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Glomalean mycorrhizal fungi from tropical Australia

II. The effect of nutrient levels and host species on the isolation of fungi

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Abstract The isolation of vesicular-arbuscular mycorrhizal (VAM) fungi from natural (savanna, rocky hill, wetland and rainforest) and disturbed (minesite) habitats in a seasonally-dry tropical region in the Northern Territory of Australia into open-pot cultures was undertaken to supplement knowledge about the diversity of these fungi. This experiment considered factors affecting the diversity of fungi obtained in trap cultures started using diluted soil from field sites and two host plants. A range of soil phosphorus and other nutrient levels from severely deficient to sufficient for maximal growth was used to determine the impact of nutrition on mycorrhizal associations of sorghum *(Sorghum* sp.) and clover *(Trifolium subterraneum)*. Soil cores taken from pots at 6-week intervals provided roots and soil to assess mycorrhiza formation and sporulation without substantial damage to plants. The identification of VAM fungi to genus by observing morphological patterns within clover roots revealed substantial differences in fungus populations between soils and a moderate effect of nutrient levels on fungal diversity. Changes in the proportion of different fungi in roots over the 31 weeks of the experiment were also observed. *Glomus* spp. were initially the most abundant fungi within roots, but *Scutellospora* spp. gradually became more dominant at later harvests, while colonisation by *Acaulospora* spp. was limited at all times. For both clover and sorghum, sporulation was limited and was dominated by single species of *Scutellospora* and *Acaulospora*. This contrasted with the much higher diversity of spore types in the original field soils.

Key words Vesicular-arbuscular mycorrhizas · Glomalean fungi · Diversity · Inoculum · Tropical fungi · Succession · Nutrition

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Introduction

The initiation and propagation of living cultures of vesicular-arbuscular mycorrhizal (VAM) fungi in association with plant roots is the only means currently available for providing material for research purposes and practical applications (Jarstfer and Sylvia 1993; Morton et al. 1993). Trap cultures, using a host plant grown in soil diluted with sterile sand, are most commonly used to isolate fungi, but spores from field soils and other inoculum sources can also be used (Jarstfer and Sylvia 1993; Morton et al. 1993; Brundrett et al. 1999). Soil trap cultures contain larger numbers of healthy spores than the soils from which they were started, but usually result in complex mixtures of species which change over time (Morton et al. 1993; Stutz and Morton 1996; Brundrett et al. 1999). Recent comparisons of methods of initiating cultures showed substantial differences in efficiency and specificity (Bever et al. 1996; Watson and Millner 1996; Brundrett et al. 1999). Earlier experiments comparing isolation methods using soil, roots, spores, or transplanted seedlings found that several methods were required to obtain cultures of most of the fungi present in soils (Brundrett et al. 1999). These pot culture methods supplemented data obtained from a spore-based population survey by revealing additional species as important components of soils.

In ecosystem studies and glasshouse experiments, host plants and soil factors can influence both the diversity of VAM fungi and overall levels of mycorrhizal

root formation and sporulation (Brundrett 1991). For example, high levels of phosphorus (P) in soil and plants can inhibit mycorrhiza formation (Menge et al. 1978; Douds and Schenck 1990) and influence the diversity of VAM fungi in field soils (Thomson et al. 1992; Cuenca and Meneses 1996). It has also been demonstrated that the presence of different host plants influences fungal diversity (Schenck and Kinloch 1980; Collins Johnson et al. 1992; Hendrix et al. 1995; Bever et al. 1996). Thus variation in host plant or nutrient levels might be expected to influence the diversity of fungi isolated from soils in trap cultures.

This paper reports one part of a study aimed to characterise the role of mycorrhizal associations in undisturbed and disturbed habitats in the Alligator Rivers Region of the Northern Territory of Australia. In other experiments using soils from these habitats, four different pot-culture isolation techniques were compared (Brundrett et al. 1999), bioassays were used to measure mycorrhizal inoculum levels (Brundrett et al. 1996a) and spores of glomalean fungi were counted and identified. The aim of the experiment presented here was to increase the diversity of fungi (species richness) isolated in soil trap cultures by using two host plant species with substantially different root systems and a range of soil nutrient levels. Nutrition was chosen as a major experimental factor because of its expected impact on mycorrhizal associations.

