al. 2013). That study suggested that the gross type of mycorrhiza formed in the seedlings could predict *E. gomphocephala* health in the field where the soil cores were taken. In particular, ECM colonization was high in seedling roots grown in soil collected from healthy sites whilst AM colonization was high in seedling roots grown in soil collected from declining sites.

Variable results have been reported regarding the relationship between ECM and stand/forest health. For instance, the proportions of ECM colonization, diversity or both, were higher in healthy trees than declining trees (Causin et al. 1996; Swaty et al. 2004; Horton et al. 2013). However, it is also reported that the state of tree health may not impact on

ECM fungi (Perrin and Estivalet 1989; Peter et al. 2008; Lancellotti and Francheschini 2013). Montecchio et al. (2004) found a higher number of ECM morphotypes associated with declining trees than asymptomatic trees, and hypothesized that some ECM morphotypes might be essential for maintaining the stability of the modified ecosystem processes, but less efficient in their mutualistic manner. Peter et al. (2008) observed Norway spruce decline due to air pollution was related to ECM, and reported the ECM colonization was high, but ECM species richness decreased in heavily polluted sites. Conversely, Swaty et al. (2004) reported both reduced ECM colonization and diversity in high mortality pinyon pine (*Pinus edulis*) sites.

Our results indicate ECM might be important for the healthy functioning of *E. gomphocephala*. Eucalypts are well known to form mycorrhizal associations (Lapeyrie and Chilvers 1985), and may benefit from this association in their natural environment especially where abiotic factors such as soil conditions or climatic factors are unfavorable for growth. The soils in the study region are generally of low fertility (Moore 1998), and increased temperature and reduced precipitation due to climate change has been an issue in Western Australia (Bates et al. 2008). The loss of ECM, therefore could have impacted on health of *E. gomphocephala* in its natural stands.

Our molecular study found a higher proportion of ECM fungi associated with seedling roots grown in soil collected from woodland with healthy canopies suggesting the possible involvement of ECM on *E. gomphocephala* health. Since our study was based on a glasshouse assay where environmental conditions within the pots and glasshouse differed from the

Fig. 2 Proportional distribution of mycorrhizal fungi colonizing the seedling roots grown in soil collected from; **a** healthy, **b** moderately healthy, and **c** declining sites
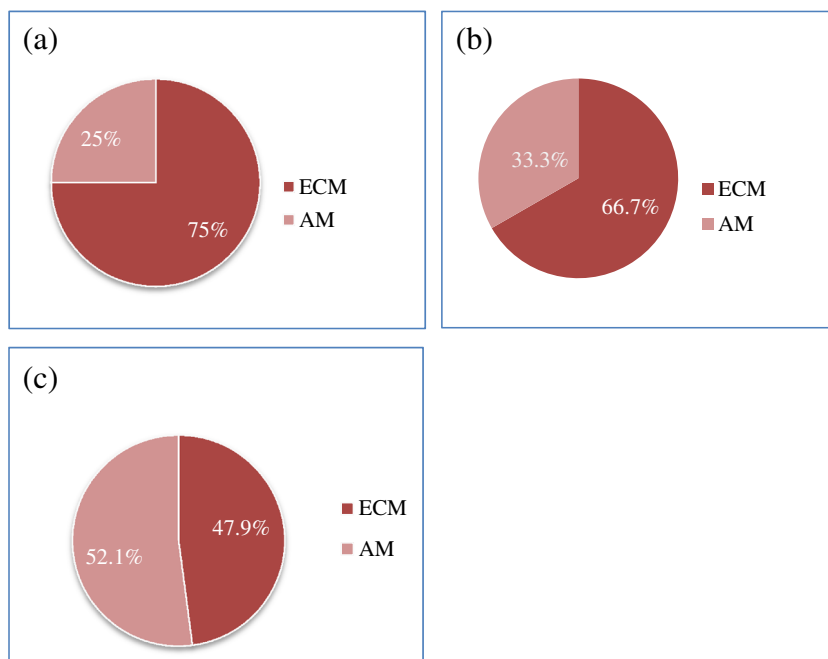
natural forest, it is possible these conditions could have influenced fungal diversity. Future studies on ECM colonization and communities should be extended to the field. Furthermore, as spatial diversity in time and place could be an issue in exploring ECM communities and diversity, repeated samplings for a longer period of time would be preferable in order to get a more representative understanding of actual ECM communities and diversity in the field.

