Plant Soil (2013) 373:699–710
DOI 10.1007/s11104-013-1838-2

REGULAR ARTICLE

Do arbuscular mycorrhizas or heterotrophic soil microbes contribute toward plant acquisition of a pulse of mineral phosphate?

Nazanin K. Nazeri · Hans Lambers · Mark Tibbett · Megan H. Ryan

Received: 21 May 2013 / Accepted: 2 July 2013 / Published online: 20 July 2013

Abstract
Aims We investigated the role of arbuscular mycorrhizal fungi (AMF) and heterotrophic soil microbes in the uptake of phosphorus (P) by *Trifolium subterraneum* from a pulse.
Methods Plants were grown in sterilised pasture field soil with a realistic level of available P. There were five treatments, two of which involved AMF: 1) unsterilised field soil containing a community of AMF and heterotrophic organisms; 2) *Scutellospora calospora* inoculum (AMF); 3) microbes added as filtrate from the field soil; 4) microbes added as filtrate from the *S. calospora* inoculum; 5) no additions, i.e. sterilised field soil. After 11 weeks, plants were harvested: 1 day before (day 0), 1 day after (day 2) and 7 days after (day 8) the pulse of P (10 mg kg^{-1}).
Results There was no difference among treatments in shoot and root dry weight, which increased from day 0 to day 8. At day 0, shoots and roots of plants in the colonised treatments had higher P and lower Mn concentrations. After the pulse, the rate of increase in P concentration in the shoots was slower for the colonised plants, and the root Mn concentration declined by up to 50 % by day 2.
Conclusions Plants colonised by AMF had a lower rate of increase in shoot P concentration after a pulse, perhaps because intraradical hyphae accumulated P and thus reduced its transport to the shoots.

Keywords Arbuscular mycorrhizal fungi · Heterotrophic soil microbes · Manganese · Phosphorus pulse · *Scutellospora calospora* · Shoot and root nutrient concentrations

Responsible Editor: Timothy Cavagnaro.

N. K. Nazeri · H. Lambers · M. H. Ryan
School of Plant Biology and Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

N. K. Nazeri · M. Tibbett · M. H. Ryan
Future Farm Industries Cooperative Research Centre, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

M. Tibbett
School of Earth and Environment, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

M. Tibbett
National Soil Resources Institute, Department of Environmental Science and Technology, School of Applied Sciences, Cranfield University, Cranfield MK43 0SZ England, UK

N. K. Nazeri (✉)
School of Plant Biology, M080, The University of Western Australia, Crawley, WA 6009, Australia
e-mail: nknazeri@gmail.com

Introduction

Arbuscular mycorrhizal fungi (AMF) have been repeatedly found to enhance plant phosphorus (P) uptake,

especially under low-P conditions (Smith et al. 2011; Koide and Mosse 2004). However, most of these studies were conducted under glasshouse conditions where plant-available P was kept constant or, presumably, gradually declined over time due to plant uptake. In the field, soil P may not be so constant, and in some situations plants may encounter a pulse of P. For instance, in a Mediterranean climate that features hot, dry summers (Lamont 1982), a pulse of P may be released into surface soil during the first autumn rains (Butterly et al. 2009). This pulse may result from P release from desiccated microbes, microbes rupturing due to osmotic shock in response to rapid rewetting of the soil and/or sudden fluctuations in soil matric potential, or rapid degradation of dry organic matter on the soil surface (Turner et al. 2002; Blackwell et al. 2010). For instance, Qiu et al. (2004) found low levels of anion exchange membrane-extractable P (AEM-P) (<2.6 mg P kg^{-1} dry soil) in surface soils (just under the litter layer) in south-western Australia before winter rainfall, but this increased 3.4–56 fold during winter; 30 % of this P increase occurred (from litter) in the first 24 h. Such a pulse also mimics the top-dressing of pasture fertiliser (Lewis et al. 1987). No studies have examined the role of AMF in capturing P from a sudden P pulse. However, the ability of AMF to store high concentrations of P in their hyphae, up to 600 mM, suggests that AMF may have the capacity to quickly absorb and store relatively large amounts of P without toxicity (Ryan et al. 2003).

A diverse community of more than 20 species of AMF may be present in field soil (Tibbett et al. 2008). Species of AMF may differ in length of external hyphae, and thus in their ability to absorb a pulse of P. Therefore, a similar percentage of root length colonised among species of AMF does not necessarily result in similar enhanced plant uptake of P or other nutrients (Jakobsen et al. 1992b; Graham et al. 1982). For example, external hyphae spread 81 mm for *Acaulospora laevis*, but only 32 mm for *Glomus* sp. and 31 mm for *Scutellospora calospora* after 28 days of growth (Jakobsen et al. 1992b). The greater spread by *A. laevis* had a positive effect on P concentration in roots which was 3.1 mg P kg^{-1} dry weight, compared with 1.7 mg P kg^{-1} dry weight for *Glomus* sp. and 2.0 mg P kg^{-1} dry weight for *S. calospora* (Jakobsen et al. 1992b). However, further studies suggested that *S. calospora* had a greater P-uptake capacity per unit of hyphal length than the other two species (Jakobsen et al. 1992a).

In view of the different capacities of species of AMF to enhance P uptake, it is not surprising that there are several reports of host-plant nutrient uptake and growth being most improved when more species of AMF are present (Van der Heijden et al. 1998; Wagg et al. 2011). To further test this, we compared the ability to capture a P pulse between inoculation with a single isolate of AMF of a species commonly used in glasshouse experiments (*S. calospora*) and inoculation with a field soil, which we assumed contained a diverse community of AMF.

