Seedling mycorrhizal type and soil chemistry are related to canopy condition of *Eucalyptus gomphocephala*

Lily Ishaq[1], Paul A. Barber[1,2], Giles E. St. J. Hardy[1], Michael Calver[1], Bernard Dell[1]

1.Centre of Excellence for Climate Change, Woodland and Forest HealthMurdoch UniversityPerthAustralia

2.Arbor Carbon Pty LtdWillage CentralAustralia

Abstract

The health of *Eucalyptus gomphocephala* is declining within its natural range in south-western Australia. In a pilot study to assess whether changes in mycorrhizal fungi and soil chemistry might be associated with *E. gomphocephala* decline, we set up a containerized bioassay experiment with *E. gomphocephala* as the trap plant using intact soil cores collected from 12 sites with *E. gomphocephala* canopy condition ranging from healthy to declining. Adjacent soil samples were collected for chemical analysis. The type of mycorrhiza (arbuscular or ectomycorrhizal) formed in containerized seedlings predicted the canopy condition of *E. gomphocephala* at the sites where the cores were taken. Ectomycorrhizal fungi colonization was higher in seedling roots in soil taken from sites with healthy canopies, whereas colonization by arbuscular mycorrhizal fungi dominated in roots in soil taken from sites with declining canopies. Furthermore, several soil chemical properties predicted canopy condition and the type of mycorrhizal fungi colonizing roots. These preliminary findings suggest that large-scale studies should be undertaken in the field to quantify those ectomycorrhiza (ECM) fungi sensitive to *E. gomphocephala* canopy decline and whether particular ECM fungi are bioindicators of ecosystem health.

Keywords: Tree decline; Soil nutrients; Ectomycorrhizae; Canopy health

Introduction

Globally, the health of many forest and woodlands is in decline. The causes of some of these declines are understood, for example, soil acidification leading to depletion of soil calcium is associated with *Acer saccharum* (sugar maple) decline in the USA and Canada (Horsley et al. 2000; St. Clair et al. 2005; Kogelmann and Sharpe 2006), and drought and heat associated with global climate change have been related to mortality and tree decline worldwide (Allen et al. 2010). However, other declines are manifested without known causes including many eucalypt declines in Australia (Jurkis 2005; Robinson 2008), most of which are poorly understood. One of these is *Eucalyptus gomphocephala* (tuart), a woodland tree endemic to the Swan Coastal Plain of Western Australia (Elridge et al. 1994), that has experienced a marked reduction in health and vitality (known as tuart decline) (Archibald et al. 2010; Cai et al. 2010) locally reaching up to 90 % mortality across all age classes (Tuart Response Group 2002). The cause is unknown, and several physical and biological factors might be involved (Close et al. 2009; Archibald et al. 2010) including soil bacteria and fungi (Scott et al. 2009; Cai et al. 2010; Scott et al. 2011). In particular, mycorrhizal fungi may be important in the health of *E. gomphocephala*. Although eucalypts can form both arbuscular mycorrhiza (AM) and ectomycorrhiza (ECM) (Lapeyrie and Chilvers 1985; Brundrett et al. 1996; Chen et al. 2000a), the latter generally dominate in Australian eucalypt forests. The importance of mycorrhizal fungi for tree physiology and ecosystem function is well established (Read and Perez-Moreno 2003; Smith and Read 2008; Plassard and Dell 2010).

Previous studies in Europe have identified significant changes in tree root systems, their mycorrhizal status, and mycorrhizal communities of declining trees compared to healthy trees such as in *Quercus* (Causin et al. 1996; Montecchio et al. 2004), *Fagus* (Power and Ashmore 1996), and *Picea* species (Peter et al. 2008). Reduction in tree vigor might impact on mycorrhizal fungi either directly through changes in resource availability and distribution of mycorrhizas or indirectly through changes in carbon allocation to roots and changes in plant species distribution (Bellgard and Williams 2011). Swaty et al. (2004) suggested that changes in mycorrhizal colonization might be

related to the stress level of the host plant. Furthermore, due to their quick response to environmental change, mycorrhizal fungi could be an important bioindicator of forest health (Cudlin et al. 2007).

As almost nothing is known about the relative importance of mycorrhizal fungi for the health of *E. gomphocephala* ecosystems nor their capacity to respond to perturbations, this pilot study aims to investigate the putative relationships between the canopy condition of *E. gomphocephala,* soil chemistry, and the type of mycorrhizal (AM or ECM) association. If the pilot study suggests that mycorrhizal fungi may associate with the canopy condition of *E. gomphocephala*, larger studies to resolve the question definitively are warranted. This may allow new approaches to manage the decline. For example, applying calcium improved the health status of sugar maple (Hugget et al. 2007) and mycorrhizal colonization (Juice et al. 2006). A bioassay approach was adopted using *E. gomphocephala* seedlings to probe for compatible mycorrhizal fungi in soil cores from 12 field study sites representative of tuart decline.

Materials and methods

Study area

The study area was in Yalgorup National Park (YNP), located between Mandurah and Bunbury on the western edge of the Swan Coastal Plain, south-western Australia. *E. gomphocephala* is the dominant tree species and has been listed as category II (National Park; protected area managed mainly for ecosystem conservation and recreation) by the International Union for the Conservation of Nature. The average annual rainfall in the last decade from the nearest weather station (Mandurah, station No. 009977) is 660.8 mm, of which around 76 % fell between May and September. The average annual maximum and minimum temperatures based on monthly mean temperatures are 23.1 and 14.6 °C, respectively (Bureau of Meteorology 2012). The soils are predominantly calcareous sands underlain by limestone which occasionally outcrops near ridges (Portlock et al. 1993).

The study area had 12 sites with different levels of *E. gomphocephala* decline ranging from healthy trees to trees with advanced canopy decline. These 12 sites are part of a long-term monitoring study and had previously been selected according to their vegetation trend class over a 15-year period (1990–2005) derived from Landsat Thematic Mapper satellite data (Cacceta et al. 2000; Cai et al. 2010). Most of the study sites are comprised of *E. gomphocephala* as overstory except sites 10 and 12 which consist of mixed eucalypt species (*E. gomphocephala*, *Eucalyptus marginata*, and *Corymbia calophylla*). The understory of most sites (1–9 and 11) consists of *Agonis flexuosa*, *Melaleuca acerosa* (Myrtaceae); *Allocasuarina fraseriana* (Casuarinaceae); *Banksia attenuata*, *Banksia grandis*, *Banksia littoralis* (Proteaceae); *Acacia pulchella*, *Acacia saligna*, *Jacksonia sternbergiana* (Fabaceae), and *Hibbertia hypericoides* (Dilleniaceace). Sites 10 and 12 contained *B. attenuata*, *A. flexuosa,* and *A. fraseriana* as understory (Portlock et al. 1993; Keighery 2002).