Other microbes were also evaluated in this study including saprophytes and pathogens. The diversity of saprophytic fungi was high for seedling roots grown in soil collected from the healthy and moderately healthy sites, but low for seedling roots grown in soil collected from the declining sites. Whether the lower proportion of saprophytes in the declining sites is related to *E. gomphocephala* health at these sites is not known.

Some saprophytes might be ecologically important for organic matter decomposition, contributing to nutrient release into the soil. Recently, it has been reported that a decrease in utilization of carbohydrates, carboxylic acid, amino acids and amines by the soil bacteria community was correlated to poor *E. gomphocephala* crown health indicating a strong relationship between the bacterial community and *E. gomphocephala* decline (Cai et al. 2010).

The proportion of pathogenic (leaf, stem and root pathogens) fungi was higher for seedling roots grown in soil collected from the declining sites compared to seedling roots grown in soil collected from the moderately healthy and healthy sites. As the soils used to grow the seedlings were collected from the field, these pathogens could either have been introduced with the soil, leaf litter in the soil or some may have been endophytes within the seed used for the trial. Furthermore, some of these organisms such as *Botryosphaeria*, *Mycosphaerella* and *Teratosphaeria*, may be putative pathogens in *E. gomphocephala* and other native woody plants such as *Acacia cochlearis*, *A. rostellifera*, *Allocasuarina fraseriana*, *Agonis flexuosa*, *Banksia grandis* and *E. marginata* that grow in association with *E. gomphocephala* (Taylor et al. 2009; Hunter et al. 2011). Whether the pathogen groups found in this study behave as opportunistic fungi or they are primary pathogens on *E. gomphocephala* remains to be determined. However, it is widely accepted that some opportunistic fungi may become pathogenic when the environmental conditions are unfavourable and trees are under stress. It is possible that there is a higher diversity and inoculum load of putative pathogens in soil from declining sites and this might be a contributing factor to the declining health of trees at these sites. Spores of these species could have been brought into these sites by wind, rain, air currents or other factors. It has been suggested that fungal spore dispersal by the wind can spread plant disease over long distances (Brown and Hovmeller 2002), and global movement/gene flow of some

pathogens causing canker and leaf spot disorders in eucalypts has been detected (Burgess et al. 2008; Hunter et al. 2008). More recently, the soil-borne plant pathogen *Phytophthora multivora* has been found to be associated with declining *E. gomphocepahala* in Yalgorup near the study site (Scott et al. 2012). However, the presence of this pathogen could not be detected by DNA sequence analysis in the present study, as the primers used are designed for true fungi only, consequently it is not surprising *Phytophthora* species or other oomycetes were not observed.

The weak relationship between ECM fungal communities and crown health of *E. gomphocephala* might be due to a relatively small number of ECM fungal communities detected by DNA sequence analysis. A key ECM family, the Russulaceae, and some key genera such as *Pisolithus*, *Russula*, *Cortinarius*, *Laccaria* and *Cenococcum* are missing from the molecular analysis. Indeed, some of the mat-forming ECM fungi like *Hysterangium* observed in the pot trial were not detected in the molecular analysis. Recently, Horton et al. (2013) investigated the relationship between ECM fungi communities, soil chemistry and *E. delegatensis* decline in Tasmania. Using ECM root tips coupled with sporocarps collected from the field to extract DNA for molecular analysis, the authors were able to detect many important families and key genera, and successfully showed a significant relationship between reduced ECM richness and declining tree health. The smaller number of ECM taxa detected in the current study compared to findings by Horton et al. (2013) could be due to differences in ecosystems between the two regions such as climate and soil. *Eucalyptus delegatensis* is a high-altitude, high-rainfall, lower temperature species with a closed canopy and rich organic soil, whereas *E. gomphocephala* forest is much lower in altitude, hotter and drier with sandy soil. However, there are other possibilities that might be relevant in explaining the smaller number of ECM taxa detected by molecular analysis in the current study. For instance, it is not known whether the plants only trapped a limited number of ECM fungi in the soil, or whether some fungi did not persist under glasshouse conditions. Besides, it might be also possible that the absence of key ECM in the sequences is due to the age of the seedlings being sampled. Generally, "late stage fungi" such as Russulaceae members tend to associate with more mature trees in undisturbed sites, thus it is less likely that seedlings or young trees could d pick up these types of fungi. Alternatively, the absence of key genera such as Russula and Cortinarius in the sequences might be also due to the sensitivity of the fungi to disturbance. For instance, in a *Phytophthora* die back-induced tree decline, Anderson et al. (2010) showed that fungal diversity and "the late stage fungi" were generally firstly impacted.