Materials and methods

The soils used in this experiment were collected from five different natural habitats in the Alligator Rivers Region during a field trip at the end of the growing season (start of the dry season) in April-May 1991 (Brundrett et al. 1996a). This region, which includes Kakadu National Park and adjacent parts of Arnhem Land, consists of lowland eucalypt savanna woodland, upland, wetland, rainforest and estuarine vegetation on lateritic and sandstone derived soils (Wilson et al. 1990). The climate consists of a long dry season and a 3–5 month wet season with periods of heavy monsoonal rainfall (Dunlop and Webb 1991). The five sites used were (a) lowland eucalypt woodland with a dense natural cover of *Sorghum* sp. on a sandy soil, (b) wetland with sedges and shrubs on a sandy soil, (c) acacia, eucalypt woodland with many understorey shrubs on a loamy soil, (d) dry hillside woodland with shrubs and grasses on a rocky loam soil and (e) dry hill side woodland (*Erythrophleum*, eucalypt) with grasses on a gravelly loam soil (sites are labelled using the same letters in Figure 3). Further details of vegetation and soils at these sites is provided in Table 1 of Brundrett et al. (1996a).

Soil samples taken from each site were dried and stored at room temperature for 7 months before use. To initiate pot cultures, 2.5 kg of soil from each site was thoroughly mixed in a plastic bag. This soil was then subdivided to provide five uniform 500-g soil portions for use as trap culture inoculum for each host plant and soil type. This inoculant soil was mixed with a steamed infertile sandy soil by first mixing it with 500 g of sand and then placing it in a band between two 250-g layers of sand to fill 1.5 kg pots. Two additional control pots with only sterile sand were included.

The complete nutrient solution used was optimised for plant growth in the sandy soil used for this experiment by previous nutritional research (Snowball and Robson 1984; Brundrett et al. 1996b). This nutrient solution was diluted to required final concentrations with deionised water to provide four increasingly weak nutrient solutions (100%, 60%, 30% and 10% of the full-strength solution). These solutions were precisely applied by watering pots to field capacity (10% w/w). The nutrient levels used resulted in final soil P concentrations of 9.3, 6.1, 3.6 and 2.0 mg kg^{-1} in these non-draining pots. Phosphorus was the main factor limiting plant growth and was provided at a rate sufficient for maximal plant growth at the highest fertilizer level used. Other nutrients were supplied at luxury levels, but were also decreased proportionally by the dilution process. A fifth set of pots was watered with deionised water only (0% treatment), but had small amounts of residual nutrients from the soils used (1.2 mg kg^{-1} of P on average). Nitrogen (50 mg kg^{-1} NH_4NO_3) was applied to sorghum plants every 2 weeks.

Clover [*Trifolium subterraneum* L. cv. Seaton Park inoculated with *Rhizobium leguminosarum* bv. *trifolii* (TA1)] and forage sorghum [*Sorghum bicolor* (L.) Moench × *sudense* (Piper) Stapf cv. Sudax] were used as host plants for pot cultures. Five pregerminated seeds of clover or sorghum were sown in each pot and thinned to 3 uniform plants after germination. The non-draining pots were watered to weight once every 1–2 days with deionised water. Soil temperature was maintained at 25 °C by immersion in a tank of temperature-regulated water. Pots were maintained in a glasshouse where a clean environment was maintained by frequent washing of the floor with water.

To assess mycorrhiza formation and sporulation in living pot cultures, 1-cm diameter × 10-cm deep (30 g) soil samples were taken approximately every 2 months using a small soil corer. Samples were processed, spores counted and roots assessed to determine the total length of mycorrhizas (Brundrett et al. 1999). On microscope slides made using randomly selected clover root segments from each sample, 100–200 random points per slide were examined under a compound microscope to identify the genera of VAM fungi within roots.

Results

Changes in soil nutrient levels (especially P) had a large impact on plant growth, substantially increasing root growth for both sorghum and clover (Figs. 1, 2). Shoot growth responses were similar to those for root growth (data not presented). Soil fertility also influenced the production of mycorrhizal roots and spores (potential propagules) in pot cultures. For clover, mycorrhiza formation, expressed as a proportion of root length, reached a peak at intermediate nutrient levels (Fig. 1A), but total root length was substantially greater at the highest fertility levels (Fig. 1B). This resulted in an increase in the overall length of mycorrhizal roots at these nutrient levels (Fig. 1C). Spores were not numerous at any nutrient level (Fig. 1D).