The diameter at breast height of *E. gomphocephala* ranges from approximately 30 cm to well in excess of 100 cm. We estimate the age ranges from less than 40 to over 150 years. The study sites have not experienced intense wildfire or prescribed burning for many decades.

Tree selection and crown health measurement

In each site, four individual *E. gomphocephala* trees were randomly selected and located by a global positioning system and assessed for crown health. Crown health indices were measured and included crown density and foliage transparency using the methods described by USDA (2005), crown dieback ratio, and epicormic index (growth from dormant bud under bark) using the methods described by Kile et al. (1981) and Wardlaw (1989). These measures have been reviewed by Stone and Haywood (2006) and recommended as measurable or semiquantitative parameters suitable for use as a generic index of eucalypt crown condition. All four indices were combined to by Evans et al. (2012a) using the following equation: TCHI=($C + F + D + I$) / 4 where C=crown density, F=100−foliage transparency, D=100−crown dieback ratio, and I=100−epicormic index. The semiquantitative measure TCHI was used by Evans et al. (2012b), in combination with spectral and textural remotely-sensed metrics, to develop a model sensitive enough to accurately predict small variations in crown health

of *E. gomphocephala* in the same sites used for the present study. The TCHI measure was therefore utilized in the present study as a reliable positively correlated measure of variation in crown health of *E. gomphocephala*.

Field soil collection

Undisturbed intact soil samples were collected for the bioassay trial using a soil corer (20-cm deep and 12-cm diameter) at locations 5 m north and south from each *E. gomphocephala* trunk in late summer (February). This method was based on previous studies that assayed mycorrhizal propagules in soils from a wide range of habitats in the region (Jasper et al. 1991; Brundrett and Abbott 1994; Brundrett et al. 1995). Each soil core was placed into a labeled polyurethane pot lined with a polyethylene bag and transferred to a glasshouse. To check for any contaminating mycorrhizal fungi in the bioassay, four intact soils were collected from around four healthy trees (site 3), then autoclaved for two consecutive days for 30 mins at 121 °C and used as a control in the glasshouse. For arbuscular mycorrhizal fungi (AMF) spore density assessment and soil chemical analysis, another soil sample, approximately 500 g, was taken adjacent to each of the above cores to a depth of 20 cm using a 4-cm diameter soil corer.

Glasshouse bioassay

The bioassay experiment was set up as a single-factor randomized complete block design. The factor was the site, and 12 levels of sites, each with eight replicates, were tested within each site. Seeds of *E. gomphocephala* were obtained from more than 20 healthy trees selected at random in YNP. Seeds were surface-sterilized with 70 % ethanol (5 s) and 3 % sodium hypochlorite (5 mins) prior to germination. The surface sterilization was confirmed by incubating the surface-sterilized seed in Petri dishes containing 0.75 % (*w*/*v*) water agar and checking for contamination. Seeds were germinated in trays containing autoclaved sand.

After 10 days, three germinated seedlings of uniform size (ea. 1 cm high) were transferred into each pot. The pots were maintained on open benches in an evaporatively cooled glasshouse with mean temperatures 25.8 °C/9.2 °C (maximum/minimum) and natural sunlight of 600–800 μmol m^{-2} s^{-1}.

Seedlings were watered overhead daily until container capacity. Holes were made into the base of each pot to allow drainage. A complete liquid fertilizer of low nutrient content was applied every 2 weeks for the second month and weekly thereafter. The composition of fertilizer used was: 500 μM N, 10.9 μM P, 214.8 μM K, 55 μM Ca, 19.5 μM Mg, 81.8 μM S, 0.63 μM Fe, 0.3 μM Mn, 0.6 μM B, 0.1 μM Zn, 0.05 μM Cu, and 0.02 μM Mo. The fertilizer regime was based on that developed for mycorrhizal eucalypts in forest nurseries (Dell and Malajczuk 1995). Seedlings were thinned to two plants per pot when they were 2 months old. When seedlings were 6 months old, two soil cores were taken midway between the two seedlings in each pot using a 2-cm diameter and 20-cm deep corer for spore density assessment.

Seedlings were harvested when they were 7 months old. The shoots were cut at the soil surface, and dried at 65 °C to constant weight. Roots were washed from the soil over a 5-mm screen with tap water, immersed for a few mins in a bucket of water, and then agitated to remove most of the organic matter and soil particles. The roots were washed again with tap water over a 2-mm screen. The fine roots were manually separated from the coarse roots over a 1-mm sieve, and any soil particles and dead roots were manually removed under a dissecting microscope. Fresh ECM root tips were fixed in 3 % glutaraldehyde in 0.025 M phosphate buffer at pH 7 for anatomical verification of ECM structures (Hartig net, fungal mantle). The bulk of the fine roots were blotted dry with paper towel, the fresh weight recorded, the roots mixed well and then divided into portions based on fresh weight. Approximately 20 % were fixed in 50 % ethanol (ETOH) for mycorrhizal assessment, and the remainder was used for root dry weight determination.

Mycorrhizal assessment

Mycorrhizal root assessment was based on the protocols described by Brundrett et al. (1996). Briefly, ETOH-fixed root samples (1–2 g) were cleared in 10 % (*w*/*v*) KOH in an autoclave for 15 mins at 121 °C, strained over 160 μm nylon mesh, rinsed with water, and stained with 0.05 % (*w*/*v*) trypan blue (CI 23850) in lactoglycerol (1:1:1 lactic acid, glycerol, and water) overnight. The roots were destained with lactoglycerol to remove excess dye.

The proportion of ECM root tips was determined under a stereomicroscope (Zeiss Stemi SV 11) using 50–100 root tips per sample. The presence of arbuscules and/or vesicles within the root was used to determine AM colonization in 25–30 cm of root length for each sample with the gridline intersect method. The presence of arbuscules was also confirmed using a compound microscope (Olympus BX 51) at ×100 and/or ×200 magnification.

