Harnessing native Fabaceae for agriculture - the importance of mycorrhizal fungi

Final Report to the Australian Flora Foundation

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Plate 1: Project field site at Katanning, WA.

Abstract

Australian native perennial *Fabaceae* have been little explored with regard to their root biology and the role played by arbuscular mycorrhizal (AM) fungi in their establishment, nutrition and long-term health. Some of these species, notably *Cullen*, are now being evaluated for use in agricultural systems. As Australian agricultural soils generally have elevated levels of P, it is likely that the mechanisms naturally used by the legumes, including their symbiosis with AMF, will be disrupted. We hypothesised that native legumes, grown in an agricultural soil, would host a different set of species of AMF than exotic legumes. We therefore investigated the colonisation morphology in roots and the AM fungi, identified by spores extracted from rhizosphere soil, of the native legumes *Cullen australasicum*, *C. tenax* and *Lotus australis* and the exotic legumes *L. pedunculatus* and *Medicago sativa*. The level and density of colonisation by AM fungi, and the frequency of intraradical and extraradical hyphae, arbuscules, intraradical spores and hyphal coils all differed between host plants. However, none of these measures consistently differed between the native and exotic legume species. Instead, there were strong similarities between species in the same genus. The three dominant species of AM fungi in rhizosphere soil differed with host plant, but one fungus (*Glomus mosseae*) was always the most dominant. Sub-dominant species of AMF were the same between species in the same genus. No consistent differences in dominant spores were observed between the exotic and native legume species. Our results suggest that plant host influences the mycorrhizal community in the rhizosphere soil and that structural and functional differences in the symbiosis may occur at the plant genus level, not the species level or due to plant provenance. When the non-dominant species are considered there was a remarkably high species diversity of around twenty species. If these represent a remnant population of native AMF, they could provide a springboard for the regeneration of a more natural symbiotic system as native plants are re-introduced and the effects of agriculture, such as high available P, are diminished over time.

Introduction

Arbuscular mycorrhizal (AM) symbioses have a crucial role in the phosphate nutrition of many plants and evidence is emerging that they play a critical but poorly recognised role in land plant evolution (Wang and Qiu 2006) and land plant biodiversity, succession and productivity (van der Heijden et al. 1998: Klironomos et al. 2000; Klironomos 2003). The processes controlling the co-evolution of, and interactions among, the diverse communities of plants and AM fungi that exist together in natural communities seem to be considerably changed in industrialised agricultural systems, resulting in a reduction or change of AMF species (Daniell et al. 2001).

We are interested in the interaction between undomesticated plants native to a low fertility environment and the, presumably depauperate, suite of AM fungi found in local agricultural systems. This interest has arisen because a large national effort is underway in Australia to identify new perennial legumes with potential for development as pastures and several native species show promise (Bennett et al. 2006; Denton et al. 2006; Robinson et al. 2006).

A major difference between the native environment of Australian perennial legumes and agricultural environments will be the higher availability of soil phosphate in the latter. In this study we addressed preliminary issues relevant to increasing our understanding of the impact on undomesticated Australian native perennial legumes of being planted into agricultural soils. Thus for three native and two exotic legumes growing in agricultural soil we investigated if: there were structural differences in mycorrhizas; the community of AM species sporulating in rhizosphere soil varied; and, whether these aspects differed for native and exotic hosts.

Materials and Methods

Roots of three native undomesticated legumes, *Cullen australasicum*, *C. tenax* and *Lotus australis* and two exotic legumes, *L. pedunculatus* and *Medicago sativa* (Table 1), were sampled from a field trial established in 2005 at Katanning, Western Australia (33.69 ºS,

117.56 ºE, 310 m elevation, 479 mm average annual rainfall, winter dominant). The trial had been established in a field that had previously been under a long-term annual pasture and crop rotation (recent history 2002 pasture, 2003 field peas, 2004 wheat, 2005 perennial legumes sown). The site consisted of replicate plots of single species of perennial members of the *Fabaceae* sown in 4m strips and spaced 4m apart. The plots also contained naturally occurring weed species, in particular they were heavily invaded by non-native grasses and *Romulea rosea* (Guildford or Onion grass). Three samples were taken on 11 October 2006 of roots and rhizosphere soils from each of three replicate plots of each of the five species (one plot of *C. australasicum* had only one sample taken). To ensure the roots were not from weeds, each sample was traced back to its origin on the plant. The soil was slightly acidic (pH 5.5 in water) and mean bicarbonate extractable phosphate (Rayment and Higginson 1992) was 55 mg kg^{-1} across the plots.