Using a field soil as inoculum is not usual and involves introduction of not only AMF but also a diverse microbial community. Organisms in the soil other than AMF can enhance plant P uptake (Richardson et al. 2009; Barea et al. 2005; Gyaneshwar et al. 2002). Effects of soil microbes and AMF may be synergistic. For instance, Singh and Kapoor (1998) found higher P uptake when mungbean (*Vigna radiata*) inoculation by AMF was accompanied by P-solubilising microorganisms (PSM) than when plants were inoculated with AMF or PSM separately. Absence of microbes may result in overestimation of the role of AMF in host plant P-uptake. For instance, Hetrick et al. (1988) found ten times more ^{32}P was absorbed by plants inoculated with AMF when grown in sterilised soil (without the presence of microbes) than when grown in non-sterilised soil. They concluded that this reduction might be due to soil microorganisms limiting mycorrhizal activity in non-sterile soil (Hetrick et al. 1988). In response to these concerns, we thought it important to tease apart the effect of AMF and accompanying microbes for both field soil inoculum and single strain AM fungal inoculum.

This study was designed to address three hypotheses: 1) inoculation with AMF and heterotrophic microbes enhances plant uptake of P from a pulse of P; 2) that this effect is greater when plants are inoculated with a field soil containing a diverse community of AMF than when inoculated with a single strain pure culture; 3) inoculation with heterotrophic soil microbes alone enhances plant P uptake from a pulse compared to the sterile-control. These hypotheses were tested using *Trifolium subterraneum* and an agricultural field soil with a moderate level of bicarbonate-extractable P (Colwell P) (18 mg P kg^{-1} dry soil) (Colwell 1965). This soil was chosen as representative of pastures in Western Australia, although its P availability is slightly lower than average

values reported for pastures across agricultural areas in Western Australia (24–47 mg P kg^{-1} dry soil) (Weaver and Reed 1998; Scanlon 2011).

Materials and methods

Experimental design

The experiment compared the impact of a single pulse of P on mycorrhizal and non-mycorrhizal plants. It also considered the effect of the wider soil microbial community on the plants. There were five treatments (Table 1), three harvests (1 day before, and 1 and 7 days after adding the P pulse) and four replicates. Pots were arranged randomly in a temperature-controlled glasshouse (mean maximum temperature ~24 °C and mean minimum temperature ~12 °C) at the Crawley campus of The University of Western Australia, Perth, Australia from 22 June 2011 until 5 September 2011; i.e. over winter, when annual pastures of *T. subterraneum* L. cv. Denmark are actively growing in the field. Five treatments were applied to sterilised field soil: 1) addition of unsterilised field soil to supply soil microbes and AMF (Microbes + AMF (F)); 2) addition of *S. calospora* inoculum (sand, spores, roots) to supply pot culture microbes and AMF (Microbes +AMF (I)); 3) addition of field soil filtrate to supply microbes but not AMF (Microbes (F)); 4) addition of *S. calospora* filtrate to supply microbes but not AMF (Microbes (I)); and 5) no additions, i.e. sterilised field soil (no microbes or AMF added) (control-sterile) (Table 1).

Field soil characteristics

Soil was collected from the top 10 cm of a weedy *T. subterraneum* clover pasture at Newdegate (33° 06′ 16″ S, 118° 49′ 50″ E, 333 m elevation) in the southern cropping zone of Western Australia. The soil was analysed for physical and chemical characteristics at CSBP Future Farm analytical laboratories, Bibra Lake, Australia. Bicarbonate-extractable phosphorus (P) was 18 mg kg^{-1} and potassium (K) was 80 mg kg^{-1} (Colwell 1965). Available P measured using the Olsen test was 11 mg kg^{-1}. Mineral N consisted of 6 mg kg^{-1} ammonium-N and 10 mg kg^{-1} nitrate-N (Searle 1984). Available sulphur (S) was 9.3 mg kg^{-1} (Blair et al. 1991). Soil pH was 5.3 ($CaCl_2$) and 6.0 (H_2O). The soil consisted of 99.8 % sand (76.0 % coarse sand and 23.9 % fine sand), 0.05 % clay and 0.11 % silt. The soil contained sufficient inoculum to cause ~50 % colonisation of roots after 10 weeks of growth (according to a preliminary experiment). A similar soil from under a pasture in this region had a high diversity of AMF (Tibbett et al. 2008).

Mycorrhizal DNA was extracted and quantified from subsamples of the bulked field soil by the South Australian Research and Development Institute (SARDI) in Adelaide, South Australia. The methodology is described by Haling et al. (2011) and Simpson et al. (2011). The field soil contained three groups of AMF; Group A (0.2 %, includes *Glomus mosseae, G. constrictum, G. coronatum, G. geosporum, G. verruculosum, G. caledonium* and *G. fragilistratum*), Group B (97 %, includes *G. claroideum* and *G. etunicatum*) and Group E (3 %, includes *Acaulospora*

Table 1 The five treatments applied to sterilised field soil

Treatment	Sterilised field soil only	Unsterile soil/inoculum/ filtrate added	AMF present
Field soil filtrate[a] (field soil microbes) – Microbes (F)	No	Yes	No
Field soil (complete soil microbial community including AMF) – Microbes +AMF (F)	No	Yes	Yes
Single strain inoculum filtrate (inoculum microbes) – Microbes (I)	No	Yes	No
Single strain inoculum (complete inoculum microbial community including *S. calospora*) – Microbes +AMF (I)	No	Yes	Yes
Sterilised soil – Control-sterile	Yes	No	No

[a] Filtrate of the field soil and *S. calospora* pure culture was made by passing a mixture of 1.5 L of deionised water and 500 g of unsterilised field soil or *S. calospora* pure culture through a series of sieves, from 1,000 μm up to 50 μm

laevis and *Entrophospora* spp.). Group C2, which includes *S. calospora*, was absent.