AMF spore density

Spores of AMF were extracted from 50-g soil subsamples using the wet-sieving and sucrose methods (Tommerup 1988; Brundertt et al. 1996). Roots and coarse debris were collected on a coarse screen (700 μm), while spores were captured on finer screens (160 and 45 μm). Total AMF spore abundance was determined under a stereomicroscope. For the field soil samples, spore density for each site was an average of eight replicates (4 trees ×2 sampling positions).

Soil analysis

Soil samples (four samples from each site) were sent to a commercial laboratory (CSBP Soil and Plant Analysis Laboratory, Bibra Lake-Perth) for chemical soil properties. The pH was determined in H_20 and $CaCl_2$, NH_4-N using KCl extractant, organic C by the Walkley-Black method, S using the KCl-40 method, and P and K using the Colwell method. Micronutrients (Fe, Cu, Zn, and Mn) were extracted with DTPA, exchangeable cations with $NH_4Cl/BaCl_2$, and boron was measured as hot water extractable B.

Statistical analyses

Given the modest sample size and the number of variables investigated, our analysis is exploratory to generate hypotheses about possible relationships that should be tested with an independent, larger data set.

Soil properties at the different sites

Differences in soil chemical properties at each of the sites were assessed visually with cluster analysis based on the Euclidean distance measure and the unweighed pair-group average clustering algorithm.

The end result is a dendrogram which uses the similarities between sites, based on the 17 soil variables measured, to group sites into clusters. This was followed by analysis of similarity (ANOSIM) to test for significant differences between sites, and, where ANOSIM was significant, a similarity percentage (SIMPER) was used to determine which soil variables contributed most to the significant result. These analyses were conducted using PAST (Hammer et al. 2001).

ANOSIM is a nonparametric technique for comparing multivariate data across two or more groups. The test is analogous to analysis of variance (ANOVA), because it involves assessing the relative amount of distance in the data that can be ascribed to differences between groups as opposed to differences within groups. An *R* statistic indicates the degree of dissimilarity between groups. As *R* approaches 1, groups become increasingly dissimilar. *R* values near 0 indicate very similar groups. The statistical significance of *R* is determined by repeated sampling permutation tests based on group membership (Hammer et al. 2001). If sample sizes are large, it is possible for small *R* values to be statistically significant. Therefore, Clarke and Warwick (2001) recommend considering *R* and *P* values together when interpreting results and not placing great weight on a significant *P* value if *R* is low (indicating statistical significance of a small dissimilarity across groups).

We assessed differences in soil composition between sites with a one-way ANOSIM based on the Bray-Curtis distance measure. If ANOSIM revealed statistically significant differences between sites, SIMPER (Clark 1993) was used to reveal which variables were mainly responsible for the differences. The contribution of each variable to the difference is interpretable most readily if expressed as a cumulative percentage, indicating the amount of variation explained as successive variables are added in order from the largest contributor to the smallest.

Canopy health at different sites

The TCHI values were placed into three groups using k-means clustering, which groups sites so that the distance between members of different groups is consistently greater than the distance between members of the same group. The number of groups is defined by the user. In this case, we selected

three groups on the assumption that we had sampled a range from of healthy, moderate, and declining sites. The analysis first places sites into groups at random and then, in successive iterations, moves sites to the group that has the closest cluster mean. The iterations cease when individual sites are no longer jumping between clusters.

Seedling growth and mycorrhizal colonization in soil from different sites

Nested ANOVA were carried out to determine differences in seedling growth (shoot biomass), spore density, and AM/ECM abundance in seedlings between the 12 sites. Soil which came from two dependent cores per tree in the field, so tree was nested inside site for these analyses, following the example and logic of Scheiner (2001). Two seedlings were grown from each of these dependent cores in the glasshouse, so the values for shoot biomass from these seedlings were averaged to avoid issues of dependence. A single value for spore density and a single value for mycorrhizal colonization were recorded for each pot. Post hoc tests were not done because the sites did not correspond to experimental treatments. Spore density and mycorrhizal colonization were $\log_{10}$ transformed before analyses to improve the fit to the normal distribution. Differences in canopy condition between sites were tested using analysis of variance, because there were no dependent multiple measurements on individual trees. Pearson's correlation coefficients were applied to determine the relationship between seedling biomass and canopy condition, and the relationship between AM and ECM colonization. These analyses were conducted using SPSS for Windows version 17.0 (SPSS Inc.).

Predicting mycorrhizal colonization from soil chemical properties

Multiple regression was used to test whether AM and ECM colonization at each of the 12 sites could be predicted from the 17 soil characteristics measured. A separate analysis was carried out for each dependent variable (ECM and AM). The analyses were exploratory, so a forward stepwise approach was used (Field 2009).

Predicting tree canopy health from soil chemical properties and mycorrhizal colonization

These analyses were also exploratory, so two separate forward stepwise multiple regressions were used (one for the soil characteristics as independent variables and one for the mycorrhizal

colonization as independent variables) to predict tree canopy health (based on four random trees at each of the 12 sites). Linear regression analysis was applied to determine the relationship between tree canopy health (TCHI) and mycorrhizal colonization. Tree canopy health was $\log_{10}$transformed before analysis to improve the fit to the normal distribution.

Results

Canopy health and site condition

The health status of the 12 sites was determined based on the TCHI value (Evans et al. 2012a, b). Analysis of variance indicated that the TCHI differed significantly ($F_{(11, 36)} = 9.15$, $P < 0.0001$) between the sites. The lowest TCHI was at site 4 (30.31 ± 6.97), while the highest TCHI were at sites 11 (80.62 ± 2.07) and 10 (80.31 ± 5.1) (Table 1). The TCHI is an index which accurately reflects the crown condition of trees. The higher the TCHI, the healthier the trees are. K-means cluster analysis grouped sites 1, 2, 3, 8, 10, and 11 into group 1. This group had TCHI values ranging from 68.4 to 80.6, and the sites within this group were considered to be the healthiest of the sites, hereafter called healthy. The second group was sites 5 and 12, TCHI values ranged from 53.1 to 56.6, and these were considered to be intermediate sites, hereafter called moderate. The third group consisted of sites 4, 6, 7, and 9, had TCHI values ranging from 30.3 to 45, and were considered to be declining sites.