Table 1 – Characteristics of the five perennial legumes sampled as part of the experiment.

	Accession no.	Background	Origin	Winter growth activity	Habit
Cullen australasicum	SA 4966	Wild accession	Australia	Moderate	Erect, multistemmed shrub
Cullen tenax	SA 35778	Wild accession	Australia	Moderate	Spreading, herbaceous
Lotus australis	SA 33610	Wild accession	Australia	Low	Low, herbaceous
Lotus pedunculatus	SA 12952	Wild accession	Tunisia	High	Low, herbaceous
Medicago sativa	SA 36325	Cultivar - “Sceptre	Exotic	High	Erect, multistemmed

Roots were carefully removed, thoroughly washed in tapwater and cleared in 10% (w/v) KOH by heating to approximately 90°C in a water bath for 30-60 min. The cooled root samples were washed, acidified in 1% HCl, and cut into ca. 1.0 cm segments and stained with methyl blue in acidified glycerol. The magnified intersections method was used for assessment of colonisation characteristics (McGonigle *et al.* 1990). Four microscope slides were made from the roots of each sample with 20 root segments mounted in acidified glycerol on each slide.

A total of 25 intersections between roots and an eyepiece crosshair arranged perpendicular to the root axis were observed for each slide at ×200 magnification. At each intersection between the root and the crosshair the incidences of intraradical hyphae, arbuscules, intraradical spores (thick-walled structures, often occluded by a septum or plug, typical of those found in *Glomus intraradices*), hyphal coils, vesicles (thin-walled sac-like structures lacking occlusion, typical of fungi in the genus *Acaulospora*), entry points and external hyphae were recorded to calculate the percentage incidence of each structure over total colonised intersections. Total proportion of root length that was colonised was based on the presence of any mycorrhizal structure.

Soils of each sample were mixed by shaking the sample bag prior to taking ca. 25 g to extract spores. Wet sieving and sucrose centrifugation were used to extract spores (Walker *et al*. 1982). Morphological features of each type of spore present were described from spores mounted in polyvinyl alcohol lacto-glycerol, with or without the addition of Melzer's reagent (4:1 v/v) (Morton, 1988; Walker *et al*. 2007). One-way ANOVAs were performed with SPSS 11.0. Percentage data were first transformed using arcsin. Differences between legume species were tested using Duncan's Multiple Range Comparison.

Results

All legume species were well colonised by AM fungi (Table 2). No clear differences were observed between the native and exotic species for percentage of root length colonised, although colonisation was highest for the native *C. australasicum* and lowest for the exotic *M. sativa* (Table 2). Density of colonisation was highest for the two *Lotus* species, which reflected a higher density of intraradical hyphae than found in the other three species (Figure 1). Intraradical spores were common in all species with an average of 1-1.5 per colonised root crosshair intersection (Figure 1). Arbuscules were markedly less common in *M. sativa* than the other species (Figure 1). Hyphal coils were uncommon in all species but did occur at a higher density in the two *Lotus* species (Table 2). Vesicles and entry points were also quite uncommon and their occurrence did not differ among species (Table 2). Extraradical

Table 2 – Percentage of root length colonised by AM fungi and density of hyphal coils, vesicles, entry points and extraradical hyphae (number per colonised root crosshair intersection). Density of intraradical hyphae, arbuscules and spores is shown in Figure 1. For each column, values followed by a different letter are significantly different at P<0.05.

	Root length colonised (%)	Hyphal coils	Vesicles	Entry points	Extraradical hyphae	Ratio spores/arbuscules
Cullen australasicum	75 [a]	0.003 [b]	0.013	0.007	0.824 [b]	2.09 [b]
Cullen tenax	52 [b]	0.002 [b]	0.000	0.049	0.836 [b]	2.32 [b]
Lotus australis	49 [b]	0.091 [a]	0.014	0.107	1.966 [a]	1.71 [b]
Lotus pedunculatus	51 [b]	0.111 [a]	0.000	0.102	1.482 [ab]	1.95 [b]
Medicago sativa	31 [c]	0.004 [b]	0.092	0.056	1.211 [ab]	8.47 [a]

Table 3 – Average number of morphologically distinct species of AM fungi and the three dominantly sporulating species of AM fungi present in rhizosphere soil for five perennial legumes (determined from spores). Average number of species of AM fungi per plot did not differ between host plants at P<0.05. The total number of species observed under the five host plants was 25.