The effect of nutrient supply on mycorrhiza formation by sorghum was similar to that for clover, except that there was a lower proportion of mycorrhizal roots overall and a more pronounced decline in VAM colonisation at higher P levels (Fig. 2A). Sorghum root growth initially greatly exceeded rates of mycorrhiza formation at the highest nutrient levels (Fig. 2B), leading to slightly reduced overall length of mycorrhizal roots during early harvests (Fig. 2C). Spores were more numerous in sorghum than in clover pot cultures, reaching a peak at an intermediate nutrient level of 30% (Fig. 2D). The overall production of mycorrhizal root length was similar for clover and sorghum, but the

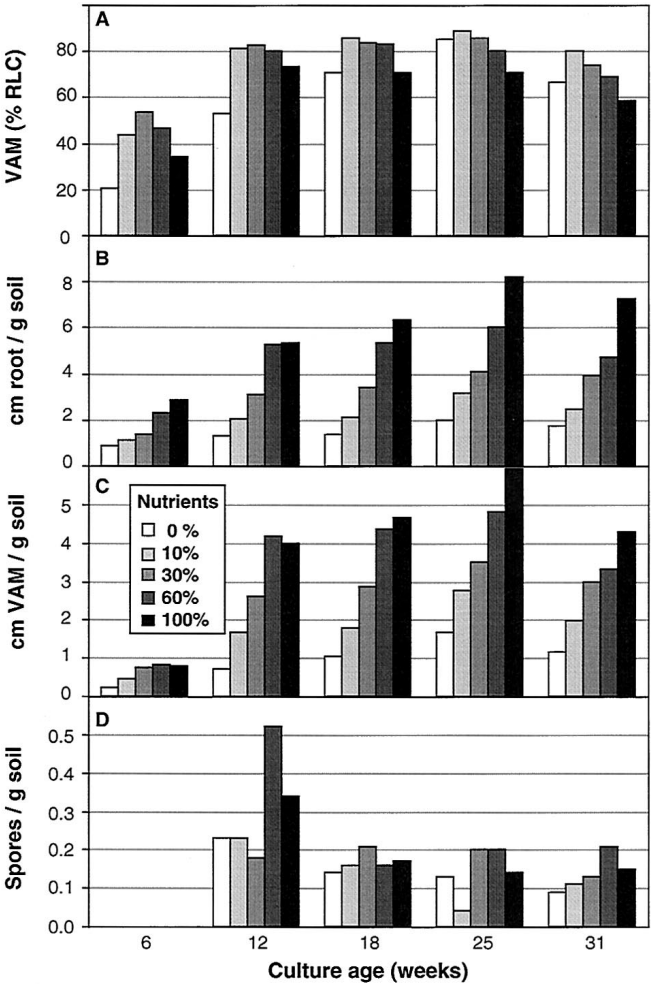

Fig. 1 The effect of increasing concentrations of soil nutrients in clover trap cultures sampled at different times on (**A**) the percentage of root length colonised (% RLC) by VAM fungi, (**B**) the total length of all roots (cm root/g soil), (**C**) the total length of mycorrhizal roots (cm VAM/g soil) and (**D**) spore production (spores/g soil). Data are the means for five soil types