Soil chemical characteristics of the study sites are presented in Table 2. Cluster analysis of these values revealed five broad groupings (Fig. 1). Site 9 separated from the others at the first step, representing a soil composition distinct from the others. The next division in the dendrogram separated sites 1, 2, 3, and 10 in a tight cluster, forming a second group. Site 6 then separated to form a third group, with sites 4, 5, and 7 forming a fourth group and sites 8, 11, and 12 in a fifth group. ANOSIM supported this picture (Table 3). Site 9 was significantly different from all other sites and also had *R* values of 0.54 or greater for paired comparisons with all other sites. This indicates substantial dissimilarity between site 9 and the other sites. Site 1 was not different from sites 2, 3, or

10 (the other sites placed alongside it in cluster analysis). Sites 8, 11, and 12 were not different in their soil composition, nor were sites 4, 5, and 7.

SIMPER based on differences across all sites was remarkable for the very similar contributions of all variables. DTPA Fe made the largest contribution, but that was only 8.9 %. The smallest, pH water, contributed 2.6 % (Table 4).

Analysis of variance indicated that AM spore density in the field differed significantly across sites ($F_{(11, 48)} = 4.25$, $P = 0.0002$). Trees were not significantly different ($F_{(36, 48)} = 1.22$, $P = 0.26$) (Table 5). Although they differed with site, there was no relationship between spore density of field soil and crown health ($r = 0.09$, $n = 48$).

Seedling growth

ANOVA showed that shoot dry weight differed significantly ($F_{(11, 48)} = 7.53$, $P < 0.0001$) between the sites (Fig. 2a). Trees were not significantly different ($F_{(36, 48)} = 1.208$, $P = 0.27$) within each site. However, crown health in the field could not be predicted by seedling growth as Pearson's correlation coefficient was not significant ($r = -0.23$, $n = 48$).

AMF spore density in bioassay trial

The AMF spore density in pots differed significantly ($F_{(11, 48)} = 2.86$, $P = 0.006$) between the sites. Trees were not significantly different ($F_{(36, 48)} = 1.22$, $P = 0.26$) within each site (Table 5). Compared to the spore density in the field soil at the time of collection, the AMF spore density tended to increase in soils from unhealthy sites, but, tended to decrease in soils from healthy sites in the bioassay trial (Table 5). Unlike for the field soil, the spore density in the bioassay soil was negatively correlated to TCHI ($P = 0.01$, $r = -0.479$, $n = 48$) and to crown density ($P = 0.01$, $r = -0.47$, $n = 48$), but positively correlated with crown dieback ratio ($P = 0.01$, $r = 0.434$, $n = 48$) and epicormic index ($P = 0.01$, $r = 0.481$, $n = 48$).

AM/ECM colonization

In the bioassay trial, analysis of variance showed that the proportion of roots colonized by AM fungi differed significantly ($F_{(11, 48)}$ =3.71, $P = 0.0007$) between the sites (Fig. 2b). Trees were not significantly different ($F_{(36, 48)} = 0.59$, $P = 0.94$) within each site. The proportion of ECM tips also differed significantly ($F_{(11, 48)} = 6.33$, $P < 0.000002$) with site (Fig. 2c). Trees were not significantly different ($F_{(36, 48)} = 1.1$, $P = 0.37$) within each site. The highest ECM colonization was in soil taken from sites 1, 2, and 10. Comparing the dual colonization of AM and ECM in roots of *E. gomphocephala* seedlings, Pearson's correlation coefficient analysis indicated that ECM colonization was negatively correlated with AM colonization ($P = 0.01$, $r = -0.43$, $n = 96$) (Fig. 2d).

Predicting mycorrhizal colonization (AM and ECM) from soil chemical properties

Forward stepwise multiple regression indicated that AM could be predicted from soil chemical properties ($F_{(10, 37)} = 4.36$, $P < 0.001$). The AM were more abundant in association with higher P, S, and exchangeable K, and with lower exchangeable Na, pH (H_2O), DTPA Zn, DTPA Cu, Boron, K, and conductivity. The ECM could also be predicted from soil chemical properties ($F_{(8, 39)} = 5.26$, $P < 0.001$). They were more abundant in association with higher conductivity, pH ($CaCl_2$), and DTPA Fe, and with lower DTPA Cu, DTPA Mn, Boron, exchangeable Ca, and pH (H_2O) (Table 6).

Predicting tree canopy health from soil chemical properties and mycorrhizal colonization

Forward stepwise multiple regression showed that tree crown health could be predicted from soil characteristics ($F_{(12, 35)} = 3.38$, $P = 0.002$). Increased tree health was related to increased pH ($CaCl_2$), exchangeable Ca, DTPA Mn, exchangeable Na, DTPA Cu, and DTPA Zn, but declined with increasing P, K, and S (Table 6). Similarly, forward stepwise multiple regression confirmed that tree crown health could be predicted from mycorrhizal colonization ($F_{(2, 45)} = 8.06$, $P = 0.001$). Increased tree health was associated with increasing proportion of ECM root tips and lower AM ($\beta = 0.421$, $P = 0.001$). In addition to forward multiple regression, linear regression showed that ECM

root tips was positively related to TCHI ($F_{(1, 46)} = 14.975$, $\beta = 0.784$, $P < 0.0001$), but negatively related to AM colonization ($F_{(1, 46)} = 7.357$, $\beta = -0.237$, $P < 0.009$) (Fig. 3).

Discussion

This pilot study found that the gross type of mycorrhiza formed on tuart seedlings grown in soil cores taken from the field predicted the canopy condition of *E. gomphocephala* at the sites where the cores were taken. In particular, ECM dominated seedling roots produced in soils taken from under healthy canopies whereas AM dominated seedling roots in soils taken from under trees with declining canopies. This raises the question as to whether ECM may be more important than AM for maintaining the health of *E. gomphocephala* in natural ecosystems, or whether the fungi are responding to another, unidentified variable, that is the real determinant of canopy condition. To address this question, in future studies, colonization of *E. gomphocephala* seedlings by AM and ECM fungi should be examined in the field to determine if the fungi have differential effects on the growth and success of seedling recruitment. The mycorrhizal status of adult trees in the field should also be assessed. Fitness in the glasshouse and in the field might not be related, so the microbial responses in the glasshouse may be independent of fitness effects in the field.