To remove AMF, field soil was steam sterilised at 80 °C for 1 h on consecutive days and then dried in a sterile environment overnight. The sterilised field soil contained 21 mg kg^{-1} bicarbonate-extractable P and 62 mg kg^{-1} of K (Colwell), 22 mg kg^{-1} ammonium-N and 5 mg kg^{-1} nitrate-N (Searle 1984).

Production of *Scutellospora calospora* inoculum

The inoculum of *S. calospora* was prepared as follows. Lancelin sand was steamed at 80 °C for 1 h each on two successive days and dried overnight at 100 °C. The dried Lancelin sand contained 10 mg kg^{-1} bicarbonate-extractable P and 32 mg kg^{-1} K (Colwell), 3 mg kg^{-1} ammonium-N, <1 mg kg^{-1} nitrate-N (Searle 1984) and had a pH ($CaCl_2$) of 5.0. A P-free complete nutrient solution was added to the Lancelin sand. The Lancelin sand was inoculated with *S. calospora* (WUM 12(3)) originally isolated from Badgingarra, Western Australia. Leek (*Allium porrum* L.) was used as the host plant.

Production of filtrate

Filtrate of the field soil and the *S. calospora* pure culture was made by passing a mixture of 1.5 L deionised water and 500 g unsterilised field soil or *S. calospora* pure culture through a series of sieves from 1,000 μm up to 50 μm. This filtrate was assumed to contain no mycorrhizal hyphae or spores, but to contain most other soil microbial biota (Hetrick et al. 1988).

Experimental setup and harvest

White non-draining pots were filled with 1.2 kg of soil. For the Microbes (F) treatment, 1.2 kg of sterile field soil was added to the pots and 20 ml of field soil filtrate was added to the top of the soil. For the microbes+AMF (F) treatment, the bottom half of pots was filled with sterilised field soil (600 g) and the top half was filled with a 50:50 mixture of sterilised field soil and field soil (300 g each). Field soil filtrate (20 ml) was added to the top of the soil. For the Microbes (I) treatment, 1.2 kg of sterilised soil was added to the pots and 20 ml of *S. calospora* pure culture filtrate was added to the top of the soil. For the microbes+AMF (I) treatment, the bottom half of the pots was filled with sterilised field soil (600 g) and the top half was filled with a 1:5 mixture of *S. calospora* pure culture (125 g) and sterilised field soil (475 g); 20 ml of *S. calospora* pure culture filtrate was added to the top of the soil. The control-sterile pots were filled with 1.2 kg of sterilised field soil with no added filtrate.

Seeds of *T. subterraneum* cv. Denmark were scarified using sandpaper and left in deionised water overnight. Imbibed seeds were then kept on moist filter paper in Petri dishes for 3–4 days until germinated. Six days after filtrates were added, one seedling of *T. subterraneum* was planted in each pot, and pots were then watered to field capacity by weight twice a week. After one week, a dense suspension of an appropriate strain of *Rhizobium trifolii* was added to the pots. Commencing at two weeks, 12 ml of Long Ashton nutrient solution (P-free) was added to each pot fortnightly. This solution contained 10 ml of 2 mM K_2SO_4, 10 ml of 1.5 mM $MgSO_4$, 10 ml of 3 mM $Cacl_2$, 10 ml of 4 mM $(NH_4)_2SO_4$, 1 ml of 0.1 mM Fe EDTA, 1 ml of micronutrients solution (i.e. 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$; 0.22 g $ZnSo_4.7H_2O$; 0.08 g $CuSo_4.5H_2O$; 0.025 g $Na_2MoO_4.2H_2O$ in one litre of deionised water) and 10 ml of 8 mM $NaNO_3$ in a litre of deionised (DI) water.

The first harvest (day 0) occurred on 5 September 2011 (week 11). Shoots were cut at the soil surface, rinsed once in DI water, dried for 72 h at 70 °C, weighed and then finely ground for P digestion. Roots were washed thoroughly, rinsed in DI water, dabbed dry with paper towels and fresh weights measured. A subsample of ~3 g was taken from each root system, fresh weight recorded, and placed in 70 % ethanol for assessment of mycorrhizal colonisation. The remaining roots were dried at 70 °C for 72 h and weighed.

A day after the first harvest (6 September), 10 mg P kg^{-1} of soil was added to all pots as KH_2PO_4 in 20 ml of solution. This is equivalent to top-dressing a pasture with 6.79 kg P ha^{-1} which is a moderate rate of P fertiliser. The second harvest (day 2) occurred 24 h later (7 September) as described above. The final harvest (day 8) occurred 7 days after adding the P pulse (13 September) as described above.

Root mass ratio was calculated as:

$$\text{Root mass ratio} = \text{Root dry weight}/(\text{root dry weight} + \text{shoot dry weight})$$

Roots to be assessed for colonisation by AMF were cleared in 10 % (w/v) KOH at room temperature for

3 days, then washed thoroughly with DI water and stained in 5 % Shaeffer black ink for 2 h. For destaining, roots were washed in DI water and stored in lactoglycerol (1:1:2 (v/v/v) lactic acid, deionised water, glycerol) (Vierheilig et al. 1998). The percentage of root length colonised by AMF was assessed using the line intersect method for at least 100 intersections per sample (Giovannetti and Mosse 1980).

Subsamples of ground root and shoot materials of ~0.2 g were digested in a 3:1 (v/v) HNO_3:$HClO_4$ mix and analysed using inductively-coupled plasma (ICP) atomic absorption with a Perkin Elmer Optima 5300 DV optical emission spectrometer (OES; Shelton, CT, USA).