As mentioned earlier, not all morphotypes observed were detected in the molecular study. For example, *Cenococcum* with its distinctive black, hairy ECMs was observed

microscopically, but was not detected by molecular analysis. Similarly, distinctive spores of the AM genera including *Gigaspora* and *Acaulospora* were observed, but were missing from the molecular analysis. Kauserud et al. (2012) compared 454 sequencing and the Sanger sequencing approach for their ability to characterize fungal communities of the ECM plant *Bistorta vivipora*, and reported that 454 performed poorly in detecting *Cenococcum* and Thelephoraceae, although the method had a higher affinity to detect Glomales than the cloning/Sanger approach. A similar study by Tedersoo et al. (2010) reported that several taxa were not captured by Cloning/Sanger and some taxa were poorly detected using 454. Sakakibara et al. (2002) on the other hand suggested that morphological techniques in conjunction with the use of molecular techniques may provide more powerful information on mycorrhizal communities.

Surprisingly, *Tuber indicum* was detected by molecular analysis. Apart from introduced *Tuber* species into commercial tuber farms, as close as 100 km from the study site, as yet there is no record of a true *Tuber* species in Australia. *Tuber indicum* is known to be native to Asia but it is widely dispersed as food around the world including Melbourne (Australia) (Wang et al. 2006; Bonito et al. 2011). The fact that *Rhizopogon* was detected by molecular analysis was quite interesting. *Rhizopogon* is usually associated with conifers such as *Pinus* species, and only formed surface colonization with a loose mantle in associations with eucalypts (Malajczuk et al. 1994). Introduced *Rhizopogon* is one of the ECM fungi commonly associated with *Pinus* in Western Australia (Dunstan et al. 1998) and is used commercially in pine nurseries in the region. Since there are *Pinus* plantations near the Yalgorup National Park, it is possible that the *Rhizopogon* spores could have been blown into this area.

When only ECM and AM fungi were considered in the calculation of proportional fungal distribution in the roots, the ECM fungi were quite high in the seedling roots grown in soil collected from healthy and moderately healthy sites, but low in the seedlings grown in soil collected from declining sites. Our results were consistent with the study of Scott et al. (2013), who investigated the relationship between crown health, fine roots and ECM density of declining and healthy *E. gomphocephala* in the Yalgorup region, and found that trees with crown decline symptoms had fewer fine roots and ECM density than healthy trees, suggesting decline of *E. gomphocepahala* is associated with absence of ECM. Similarly, it was previously reported that diversity and abundance of ECM in the jarrah (*Eucalyptus marginata*) forest in southwestern Australia were greatly reduced in die-back affected areas (Anderson et al. 2010).

We cannot determine whether the reduced ECM colonization and diversity are the drivers of the state of *E. gomphocephala* health, or vice versa, however, given the variety of services individual ECM fungi may provide to the host plants (Smith and Read 2008), and the variety of their response to environmental variables (Cairney 1999), a decrease in ECM colonization and diversity may lead to a corresponding loss of some ecosystem functions. Since this finding was based on glasshouse conditions, future studies need to be extended to the field by investigating colonization patterns in seedlings and also the molecular analysis of mycorrhizal fungi populations in sites with a range of canopy conditions. Furthermore, other soil fungal communities associated with *E. gomphocephala* such as saprophytes, pathogens and endophytes need to be considered. Indeed, the presence of *Phytophthora multivora* has been recently related to *E. gomphocephala* decline (Scott et al. 2012). An approach for managing the health of *E. gomphocephala* can be gained from these studies.

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