It is well established that ECM fungi are important for the growth of many trees in nutrient-poor soil (Marschner and Dell 1994, Plassard and Dell 2010). Furthermore, where compatible ECM fungi were absent, inoculation with some ECM fungi in the nursery significantly improved the growth of outplanted eucalypts in plantations (Malajczuk et al. 1994; Chen et al. 2000b; Xu et al. 2001; Chen et al. 2006). In the absence of compatible ECM fungi, eucalypts become colonized by AM fungi (Zambolim and Barros 1982; Oliveira et al. 1997; Santos et al. 2001; Pagano and Scotti, 2008; Campos et al. 2011).

We anticipated that *E. gomphocephala* seedlings in containers would have initially been colonized by AM fungi and that new roots would increasingly have become colonized by ECM fungi over time if suitable inocula were present in the soil cores. For this reason, we harvested the plants after 7 months

to allow adequate time for ECM colonization to occur. This succession in containerized seedlings has been described previously (Chen et al. 2000a; Santos et al. 2001; Egerton-Warburton and Allen, 2001). The lack of ECM in seedlings grown in cores taken from under trees with declining canopies may result from a lack of suitable ECM fungi inoculum at these sites.

Factors that may influence ECM fungi inoculum loads in forest soil include spore dispersal and longevity, distribution of host plants across sites, and the abundance of sporocarps above and below ground. While seasonal sampling for viable spore loads of mycorrhizal fungi was not conducted, we are aware that ECM fungi such as *Pisolithus* and *Scleroderma,* which are common in the region, persist in soil for several years and remain viable (unpublished data).

The distribution of host plants did not seem to be a factor as the under story of healthy and declining sites contained species within the Myrtaceae, Casuarinaceae, and Fabaceae that are known to form ECM (Brundrett 1991).

The presence of soil-borne pathogens such as *Phytophthora* may have an impact on ECM fungi and hence spore production. *Phytophthora multivora* has been isolated from declining *E. gomphocephala* in the same region as this study (Scott et al. 2009), and demonstrated to cause loss of fine roots in *E. gomphocephala* seedlings in containers (Scott et al. 2011). Recently, fewer ECM fungal mats have been observed in declining *E. gomphocephala* stands (Scott et al. 2012). We baited for *P. multivora* in the pot trial but did not detect *P. multivora* or *P. cinnamomi* which is widespread in south-western Australia. Whether the distribution and abundance of ECM fungi can be differentially affected by *P. multivora* in the field as shown for infested *Castanea sativa* stands (Blom et al. 2009; Scatollin et al. 2012) remains to be determined. Also, the canopy condition of *E. gomphocephala* in the field may impact on spore production by mycorrhizal fungi. It is generally assumed that ECM associations obtain more energy from host plants than AM associations (Smith and Read 2008) and the amount of carbon allocated below ground can influence populations of mycorrhizal fungi (Peter et al. 2008).

Soil conditions have been suggested to be important factors affecting mycorrhizal fungi (Erland and Taylor 2002; Toljander et al. 2006; Scatollin et al. 2008; Alzetta et al. 2012; Newbound et al. 2012). In the bioassay study, we found AM colonization was more extensive in *E. gomphocephala* seedlings grown in soil with higher availability of P, S, and exchangeable K. It is not known whether a causal relationship was present. However, it is possible that some AM fungi are less sensitive than ECM to changes in soil chemical composition (Na Bhadalung et al. 2005). Recently, a study by Karlinski et al. (2010) on poplar (*Populus* sp.), a dual AM/ECM genus, found that AM fungi predominated over ECM fungi particularly in polluted soil which was high in carbon, nitrogen, phosphorus, and potassium. These authors suggested that the proportion of the two mycorrhizal types was strongly influenced by environmental conditions particularly site and soil.

In the present study, cluster analysis showed that the sites were quite different in their soil chemical composition. Interestingly, from the six sites where trees were mostly healthy, four of them clustered into one group, suggesting that they had similar soil chemical properties. Sites 9 and 6 were quite different in their soil chemical composition, and at these sites trees were declining. Sites 4, 5, and 7 were similar in their soil chemical composition, and trees had poor growth at sites 4 and 7, but were moderately healthy at site 5. The most probable explanation for the differences in soil chemical composition between the sites included geological origin, fire and adjacent land-use history (pasture and fertilizer), and the structure of the understory, which might affect litter quantity, quality, and heterogeneity.

Statistical analysis also showed that soil chemical properties can be used to predict canopy condition (TCHI) of *E. gomphocephala* in the field. In particular, tree health improved with increasing pH ($CaCl_2$), exchangeable Ca, exchangeable Na, Mn, Cu, and Zn, but declined with increasing P, K, and S. Whether these relationships are causal remains to be established. *E. gomphocephala* might be classified as a calcicole plant as it is largely confined on calcareous soil profiles, thus it is likely that the species could be impacted when the soil pH decreases with perturbations. The canopy condition of *E. gomphocephala* tended to improve with increasing DTPA extractable levels of Mn, Cu, and Zn, nutrients which are poorly available in calcareous soil (Bell and Dell, 2008). Relationships between

edaphic factors and tree decline have been reported in sugar maple decline (Wilmot et al. 1995; Horsley et al. 2000; Schaberg et al. 2006) as well as eucalypt decline (Czerniakowski et al. 2006; Parsons and Uren 2007; Grigg et al. 2009).

In the present study, shoot dry weights of seedlings differed significantly between the sites, but there was no correlation with crown health in the field. This is not surprising as we added a minimal nutrient regime to the seedlings to make sure that carbon was not a limitation for mycorrhizal colonization as the main point of this study was to trap mycorrhizal fungi. The level of fertilizer added was based on levels that promoted ECM formation in nursery containers (Dell and Malajczuk 1995).

The results of the present work indicate a probable link between mycorrhizal type and soil chemistry to the canopy condition of *E. gomphocephala*, justifying further work to determine if causal relationships exist using larger sample size than those in this study to permit more sophisticated analyses such as path analysis. More in-depth studies are required to determine how canopy decline interacts with mycorrhizal fungi and mycorrhizal loads in the field. In particular, detailed molecular characterization of the fungi colonizing roots is required in order to identify those ECM species that are sensitive to canopy decline of *E. gomphocephala*. Previous studies in other forest systems have demonstrated that defoliation might affect ECM fungi through carbon allocation to roots (Markkola et al. 2004; Peter et al. 2008). The situation is complex as ECM fungi may contribute to forest resilience, recovery, and vigor (Amaranthus 1998) as well as plant biodiversity and productivity in natural ecosystems (van der Heijden et al. 1998; van der Heijden and van Straalen 2008).