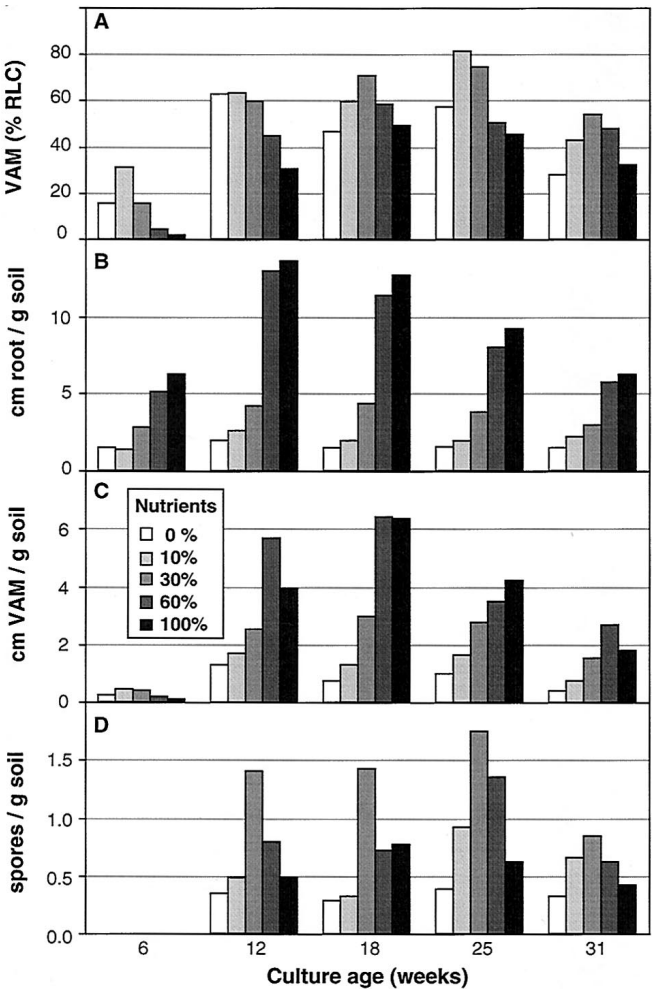

Fig. 2 The effect of increasing concentrations of soil nutrients in sorghum trap cultures sampled at different times on (**A**) the percentage of root length colonised (% RLC) by VAM fungi, (**B**) the total length of all roots, (C) the total length of mycorrhizal roots and (**D**) spore production. Data are the means for five soil types. Units as in Fig. 1

mycorrhizal biomass for clover was probably larger as clover roots were thicker.

Spore production was very limited in all samples until 12 weeks, when there was a modest increase in sporulation, especially in pots with sorghum (Figs. 1D, 2D). Limited quantities of spores were recovered in the small soil core samples used, but it was still possible to identify the main sporulating fungi. In all samples from both clover and sorghum trap cultures, spores were produced by a very low diversity of fungi. In both hosts, these were dominated by one *Scutellospora* species (*S.* sp. 7 in Brundrett et al. 1999) and one *Acaulospora* species (*A.* sp. 5), each of which produced approximately 43% of all spores in sorghum cultures. The remaining sporulation (14%) was primarily by other species of *Scutellospora*. The two species listed above were often found in trap cultures started using soil or roots from the same habitats, but were not observed as spores from field soils (Brundrett et al. 1999). Despite substantial levels of colonisation in roots, there was no sporulation by *Glomus* in pots. However, *Glomus* vesicles were often abundant in roots and had a similar structure to spores produced by these species after 2 or 3 culture generations (cycles). Spore numbers in pot cultures did not continue to increase in late harvests, and the incidence of spore loss by parasitism increased with time. Parasitism was evidenced by discolouration and/or changes in the appearance of spore contents and eventually resulted in the collapse of many spores.

The identity of mycorrhizal fungi within roots was only determined for clover (Fig. 3), because identification of fungi in thin sorghum roots proved too difficult. There were large differences in the proportions of roots colonised by genera of VAM fungi between the five soils/habitats. Colonisation by *Scutellospora* spp. was greatest in soils B and C, while *Glomus* spp. were most abundant in soils D and E. *Acaulospora* spp. were more prevalent in soils A and B than in other soils (Fig. 3).