Statistical analyses

The experiment was a fully randomised design which was analysed in Genstat version 9.2 using general ANOVA to assess the effect of treatment (Microbes + AMF (F), Microbes +AMF (I), Microbes (F), Microbes (I), control-sterile) and harvest (day 0, day 2, day 8) on response variables. All interactions were examined. Normality was checked and no transformations were required. For a small number of variables (root dry weight, root-shoot ratio, shoot Fe and Cu, root K, Zn and Fe), up to three outliers were removed. In general, the interaction of treatment and harvest is presented when significant at $P<0.05$, along with the LSD at $P=0.05$. If the interaction was not significant, the marginal means for treatment and harvest are presented along with their associated LSD at $P=0.05$ if they were significant at $P<0.05$.

Results

Colonisation by AMF

At the harvest in week 11, all plants inoculated with AMF (i.e. microbes +AMF (F) and microbes +AMF (I)) were highly colonised by AMF, while all other plants were uncolonised (Table 2). There was no difference in the percentage of root length colonised between plants inoculated with field soil (44 %) and with *S. calospora* (47 %). Harvest day affected percentage of root length colonised as colonisation decreased by ~13 percentage points between day 0 and day 8 (Table 2).

Plant growth

There was no treatment effect on shoot and root dry weights or root mass ratio at the harvest in week 11 (Table 2). However, shoot and root dry weights increased between day 0 and day 8; shoot weight by 47 % and root weight by 44 %.

Phosphorus

For shoot P concentration, there was an interaction between harvest day and treatment (Fig. 1a). The two mycorrhizal treatments had a higher shoot P concentration at day 0 and day 2 than the other treatments (~0.4 mg P g^{-1} higher at day 0 and ~0.5 mg P g^{-1} higher at day 2). However, shoot P concentrations in the control and microbes treatments increased rapidly between days 2 and 8; by day 8 all treatments had similar shoot P concentrations.

Root P concentration was consistently higher than shoot P concentration. For root P concentration, there was an effect of harvest day and treatment, but no interaction was found (Table 2). However, results were graphed to allow easily comparison with shoot P concentration (Fig. 1b). The two colonised treatments had a P concentration around ~1.4 times higher than the other treatments at day 0 and this difference was maintained over time. For shoot P content, there was no effect of treatment and no interaction between harvest day and treatment (Table 2). Whilst there was a trend towards higher shoot P content at day 0 in the mycorrhizal treatments, this was not significant and by day 8, by which time shoot P content had nearly doubled, shoot P content was very similar among treatments. Root P content was affected by both treatment and harvest day, being higher in the mycorrhizal treatments and increasing ~2.7 times from day 0 to day 8 (Table 2). Total plant P content was also affected by treatment and harvest time (Table 2).

Manganese

For shoot Mn concentration, there was an effect of treatment and harvest day as the two mycorrhizal treatments had consistently lower Mn concentrations from day 0 to day 8 and Mn concentration increased slightly, from day 0 to day 8 (Table 3, Fig. 2a; interaction presented to allow easy comparison with root Mn).

Table 2 Marginal means for measures of *Trifolium subterraneum* growth for treatment (control-sterile, Microbes+AMF (F), Microbes+AMF (I), Microbes (F), Microbes (I)) and harvest (day 0, day 2 and day 8) for which there was no interaction (ns, no significant difference)

	AMF%[a]	Shoot dry weight (g)	Root dry weight (g)	Root mass ratio	Shoot P content (mg)	Root P content (mg)	Root P concentration (mg g^{-1})	Plant P content (mg)
Treatment								
Control-sterile	Uncolonised	1.43	0.70	32.9	3.71	3.05	4.19	6.76
Microbes+AMF (F)	46.4	1.52	0.63	30.2	4.05	4.15	6.43	8.21
Microbes+AMF (I)	48.7	1.45	0.66	31.1	4.11	4.36	6.34	8.47
Microbes (F)	Uncolonised	1.51	0.70	32.1	3.74	3.29	4.47	7.03
Microbes (I)	Uncolonised	1.53	0.70	32.1	3.73	3.18	4.28	7.05
LSD at P=0.05	ns	ns	ns	ns	ns	0.44	0.4	0.8
P-value	–	–	–	–	–	<0.001	<0.001	<0.001
Harvest								
Day 0 (no added P)	50.7	1.23	0.56	31.9	2.71	2.06	3.67	4.85
Day 1 (P added)								
Day 2	54.9	1.42	0.66	32.2	3.38	3.12	4.79	6.51
Day 8	36.9	1.82	0.81	30.9	5.51	5.64	6.96	11.1
LSD at P=0.05	5.6	0.15	0.05	ns	0.37	0.34	0.3	0.62
P-value	0.012	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001

[a] AMF% = the percentage of root length colonised by AMF

Root Mn concentration was in a similar range to shoot Mn concentration. For root Mn concentration, there was an interaction between treatment and harvest (Fig. 2b). Similar to shoots, Mn concentration in the roots of the mycorrhizal treatments was lower than that of non-mycorrhizal treatments and changed little over time. However, one day after the P pulse (day 2) a sharp decrease occurred in the non-mycorrhizal treatments with the concentration decreasing by ~50 %.

Other nutrients

The marginal means for treatment and harvest day for concentrations of the other nutrients measured are provided in Table 3 for shoots and in Table 4 for roots. For K, Mg, Ca, Zn, Fe and Mn, the mycorrhizal treatments generally had lower shoot concentrations than the other treatments, while for Na and Cu the concentration was higher for the mycorrhizal treatments. Microbes treatments generally had shoot nutrient concentrations similar to the control. In the roots, the concentration of Na was higher in the mycorrhizal treatments while concentrations of Mg, Cu and Zn were lower. Small changes occurred over time.