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Table 1 Canopy condition of *E. gomphocephala* prior to sampling soils in 2009

Sites	Total crown health index#	Crown density#	Foliage transparency#	Crown dieback ratio#	Epicormic index#
1	70.4 ± 8.8	51.2 ± 7.4	27.5 ± 6.3	38.0 ± 9.6	20.0 ± 12.4
2	69.1 ± 6.7	50.0 ± 6.1	32.5 ± 4.8	44.0 ± 11.6	13.7 ± 5.5
3	73.4 ± 3.0	53.7 ± 4.3	40.0 ± 2.9	22.0 ± 5.5	6.2 ± 1.2
4	30.3 ± 7.0	23.7 ± 6.6	52.5 ± 7.5	92.0 ± 11.1	92.5 ± 5.9
5	53.1 ± 8.7	42.5 ± 4.3	50.0 ± 2.9	54.0 ± 11.1	46.2 ± 22.5
6	40.3 ± 2.2	30.0 ± 0	42.5 ± 2.5	72.0 ± 6.4	81.2 ± 5.1
7	41.9 ± 2.3	38.7 ± 6.2	60.0 ± 2.9	62.0 ± 3.7	68.7 ± 6.6
8	68.4 ± 3.0	46.2 ± 2.4	37.5 ± 4.8	30.0 ± 3.7	16.2 ± 4.7
9	45.0 ± 6.7	40.0 ± 8.9	45.0 ± 4.1	64.0 ± 7.4	75.0 ± 16.7
10	80.3 ± 5.1	58.7 ± 4.3	25.0 ± 9.1	10.0 ± 3.1	6.2 ± 4.7
11	80.6 ± 2.1	62.5 ± 3.2	35.0 ± 4.1	8.0 ± 2.0	0
12	56.6 ± 4.6	38.7 ± 3.7	37.5 ± 2.5	52.0 ± 4.3	42.5 ± 13.6

#Each value is a mean ($n = 4$) ± standard error

Table 2 Soil chemical characteristics of the twelve sites in Yalgorup National Park. Each value is an average of four replicates. Soil was collected adjacent to soil core collected for bioassay trial for mycorrhizal assessment

Site	NH4-N	P	K	S	Organic	Conductivity	pH	pH	DTPA Cu	DTPA Fe	DTPA Mn	DTPA Zn	Ex.Ca	Exc.Mg	Exc.K	Exc.Na	B
	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	C (%)	(ds/m)	CaCl2	H2O	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
1	1.75	12	60.5	7.05	2.32	0.15	7.25	7.88	0.14	22.79	2.41	0.24	15.14	1.39	0.15	0.25	1.18
2	0.25	5.5	39.5	6.42	1.52	0.14	7.37	8.15	0.22	10.53	1.89	0.18	10.65	0.87	0.1	0.18	0.73
3	0.75	16.25	64.25	7.12	1.58	0.12	7.05	7.85	0.28	12.95	2.36	0.22	7.85	1.44	0.16	0.35	0.86
4	0.25	6.5	29	2.95	0.99	0.05	6.77	7.58	0.3	5.89	2.9	0.17	3.87	0.53	0.07	0.09	0.33
5	0.5	7.25	31.5	3.9	1.04	0.05	6.62	7.38	0.31	3.16	4.42	0.22	4.46	0.79	0.08	0.15	0.39
6	0.5	27.75	51.75	3.87	2.24	0.08	6.7	7.55	0.64	10.77	2.65	0.19	11.75	1.38	0.13	0.14	0.81
7	0.5	8.25	25.75	4.17	1.5	0.06	6.87	7.55	0.49	8.21	4.13	0.12	8.65	0.76	0.07	0.08	0.58
8	0.75	8.25	28.5	4.45	1.52	0.05	5.85	6.83	0.53	26.19	3.14	0.17	3.79	0.96	0.07	0.19	0.44
9	1.5	8.25	115.75	6.6	3.23	0.1	6.67	7.38	0.43	31.33	3.21	0.01	16.23	2.57	0.29	0.36	1.35
10	0.5	4.5	35.5	6.2	1.71	0.11	7	7.58	0.53	15.87	2.47	0.08	11.07	0.87	0.09	0.15	0.65
11	1.25	12	38	6.5	1.43	0.09	6.32	6.83	0.59	19.97	3.03	0.12	6.35	1.06	0.1	0.28	0.5
12	0.75	3	31.75	3.67	1.42	0.04	5.62	6.43	0.57	17.99	2.47	0.09	3.76	0.62	0.08	0.11	0.31

Table 3 ANOSIM *P* and *R* values for pair-wise comparisons between different sites using soil variables from Table 2

Site	***P* and *R* values**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	0	0.108	0.17	**0.029**	**0.027**	0.085	0.054	**0.028**	**0.028**	0.058	0.12	**0.028**
2	*0.417*	0	**0.027**	**0.026**	**0.027**	**0.03**	**0.031**	**0.028**	**0.026**	**0.028**	0.056	**0.028**
3	*0.146*	*0.552*	0	**0.029**	**0.03**	0.083	**0.027**	**0.028**	**0.031**	**0.039**	**0.031**	**0.029**
4	*0.865*	*0.99*	*0.917*	0	0.18	**0.026**	0.141	**0.025**	**0.031**	**0.029**	**0.027**	**0.027**
5	*0.865*	*0.792*	*0.604*	*0.24*	0	**0.029**	0.292	**0.028**	**0.027**	**0.03**	**0.028**	**0.027**
6	*0.365*	*0.729*	*0.292*	*0.75*	*0.51*	0	0.403	**0.03**	**0.03**	**0.027**	0.205	**0.029**
7	*0.469*	*0.531*	*0.531*	*0.271*	*0.094*	*0.042*	0	0.058	**0.031**	**0.03**	0.145	0.086
8	*0.844*	*1*	*0.76*	*0.875*	*0.646*	*0.49*	*0.302*	0	**0.026**	**0.029**	0.145	0.287
9	*0.375*	*1*	*0.74*	*1*	*1*	*0.615*	*0.719*	*0.917*	0	**0.031**	**0.027**	**0.028**
10	*0.531*	*0.844*	*0.708*	*1*	*0.76*	*0.427*	*0.312*	*0.75*	*0.917*	0	0.061	**0.03**
11	*0.344*	*0.594*	*0.375*	*0.792*	*0.594*	*0.177*	*0.208*	*0.125*	*0.542*	*0.5*	0	0.111
12	*0.937*	*1*	*0.937*	*0.792*	*0.823*	*0.51*	*0.281*	*0.146*	*0.885*	*0.688*	*0.271*	0