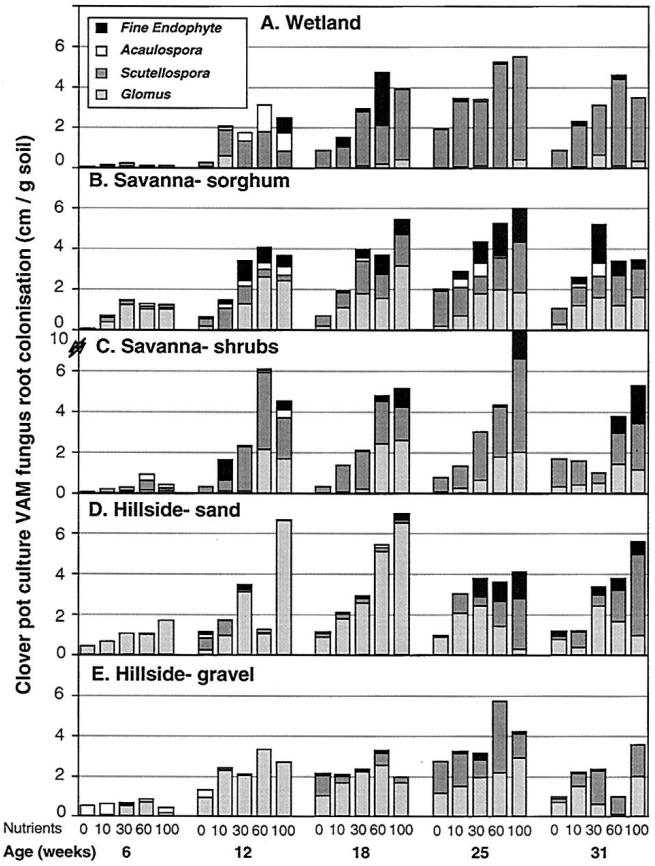

Fig. 3A–E Change with time in mycorrhiza formation by different genera of VAM fungi with varying levels of soil nutrients (0–100%). Results are from clover pot cultures started using soil from five different habitats (**A**–**E**) in tropical Australia

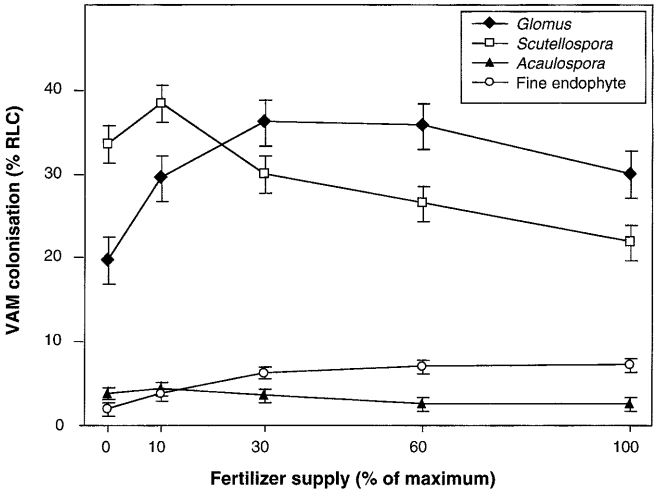

Fig. 4 Change in the proportion of roots colonised by different fungi with fertilizer supply averaged for all sampling times (*RLC* root length colonised, *error bars* SE of the group mean)

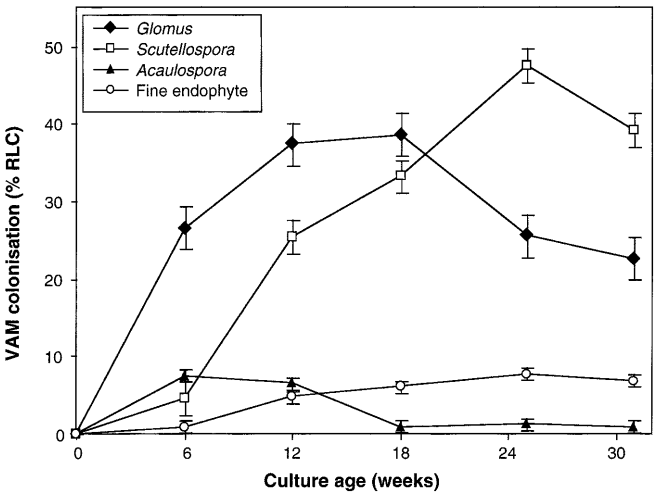

Fig. 5 Change in the proportion of roots colonised by different fungi with time averaged for all fertilizer levels (*RLC* root length colonised, *error bars* SE of the group mean)

Figures 4 and 5 show averaged results for all five soil types to illustrate general trends. Overall, there was a moderate reduction in the proportion of roots colonised by the genera *Scutellospora, Glomus*, and *Acaulospora* at higher rates of fertilizer supply, but fine endophyte colonisation was not adversely effected by higher nutrient levels (Fig. 4). Root colonisation was dominated by *Glomus* and *Scutellospora* and nutrient supply influenced the proportion of roots occupied by these genera. At the lowest fertility levels, *Scutellospora* occupied a greater proportion of roots than *Glomus*, but this was reversed at higher soil fertility levels (30–100%).