	Number of AM fungal species		Dominant species		
	Per plot[a]	Per plant[b]	First	Second	Third
Cullen australasicum	11[c]	16	*Glomus mosseae*	*G. cf.* 'monosporum'	No species clearly dominant
Cullen tenax	7	13	*G. mosseae*	*G. cf. 'monosporum'*	No species clearly dominant
Lotus australis	7	11	*G. mosseae*	*Archaeospora trappei ?*	*G. cf* 'monosporum'
Lotus pedunculatus	7	12	*G. mosseae*	*Ar. trappei ?*	*G. cf.* 'monosporum'
Medicago sativa	7	12	*G. mosseae*	*G. cf* 'monosporum'	*Ac.* sp.

a Results from the three replicate samples per plot were combined
b Results from the three replicate plots of each species were combined
c *C. australasicum* had only one replicate, not three, in one of the plots. This plot was included as missing data in the analysis on number of AM fungal species per plot

hyphae were present, on average, at most colonised intersections and were least abundant in the two *Cullen* species and most common in *L. pedunculatus* and *M. sativa* (Table 2). The ratio of spores to arbuscules was approximately four times higher for *M. sativa* than the other species (Table 2).

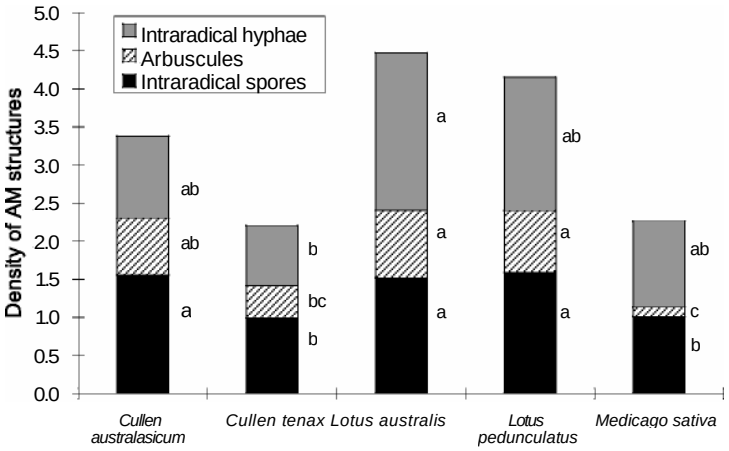

Figure 1 – Density of intraradical hyphae, arbuscules and intraradical spores (number per colonised root crosshair intersection). For each parameter, column segments followed by a different letter are significantly different at P<0.05.

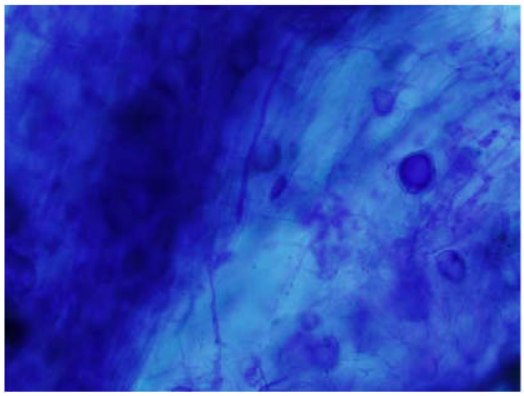

Plate 2 – Colonisation of *Cullen australasicum*. Intraradical spores, intraradical hyphae and finely branched hyphae are evident.

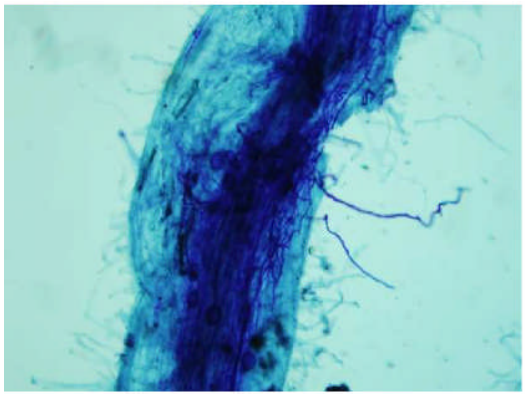

Plate 3 – Colonisation of *Lotus corniculatus*. Dark blue hyphae are evident entering the root through roots hairs and intraradical hyphae and intraradical spores are present in the root cortex.