Discussion

Plant growth was unaffected by treatment

The five treatments did not differ in their effect on any measure of plant growth. The lack of an effect of inoculation with AMF may reflect the moderate level of soil bicarbonate-extractable P (18 mg kg^{-1}). While this is at the lower end of what can be expected in pastures in Western Australia (Weaver and Reed 1998; Scanlon 2011), shoot P concentrations >2.0 mg g^{-1} suggest P was not limiting growth of *T. subterraneum* plants (Snowball and Robson 1988). However, the plants did respond over 7 days to the addition of 10 mg kg^{-1} of P in the pulse with an increase in shoot dry weight of 47 % and in root dry weight of 44 %, suggesting some degree of P limitation. The similar root dry weights among treatments mean that the effect of AMF on uptake of the pulse was not

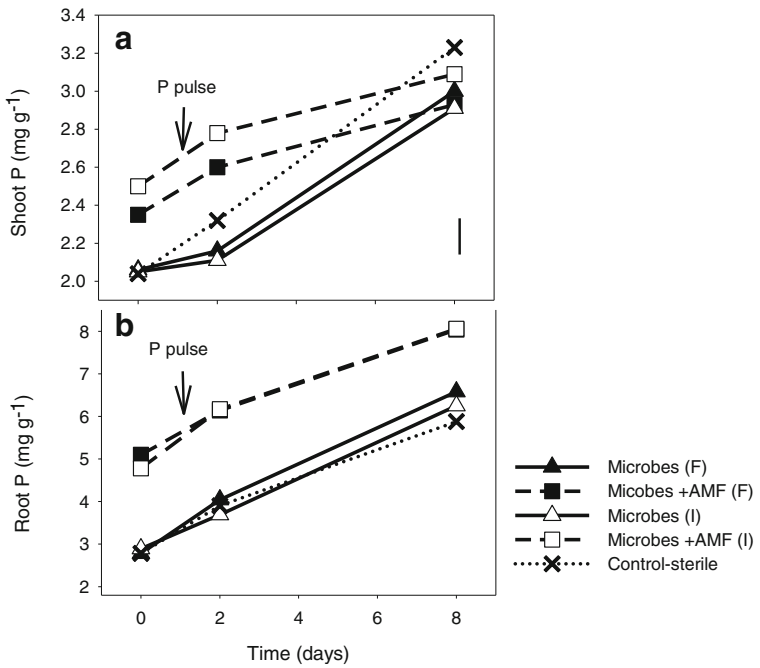

Fig. 1 Phosphorus concentration in the shoots (**a**) and roots (**b**) of *Trifolium subterraneum* grown for 11 weeks under five soil treatments (Microbes (F), Microbes +AMF (F), Microbes (I), Microbes +AMF (I), Control-sterile) (see Table 1) with three harvest times (day 0, day 2, day 8). Phosphorus (10 mg P kg^{-1} dry soil) was added at day 1. For shoot P concentration, the interaction between treatment and harvest was significant ($P<0.001$), and is presented with the l.s.d at $P=0.05$. For root P, there was no interaction (Table 2)

confounded by differences in the size of the root system.

Inoculation with AMF does not increase uptake of P applied in a pulse after 7 days

At day 0 (a day before the P pulse), the mycorrhizal treatments had the highest P concentrations in both shoots and roots. This is consistent with other reports of AMF enhancing uptake of P by *T. subterraneum* (Bolan et al. 1987; Smith 1982; Abbott and Robson 1977). However, hypothesis 1—that AMF enhance plant uptake of P from a pulse—was not supported; between days 2 and 8 (7 days after adding the pulse), the rate of increase in shoot P concentration was lower in the mycorrhizal treatments than in the microbes and control treatments. Indeed, by day 8, the mycorrhizal and non-mycorrhizal treatments had similar shoot P concentrations. In contrast, the roots in all treatments accumulated P at a similar rate between days 2 and 8 and the higher concentrations of P in the mycorrhizal treatments were maintained. Thus it appears that in the mycorrhizal treatments, a smaller proportion of the P absorbed from the P pulse by the roots was then translocated to the shoot than in the microbes only and control treatments.

We contend that the retention of P in the roots of mycorrhizal treatments may be due to the primary path of P uptake being through the external hyphae of AMF and storage of a high proportion of absorbed P therefore being in the intraradical hyphae of the AMF in poorly soluble forms (Ryan et al. 2003; Ryan et al. 2007). Since the roots were heavily colonised (~47 % of root length), the P content of the intraradical mycorrhizal hyphae could be a large proportion of root P content. In contrast, in the non-mycorrhizal treatments, P may have been primarily absorbed by plant uptake pathways, stored in the root cell vacuoles in readily soluble forms and hence readily transported to the shoots. These results are consistent with recent evidence that colonisation by AMF leads to a 'switch–off' of the direct pathway (which occurs through root hairs of the plant) and that the mycorrhizal pathways prevail over the direct pathway even when plants do not exhibit better growth when colonised by AMF (Smith et al. 2011). For instance, in an experiment performed by Facelli et al. (2010), inoculation with AMF reduced P uptake via the direct uptake pathway in two genotypes of tomato. P uptake via the AM pathway was 0.7236 mg P $plant^{-1}$ and via the direct uptake pathway was 0.0001 mg P $plant^{-1}$. There was no significant difference between mycorrhizal and non-mycorrhizal treatments in shoot dry weight (Facelli