The proportion of roots occupied by different fungal genera changed with time (Fig. 5). Colonisation by *Glomus* increased more rapidly than other genera, reached a plateau after 12 weeks and then declined. There was a 1- to 2-month lag in *Scutellospora* colonisation then a rapid increase. *Scutellospora* occupied more roots than *Glomus* after 18 weeks. Colonisation by *Acaulospora* and fine endophyte averaged less than 10% of root length at all times. *Acaulospora* increased initially then declined after 12 weeks. The presence of fine endophyte in pots was sporadic and probably resulted from contamination, as this fungus was rare in root samples from the field. Overall, there was a clear successional trend within roots, resulting in the partial replacement of *Glomus* by *Scutellospora*.

Statistical analysis using soils types as replicates indicated a significant effect of nutrient levels on the length of roots occupied by all categories of fungi except *Acaulospora* and changes in root colonization with time were highly significant for all fungi (ANOVA P range <0.001–0.014). There were no significant time × nutrient level interactions.

Discussion

When changes in the relative abundance of different genera of glomalean fungi in roots were quantified, differences between soils were found to be more impor-

tant than the influence of host plants or extremes in soil fertility. Increasing levels of fertilizers applied to plants substantially increased root growth, but only caused a minor reduction in colonisation by VAM fungi. Very high levels of P in soil and plants can inhibit mycorrhiza formation (Menge et al. 1978; Douds and Schenck 1990), but these effects occur at much higher nutrient levels than those used in the present study. In this study, changes in root colonisation suggest that *Scutellospora* was favoured by higher nutrient levels, while *Glomus* was more competitive at lower fertility levels. Fertilizer levels can be optimised for mycorrhizal root production, or sporulation, but not for both, as maxima for these can occur at different nutrient levels. The use of P levels lower than that required for maximal plant growth was found to have practical advantages. Smaller plants were produced which required less frequent watering and did not outgrow root space in pots or reach reproductive maturity as rapidly as plants provided with more fertilizer.

Sampling over time revealed successional changes in fungal dominance in soil trap cultures: 1) *Glomus* root colonisation increased more rapidly than that by other genera, reached a plateau and then declined, 2) *Scutellospora* colonisation began slowly and then increased in importance, eventually overtaking *Glomus*, 3) moderate early *Acaulospora* colonisation occurred then declined at later harvests. Rapid initial *Glomus* colonisation probably resulted from the dominance of propagules of these fungi in soils. Living *Glomus* spores were not observed in most soils used, but root fragments or other propagules were likely abundant. Another possible explanation for the slow initial VAM development by *Acaulospora* and *Scutellospora* is delayed germination by their propagules. Healthy looking spores of these fungi were common in soils and may have been important propagules. Delayed germination has been reported for some Australian *Acaulospora* species (Tommerup 1992), but not for *Scutellospora*. Storage of dry soil for 7 months before use should have ensured that spores were not dormant. *Scutellospora* and *Acaulospora* may fail to grow from dried root fragments (Biermann and Linderman 1983; Brundrett et al. 1999) or hyphae in dry soil (Jasper et al. 1993) and thus may depend on spores as inoculum. More information about the types of propagule formed by different VAM fungi, their relative abundance in soils, responses to environmental conditions and efficiency at mycorrhizal formation will help explain the observed functional differences between groups of glomalean fungal.

The increasing dominance by *Scutellospora* in older cultures may reflect their higher competitiveness over *Glomus* under these conditions. In an experimental study, root colonisation by an isolate of *Scutellospora* was found to inhibit colonisation by an isolate of *Glomus*, even when they were physically separated by a split-pot system (Pearson et al. 1993). Limitations in the availability of carbon from the plant to fungi competing for space in roots may result in a competitive advantage for fungi that are initially most successful and suppression of other "less aggressive" fungi. However, more direct interaction between competing fungi cannot be ruled out. Changes in the relative dominance of different groups of fungi within roots over time may also reflect different phenological or reproductive strategies by fungi. This may divert energy to reproductive activities, result in quiescence at different times, or different responses to environmental factors. Fungi may also be affected by changes in the physiology of host plants, e.g. reproductive maturation towards the end of an experiment may divert energy from roots once flowering has commenced. Changes in fungus diversity with time suggest that selecting the age of pot cultures is one way of controlling the outcome of isolation experiments by favouring fungi which are dominant earlier or later in the culture cycle. However, there are problems keeping cultures growing for prolonged periods, e.g. the incidence of spore parasitism increases with time and sorghum plants tend to stop growing after flowering. Parasitism of fungal spores is common in soils (Daniels and Menge 1980; Lee and Koske 1994) and is, therefore a potential problem in pot cultures.