Significantly different sites are in bold. *P* values are in the upper half of the table in normal font. *R* values for the same analysis are shown in the lower half of the table in italics. *R* values corresponding to significant *P* values are underlined

Table 4 SIMPER analysis showing the percentage contribution of each soil variable to the differences between sites

Soil properties	Contribution	Contribution (%)	Cumulative (%)	Mean abund.1	Mean abund.2	Mean abund.3	Mean abund.4	Mean abund.5	Mean abund.6	Mean abund.7	Mean abund.8	Mean abund.9	Mean abund.10	Mean abund.11	Mean abund.12
Fe	2.993	8.869	8.87	0.596	0.402	0.517	0.462	0.253	0.321	0.123	0.047	0.26	0.188	0.691	0.835
Conductivity	2.817	8.347	17.21	0.656	0.474	0.341	0.043	0.614	0.482	0.101	0.129	0.285	0.178	0.106	0.41
Exc. Ca	2.643	7.831	25.05	0.614	0.418	0.191	0.066	0.398	0.263	0.071	0.099	0.451	0.301	0.068	0.667
Cu	2.555	7.571	32.62	0.159	0.653	0.731	0.703	0.266	0.344	0.366	0.372	0.791	0.6	0.653	0.528
Zn	2.329	6.901	39.52	0.481	0.081	0.169	0.106	0.338	0.425	0.313	0.431	0.356	0.169	0.306	0.119
Exc. Na	2.308	6.839	46.36	0.396	0.212	0.458	0.137	0.269	0.585	0.104	0.212	0.189	0.075	0.288	0.613
Mn	2.26	6.697	53.06	0.197	0.205	0.281	0.205	0.127	0.19	0.264	0.472	0.23	0.432	0.296	0.306
N (NH4)	2.121	6.285	59.34	0.357	0.143	0.429	0.357	0.036	0.214	0.071	0.214	0.286	0.179	0.25	0.643
B	2.04	6.045	65.39	0.519	0.249	0.174	0.075	0.288	0.358	0.089	0.117	0.331	0.216	0.143	0.603
S	1.948	5.772	71.16	0.399	0.328	0.353	0.116	0.347	0.405	0.055	0.134	0.132	0.158	0.161	0.361
Exc.Mg	1.762	5.221	76.38	0.312	0.162	0.215	0.091	0.162	0.329	0.066	0.138	0.308	0.131	0.188	0.646
Org.C.	1.613	4.779	81.16	0.331	0.188	0.122	0.12	0.145	0.159	0.02	0.031	0.312	0.139	0.143	0.547
P	1.551	4.595	85.75	0.208	0.052	0.208	0.021	0.073	0.297	0.094	0.109	0.536	0.13	0.13	0.13
Exc. K	1.548	4.587	90.34	0.299	0.152	0.171	0.116	0.171	0.329	0.11	0.122	0.25	0.091	0.098	0.64
K	1.494	4.427	94.77	0.277	0.125	0.14	0.102	0.349	0.3	0.085	0.101	0.224	0.065	0.082	0.614
pH Ca	0.884	2.619	97.39	0.291	0.257	0.166	0.071	0.307	0.264	0.226	0.206	0.216	0.24	0.101	0.213
pH H2O	0.881	2.61	100	0.982	0.939	0.832	0.775	1.02	0.979	0.939	0.911	0.936	0.936	0.832	0.911

The higher the percentage contribution for an individual variable, the more it contributes to the overall differences between sites

Table 5 Spore density of AMF per 100 g soil at twelve sites with standard error

Sites	Spore density[a]	Spore density[b]
1	135.5 ± 46.2	45.5 ± 3.2
2	24.7 ± 4.5	17.7 ± 3.8
3	71.2 ± 23.9	68.2 ± 16.3
4	52.2 ± 13.7	59.7 ± 13.0
5	36.5 ± 6.4	69.0 ± 13.31
6	17.5 ± 4.9	68.5 ± 12.45
7	27.2 ± 4.2	54.5 ± 13.75
8	15.5 ± 4.1	50.2 ± 25.6
9	35.0 ± 13.0	86.2 ± 50.3
10	40.2 ± 10.4	15.0 ± 3.2
11	22.2 ± 6.9	13.7 ± 5.3
12	38.2 ± 9.9	71.5 ± 21.5

Each value is an average of eight replicates

[a]Soil collected from field

[b]Soil collected from bioassay trial

Table 6 Soil chemical properties as predictors for canopy health (TCHI) and mycorrhizal (AM and ECM) colonization using forward stepwise multiple regression

Soil variables measured	Soil variables in the final model for canopy health (TCHI)	Soil variables in the final model for AM colonization	Soil variables in the final model for ECM colonization
NH_4–N	N (β = 0.15)	P (β = 0.61)[a]	DTPA Cu (β = −0.41)[a]
P	P (β = −0.49)[a]	S (β = 0.689)[a]	Conductivity (β = 0.4)
K	K (β = −1.5)[a]	Exc. K (β = 4.52)[a]	pH $CaCl_2$ (β = 0.985)[a]
S	S (β = −0.95)[a]	Exc. Na (β = −1.4)[a]	pH H_2O (β = −0.82)[a]
Organic C	pH $CaCl_2$ (β = 2.02)[a]	B (β = −0.58)	DTPA Mn (β = −0.16)
Conductivity	pH H_2O (β = −0.82)	K (β = −3.2)	DTPA Fe (β = 0.469)[a]
pH ($CaCl_2$)	Exc. Ca (β =0.361)[a]	pH H_2O (β = −0.25)	Exc. Ca (β = −0.43)
pH (H_2O)	Exc. Na (β = 0.969)[a]	Conductivity (β = −0.33)	B (β = −0.38)
DTPA Cu	Exc. Mg (β =0.919)	DTPA Cu (β = −0.41)[a]	
DTPA Fe	DTPA Mn (β = 0.499)[a]	DTPA Zn (β = −0.18)	
DTPA Mn	DTPA Cu (β = 0.634)[a]		
DTPA Zn	DTPA Zn (β = 0.337)[a]		
Exc. Ca			
Exc. Mg			
Ecx. K			
Exc. Na			
B			