Table 3 Marginal means for shoot nutrient concentrations in *Trifolium subterraneum* for treatment (Control-sterile, Microbes+AMF (F), Microbes+AMF (I), Microbes (F), Microbes (I)) and harvest (day 0, day 2 and day 8) for which there was no interaction (ns, no significant difference). The elements with * had a very small interaction which is not presented

	K ($g\ kg^{-1}$)	Mg ($g\ kg^{-1}$)	Ca ($g\ kg^{-1}$)	Na * ($g\ kg^{-1}$)	Zn ($mg\ kg^{-1}$)	Cu * ($mg\ kg^{-1}$)	Fe * ($mg\ kg^{-1}$)	Mn ($mg\ kg^{-1}$)
Treatment								
Control-sterile	18.3	4.2	15.9	5.5	68	8.6	103	156.6
Microbes+AMF (F)	17.3	2.8	12.4	8.1	41	10.5	81.7	83.6
Microbes+AMF (I)	17.4	3.1	12.3	7.3	54	10.3	75.9	99.2
Microbes (F)	19.1	4.0	14.8	5.0	64	8.7	118.1	130.5
Microbes (I)	18.7	3.9	15.4	5.0	69	8.8	112.6	143.8
LSD at P=0.05	1.1	0.1	0.066	0.4	3.93	0.75	8.77	8.19
P-value	0.009	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Harvest								
Day 0 (no added P)	18.6	3.7	14.2	6.4	61	9.2	102.4	118.2
Day 1 (P added)								
Day 2	17.9	3.5	14.1	6.1	61	9.8	102.5	120.6
Day 8	17.9	3.6	14.3	6.0	57	9.2	89.9	129.3
LSD at P=0.05	ns	0.1	ns	ns	3.04	ns	6.80	6.34
P-value	–	0.009	–	–	0.018	–	<0.001	0.002

et al. 2010). Thus, even when there is no net increase in plant P uptake as a result of inoculation with AMF, fungi may still play a major role in plant P uptake. In the current experiment no other elements showed this trend which is

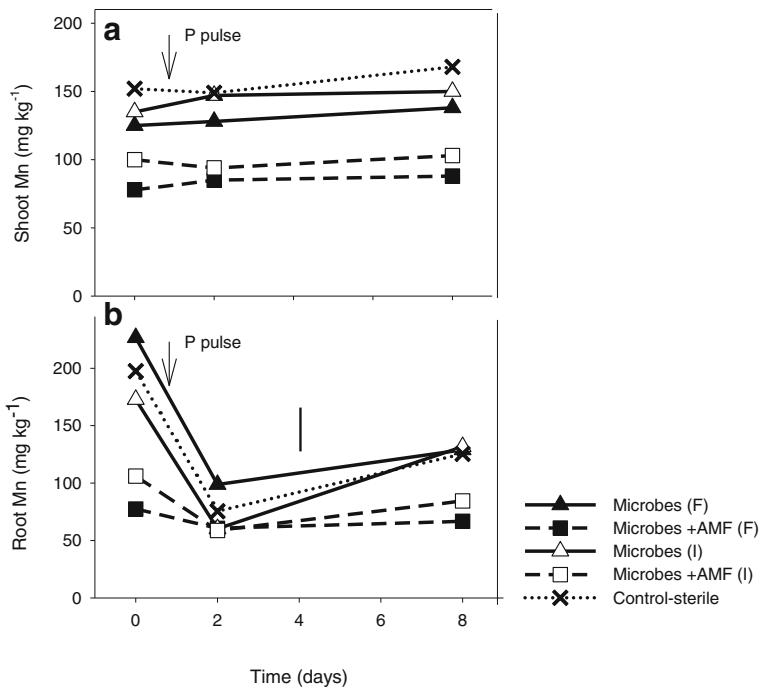

Fig. 2 Manganese concentrations in the shoots (**a**) and roots (**b**) of *Trifolium subterraneum* grown for 11 weeks under five soil treatments (Microbe (F), Microbes +AMF (F), Microbes (I), Microbes +AMF (I), Control-sterile) (see Table 1) with three harvest times (day 0, day 2, day 8). Phosphorus (10 mg P kg^{-1} dry soil) was added at day 1. For root Mn, the interaction between treatment and harvest was significant (P<0.001), and is presented with the l.s.d at P=0.05. For shoot Mn, there was no interaction between treatment and harvest (Table 2)

Table 4 Marginal means for root nutrient concentrations in *Trifolium subterraneum* for treatment (Control-sterile, Microbes+AMF (F), Microbes+AMF (I), Microbes (F), Microbes (I)) and harvest (day 0, day 2 and day 8) for which there was no interaction (ns, no significant difference). The elements with * had a very small interaction which is not presented. The treatment × harvest interaction for root Mn concentration is presented in Fig. 2

	K (g kg^{-1})	Mg (g kg^{-1})	Ca (g kg^{-1})	Na * (g kg^{-1})	Zn (mg kg^{-1})	Cu * (mg kg^{-1})	Fe * (mg kg^{-1})
Treatment							
Control-sterile	11.5	3.2	4.4	20.2	103	253	641
Microbes+AMF (F)	13.5	3.1	4.5	24.7	73	187	606
Microbes+AMF (I)	12.9	2.5	4.5	22.4	85	196	745
Microbes (F)	13.2	3.4	4.4	23.3	94	269	655
Microbes (I)	13.3	3.2	4.6	22.1	98	259	615
LSD at P=0.05	ns	0.3	ns	2.7	14.78	48.7	94.7
P-value	–	<0.001	–	<0.001	0.002	0.002	0.03
Harvest							
Day 0 (no added P)	13.7	3.5	4.3	25.1	91	217	563
Day 1 (P added)							
Day 2	12.3	2.7	4.7	17.7	85	214	712
Day 8	12.6	3.0	4.4	25.0	97	266	683
LSD at P=0.05	ns	0.2	0.2	2.1	ns	37.8	73.4
P-value	–	<0.001	0.014	<0.001	–	0.013	<0.001

consistent with Ryan et al. (2007) who found K, Na, Ca, S and Mg were not present in mycorrhizal hyphae in insoluble forms. Since the present experiment ran for only 7 days after adding the P pulse, further research over a longer time is now needed to investigate the role of AMF in regulating the release of P provided in a pulse to the plant and whether colonisation by AMF can provide a protective role when large P pulses are encountered by reducing plant access to the P.