Approximately 20 morphologically different AM fungi were identified from the extracted spores. No differences were observed in the number of AMF species present under each host plant (Table 3), but differences were apparent in the 2-3 species of AM fungi that were dominant. *Glomus mosseae* was the most common fungus present for all plants, with a fungus similar to, but probably not identical with, *G. monosporum*, also very common for all species (Table 3). While there was no dominant third species under *C. tenax*, a small, hyaline-spored fungus, probably *Archaeospora trappei*, was common under *L. australis* and *L. pedunculatus* and a probably undescribed *Acaulospora* sp. was common under *C. australasicum* and *M. sativa* (Table 1). The presence of less common species also differed among hosts (data not shown, for photomicrographs see Tibbett et al. 2008).

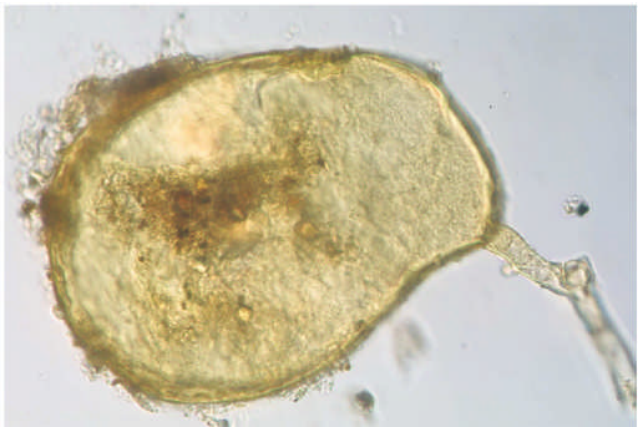

Plate 4 – Photomicrograph of a spore of *Glomus mosseae*.

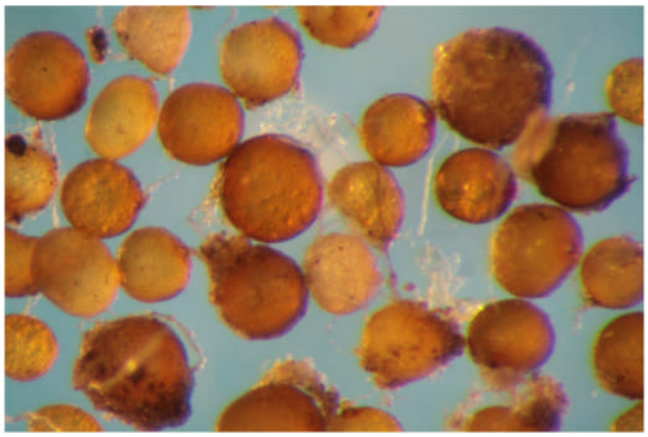

Plate 5 – Photomicrograph of spores and attached extraradical hyphae.

Discussion

Root colonisation morphology

The level and density of colonisation by AM fungi, and the frequency of intraradical and extraradical hyphae, arbuscules, intraradical spores and hyphal coils all differed among hosts. In general the data do not show a differentiation between the native and exotic perennial legumes. Indeed the species seem to group according to genera. This is particularly noticeable for the two *Lotus* species, one native and one exotic to Australia, with their similar percentage of root length colonised and similar density of all colonisation components that were measured. These similarities are also present for the two *Cullen* species. Although, *Cullen australasicum* had a higher percentage of root length colonised and a greater density of spores. Thus it seems that native legumes grown in agricultural soils will readily form arbuscular mycorrhizas with the species of AMF that are present in the soil. Whether these associations are as effective as the ones formed under natural conditions, remains to be investigated.

The host plants which differed most from all other species was *M. sativa* which had a very low density of arbuscules and consequently a high ratio of spores to arbuscules. Spores are likely to be full of energy rich lipids (Kinden and Brown 1975) which must have been formed

using carbon compounds of host plant origin (Ho and Trappe 1973; Pfeffer et al. 1999). This result could reflect a number of factors. Perhaps the arbuscules in *M. sativa* were present earlier in the season and had deteriorated, leaving hyphae and spores behind. Indeed, arbuscules have been found to have a lifecycle of only two days in annual species (Alexander et al. 1989). Alternatively, the different morphology of the colonisation in *M. sativa* may have reflected the impact of the host plant or a different suite of colonising fungi. While the spore data does not support the idea of *M. sativa* being colonised by predominantly different fungi to the other host species, other studies have shown that morphology of colonising AM fungi can be greatly affected by host plant (Cavagnaro et al. 2003, Burleigh et al. 2002).