The soil variables included in the most parsimonious models in each case are shown, together with an indication of which variables are significant

[a]Significant predictor

Fig. 1 Cluster analysis for the twelve sites based on soil characteristics

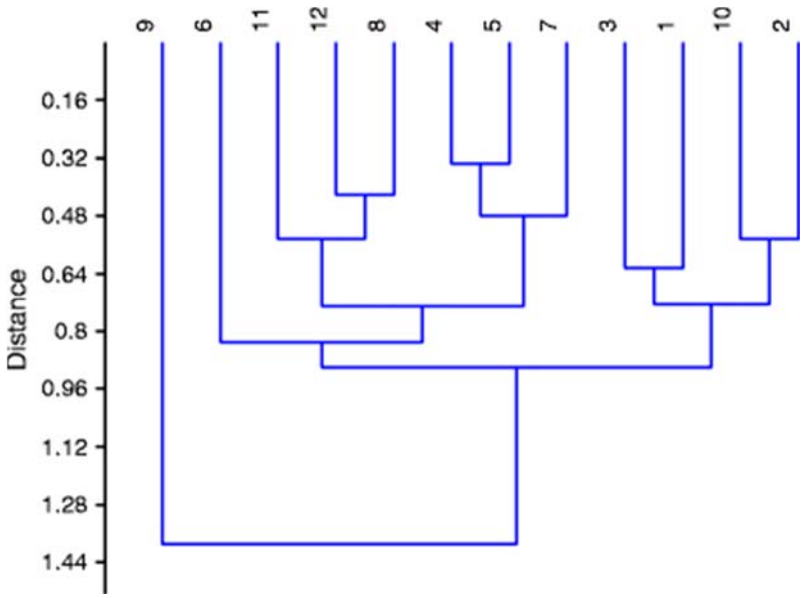

Fig. 2 *E. gomphocephala* seedling biomass (**a**); proportion of root colonized by AM (**b**), ECM (**c**), and correlation between ECM tips and AMF colonization (**d**) growing at 12 sites. *Bars* represent standard errors of the mean. Each value is an average of eight replicates

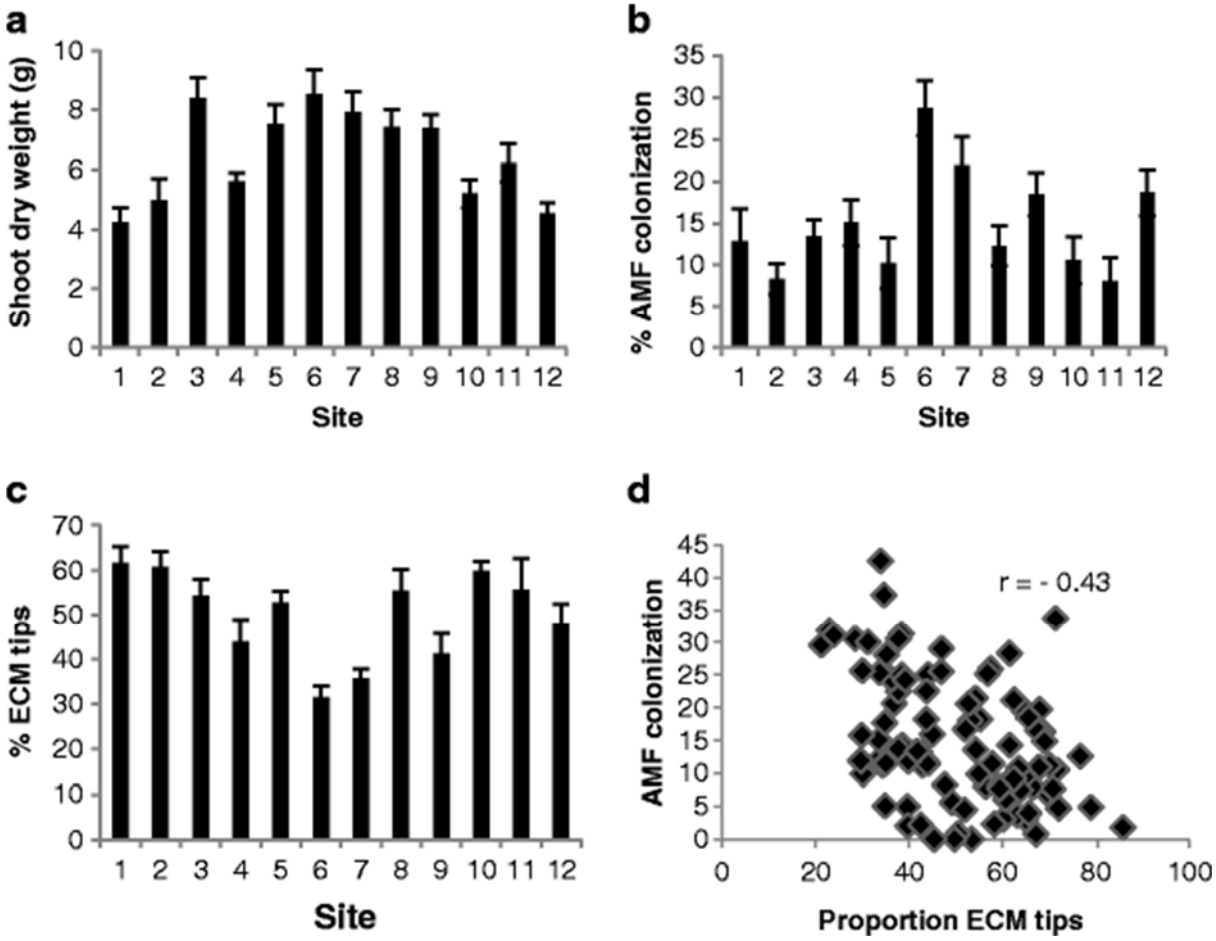

Fig 3 Relationship between crown health and ECM tips (a); crown health and AMF colonization (b)