Little difference in P uptake between AMF from the field soil (diverse mycorrhizal and heterotrophic community) and single species culture of AMF

There was little difference in plant P uptake when *T. subterraneum* was inoculated with field soil or *S. calospora*; hence hypothesis 2 was not supported. This was unexpected as species of AMF are known to differ in their effects on P uptake and growth of host plants (Jansa et al. 2005), and improvement in plant nutrient uptake and growth has been reported when more AMF species are present (Van der Heijden et al. 1998; Wagg et al. 2011). However, the findings of Bainard et al. (2012) may help to explain our results. They suggested that plant productivity was not affected by higher mycorrhizal richness, because all AM fungal taxa present in their experiment belonged to a single family (Glomeraceae) and they all have similar functions. Certainly, >97 % of the DNA detected in our soil came from one group of fungi. Thus while a large diversity of species may have been present in the soil, as found nearby by Tibbett et al. (2008), the community of AMF may have been dominated by a small number of *Glomus*. However, it could still be expected that the effect on plant growth of the *Glomus*-dominated field soil would differ from the *S. calospora* inoculum. A combination of factors may explain the lack of difference. First, *S. calospora* is an "aggressive coloniser" and particularly effective at enhancing P uptake (Graham and Abbott 2000). Use of a pure culture of a "non-aggressive coloniser" may have changed the results. Second, roots inoculated with the field soil and with *S. calospora* were both highly colonised. Third, plant-available P was moderately high. Perhaps these factors resulted in a similar length of hyphae present in both treatments, with differences in hyphal characters being of little importance due to the moderately high soil P.

Microbes alone had little effect on uptake of P and other nutrients

Rarely did the effects of the microbes treatments differ from that of the control, both for plant growth and plant nutrient concentration. Thus, our third hypothesis is rejected. This supports the findings of Richardson (2001) and Richardson et al. (2009) who found that, although the presence of PSM under controlled growth conditions and laboratory media increased P nutrition of the plants, in more complex soil environments and in the field the results were inconsistent and the performance of microorganisms changed. Also, as the level of soil P increases, the activity of microorganisms involved in P mobilisation may or may not be reduced (Richardson 2001). On the other hand, our finding is not consistent with Hetrick et al. (1988) who found addition of non-sterile soil sievings or filtrates to sterilised soil increased growth of non-mycorrhizal plants. However, that experiment was performed under low concentrations of P soil (5 mg kg^{-1}). The moderate levels of soil P in the present experiment might be the reason for this contrast.

Manganese concentration is greatly decreased by inoculation with AMF and addition of a P pulse

Manganese was consistently and substantially lower in the mycorrhizal treatments except for day 2 in the roots. This result is consistent with numerous studies such as those of Kothari et al. (1991), Posta et al. (1994), Liu et al. (2000), Fageria et al. (2002) and Ryan and Angus (2003), which reported mycorrhizal treatments had lower Mn concentrations. According to Posta et al. (1994), the lower uptake of Mn in mycorrhizal plants might be due to less Mn-reducing microbial communities and also less Mn-solubilising root exudates by mycorrhizal plants. An alternative or additional explanation involves the effect of AMF on plant exudation of carboxylates. Carboxylates enhance the solubilisation of P (Godo and Reisenauer 1980) and make it easier for the plant to access P (Shane and Lambers 2005). Inoculation with AMF, which increases P uptake, reduces the amount of rhizosphere carboxylates (Ryan et al. 2012). Such a reduction is likely to result in Mn becoming less mobile in the soil and less able to move through the soil to plant roots (Godo and Reisenauer 1980). Thus, a reduction in rhizosphere carboxylates in response to higher root P concentrations could have contributed to lower Mn concentrations in the mycorrhizal plants, as also observed in plants with elevated P concentrations (Shane and Lambers 2005; Shane et al. 2004).

In addition to reduced Mn concentrations in the mycorrhizal treatments, root Mn concentration in the microbes and control treatments (which had the lowest shoot and root P concentrations and the highest Mn concentrations) declined 50 % between day 0 and day 2. As there was no indication that the Mn was transported to the shoots, it seems likely that the roots released Mn into the rhizosphere (Pittman 2005).

In conclusion, there was no difference among treatments in shoot and root dry weights. The two filtrate treatments and the control generally did not differ for any parameter measured, nor did the two mycorrhizal treatments. The colonised treatments had higher P and lower Mn concentrations in shoots and roots before the pulse. After the pulse, the rate of increase in P concentration, in the shoots only, was slower for the colonised treatments, and root Mn concentration immediately (i.e. by day 2) declined by up to 50 %. We hypothesise that absorption of P by the intraradical hyphae of AMF in the 7 days following the pulse reduced its transportation to the shoots.

Acknowledgments This project was funded by the Future Farm Industries CRC. We wish to thank Tamara Edmonds-Tibbett for technical support of the preliminary experiment.