It should be noted that this survey was carried out from a single sampling date, and thus does not take into account the dynamic nature of the mycorrhizal symbioses. Each host may have different interactions with the AMF, and such may be reflected by different phenologies (Pringle and Bever 2002). Further experimentation is required to explore this issue and to determine the potential contribution of AM fungi to the growth and P nutrition of these species.

Rhizosphere populations of spores

The community of AM fungi as explored through the three dominantly sporulating species also differed with host plant. Again, no consistent differences were observed between the exotic and native legumes, and the dominant spore type did not differ between the two *Lotus* species or the two *Cullen* species. Thus, in spite of the large diversity of AM fungal species present, greater than 20, the native and exotic species appeared to favour (as indicated by number of spores in their rhizospheres) the same species. Again, this suggests that native legumes, if grown agriculturally, will not experience difficulties in forming mycorrhizal symbioses with the species of AMF present in the soils.

The snapshot nature of this survey, taken as it was on a single date, can only be used in the broadest sense (Walker et al. 1982), as it cannot provide dynamic information. The survey was designed merely as a preliminary investigation that might lead to future work during which a more sophisticated analysis over time, space and plant species can be carried out. This will require isolation of AMF and the determination of species (including species-specific DNA sequence motifs) so that specific combinations of host and AMF can be compared. Changes to the symbiotic cohorts in WA paddocks are likely to have taken place over the many decades of agriculture. Identifying such changes, and reversing them if possible (and if desirable), would not be a trivial task, and would require a long-term commitment by the scientific and agronomic community alike.

The determination of species in this study was carried out by Chris Walker, with the benefit of his long experience with this group of fungi (Walker & Rhodes 1981; Walker 1983, Walker & Sanders 1986, Walker & Vestberg 1998, Schüßler et al. 2001, Walker et al. 2007). The expressions of doubt in identifying the species concerned represents the difficult, and still rather primitive, state of the taxonomy of the organisms in the *Glomeromycota*. There are many problems associated with identification of these organisms, not the least being the inadequate quality of species descriptions, and the very poor state of preservation of some of the type material, thus making it almost impossible to interpret the already poor descriptions in a modern framework of phylogenetic taxonomy. Consequently, the identifications are a combination of re-interpretations of protologue and other literature and more than three decades of observations of collections from around the world.

At least one of the fungi (a species in *Acaulospora*) is likely to be new to science, and the fungus described as similar to *G. monosporum* may also actually be an undescribed Australian fungus. These issues are further discussed in Tibbett et al. (2008).

The pattern of dominance in terms of species is in general agreement with other work that

shows a limited number of common species in agricultural environments worldwide. The predominant species throughout, *Glomus mosseae*, is frequently recorded as dominant in agricultural environments, and it seems this fungus is remarkably tolerant to the disruptive forces associated with farming. The second-most abundant species is also morphologically very closely related to *G. mosseae*, and probably belongs in the same clade. Fungi in this clade were among the first to be isolated and widely used experimentally, mostly from agricultural, horticultural or orchard environments (Mosse 1953; 1956). *Archaeospora trappei* has a worldwide distribution. It also was originally isolated and identified from horticulture (Ames & Linderman 1976) and is very common as an invasive pest of pot cultures of AMF (Walker, pers. obs.).

Conclusions

This work provides a platform for further analysis of the contribution of AMF to growth and nutrition of perennial legumes that might be used in Australian agricultural systems. When the non-dominant species are considered, there was a remarkably high species diversity. Compared with previous studies on Western Australian soils (*e.g.* Abbott and Robson 1977) the twenty or so species found is very high. Although their occurrence is somewhat patchy, if these represent a remnant population of native AMF, they could provide a springboard for the regeneration of a more natural symbiotic system as native plants are re-introduced and the effects of agriculture, such as high plant available P, are diminished over time. An in-depth study is now needed, including some investigation of native plants in their natural environment, and some longer term examination of the effects of re-introduction of both native plants and indigenous organisms isolated from native plants.

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