The diversity of fungi sporulating in trap cultures was very low and did not reflect the diversity of fungi present in roots. Spores of two fungi (an *Acaulospora* and a *Scutellospora* species) were very rare in field soils from these habitats, but were common in pot cultures started from the same soil samples (Brundrett et al. 1999). Fungi which sporulate rapidly in pot cultures may be more aggressive colonisers of roots, and/or are faster to adjust to changes in soil conditions than other fungi. They are thus ecologically similar to weedy plants that rapidly invade new habitats, but are later often out-competed by other species. Morton et al. (1993) and Stutz and Morton (1996) reported that additional fungi sporulated in second and third pot culture cycles started using soil from the original cultures.

A link between mycorrhizal colonisation level and timing of sporulation has been observed in experiments with single isolates of VAM fungi. The observations suggest that: (i) colonised root length can be used to predict sporulation (Douds 1994), (ii) there may be minimal colonisation levels for sporulation (Gazey et al. 1992), (iii) mycorrhiza formation may decline after sporulation begins (Pearson and Schweiger 1993), and (iv) the viability of hyphae in soil may also decline after sporulation (Jasper et al. 1993). Most of these experiments used isolates of *Acaulospora* and *Scutellospora* sporulating readily in culture and the results may not be applicable to other fungi with different life cycles. Mycorrhiza formation by *Glomus* species in the current study was observed to decline after 18 weeks. This decline was not related to spore production in soil, but may have been associated with the gradual formation and maturation of vesicles within roots. These vesicles were structurally similar to spores produced in older cultures and probably function as the main perenniating structure.

Analysis of the patterns of mycorrhizal colonisation within clover roots allowed the effects of experimental factors on fungal diversity to be investigated without reliance on spore production. This was especially important because spore production by glomalean fungi in first-generation cultures did not accurately reflect the composition of VAM fungus populations within roots. The analysis of morphology patterns is an extremely valuable tool for mycorrhizal research and has allowed the relative importance of different glomalean fungi to be established in natural ecosystems (McGee 1989; Thomson et al. 1992; Brundrett and Abbott 1995; Merryweather and Fitter 1998) and in glasshouse experiments (Abbott and Robson 1984; Lopez-Aguillon and Mosse 1987; Pearson et al. 1993). This method provides more reliable information about fungal populations than the quantification of spores, because important species in soils may not sporulate and there are very large differences in levels of spore production between species (Brundrett et al. 1999). Species-level identification of fungi in roots is possible in some cases (Abbott 1982; McGee 1989; Merryweather and Fitter 1998).

Grasses like sorghum are generally considered to be better host plants for inoculum production because they produce a greater length of mycorrhizal roots, which should result in greater sporulation than with other hosts (Simpson and Daft 1990; Douds 1994). When the two host plants were compared, sorghum generally produced more spores in a given volume of soil than clover, but the length of mycorrhizal roots was similar for both hosts. Sorghum root growth responded more dramatically to increased fertilization than clover. The highest nutrient levels resulted in sorghum root growth that initially exceeded rates of mycorrhiza formation, while mycorrhizal formation in clover was better able to keep pace with increases in root production. In a number of experiments (Brundrett et al. 1999), sorghum has been found to be the most successful host for pot cultures in the summer, but clover grew better during winter months, when low glasshouse light and temperature levels affected sorghum more than clover. We now routinely use sorghum grown at a soil P level capable of sustaining 60% of maximal growth as a host plant for pot cultures.

Results of isolation experiments provide evidence for biological (functional) diversity within the glomales (Brundrett et al. 1999). The greatest functional division appears to separate fungi which sporulate readily, including many species of *Scutellospora* and *Acaulospora*, from those which sporulate infrequently and rely on other propagules, including some isolates of *Glomus*. There is much to learn about the basic biology of glomalean fungi, their life-cycles in soils and how these vary between fungi and habitats.

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