References

Abbott LK, Robson AD (1977) Growth stimulation of subterranean clover with vesicular arbuscular mycorrhizas. Aust J Agric Res 28:639–649

Bainard LD, Koch AM, Gordon AM, Klironomos JN (2012) Growth response of crops to soil microbial communities from conventional monocropping and tree-based intercropping systems. Plant Soil 363:1–12

Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Blackwell MSA, Brookes PC, de la Fuente-Martinez N, Gordon H, Murray PJ, Snars KE, Williams JK, Bol R, Haygarth PM (2010) Phosphorus solubilization and potential transfer to surface waters from the soil microbial biomass following drying–rewetting and freezing–thawing. Adv Agron 106:1–35

Blair GJ, Chinoim N, Lefroy RDB, Anderson G, Crocker G (1991) A soil sulfur test for pastures and crops. Aust J Soil Res 29:619–626

Bolan NS, Robson AD, Barrow NJ (1987) Effects of vesicular-arbuscular mycorrhiza on the availability of iron phosphates to plants. Plant Soil 99:401–410

Butterly CR, Bünemann EK, McNeill AM, Baldock JA, Marschner P (2009) Carbon pulses but not phosphorus pulses are related to decreases in microbial biomass during repeated drying and rewetting of soils. Soil Biol Biochem 41:1406–1416

Colwell JD (1965) An automatic procedure for the determination of phosphorus in sodium hydrogen carbonate extracts of soils. Chem Ind 10:893–895

Facelli E, Smith SE, Facelli JM, Christophersen HM, Smith FA (2010) Underground friends or enemies: model plants help to unravel direct and indirect effects of arbuscular mycorrhizal fungi on plant competition. New Phytol 185:1050–1061

Fageria NK, Baligar C, Clark RB (2002) Micronutrients in crop production. Adv Agron 77:185–268

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Godo GH, Reisenauer HM (1980) Plant effects on soil manganese availability. Soil Sci Soc Am J 44:993–995

Graham J, Abbott L (2000) Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. Plant Soil 220:207–218

Graham JH, Linderman RG, Menge JA (1982) Development of external hyphae by different isolates of mycorrhizal *Glomus* spp. in relation to root colonisation and growth of Troyer citrange. New Phytol 91:183–189

Gyaneshwar P, Naresh Kumar G, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245:83–93

Haling RE, Simpson RJ, McKay AC, Hartley D, Lambers H, Ophel-Keller K, Wiebkin S, Herdina, Riley IT, Richardson AE (2011) Direct measurement of roots in soil for single and mixed species using a quantitative DNA-based method. Plant Soil 348:123–137

Hetrick BAD, Wilson GT, Kitt DG, Schwab AP (1988) Effects of soil microorganisms on mycorrhizal contribution to growth of big bluestem grass in non-sterile soil. Soil Biol Biochem 20:501–507

Jakobsen I, Abbott LK, Robson AD (1992a) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 2. hyphal transport of ^{32}P over defined distances. New Phytol 120:509–516

Jakobsen I, Abbott LK, Robson AD (1992b) External hyphae of vesicular–arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.1. spread of hyphae and phosphorus inflow into roots. New Phytol 120:371–380

Jansa J, Mozafar A, Frossard E (2005) Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176

Koide RT, Mosse B (2004) A history of research on arbuscular mycorrhiza. Mycorrhiza 14:145–163

Kothari SK, Marschner H, Römheld V (1991) Effect of a vesicular–arbuscular mycorrhizal fungus and rhizosphere micro-organisms on manganese reduction in the rhizosphere and manganese concentrations in maize (*Zea mays* L.). New Phytol 117:649–655

Lamont B (1982) Mechanisms for enhancing nutrient uptake in plants, with particular reference to mediterranean South Africa and Western Australia. Bot Rev 48:597–689

Lewis DC, Clarke AL, Hall WB (1987) Accumulation of plant nutrients and changes in soil properties of sandy soils under fertilized pasture in southeastern South-Australia.I. Phosphorus. Aust J Soil Res 25:193–202

Liu A, Hamel C, Hamilton RI, Ma BL, Smith DL (2000) Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. Mycorrhiza 9:331–336

Pittman JK (2005) Managing the manganese: molecular mechanisms of manganese transport and homeostasis. New Phytol 167:733–742

Posta K, Marschner H, Römheld V (1994) Manganese reduction in the rhizosphere of mycorrhizal and nonmycorrhizal maize. Mycorrhiza 5:119–124

Qiu S, McComb AJ, Bell RW, Davis JA (2004) Phosphorus dynamics from vegetated catchment to lakebed during seasonal refilling. Wetlands 24:828–836

Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Funct Plant Biol 28:897–906

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305–339

Ryan MH, Angus JF (2003) Arbuscular mycorrhizae in wheat and field pea crops on a low P soil: increased Zn-uptake but no increase in P-uptake or yield. Plant Soil 250:225–239

Ryan MH, McCully ME, Huang CX (2003) Location and quantification of phosphorus and other elements in fully hydrated, soil-grown arbuscular mycorrhizas: a cryo-analytical scanning electron microscopy study. New Phytol 160:429–441

Ryan MH, McCully ME, Huang CX (2007) Relative amounts of soluble and insoluble forms of phosphorus and other elements in intraradical hyphae and arbuscules of arbuscular mycorrhizas. Funct Plant Biol 34:457–464

Ryan MH, Tibbett M, Edmonds-Tibbett T, Suriyagoda LDB, Lambers H, Cawthray GR, Pang J (2012) Carbon trading for phosphorus gain: the balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition. Plant Cell Environ 35:2170–2180

Scanlon T (2011) Decline in subterranean clover (*Trifolium subterraneum L.*)-based pasture systems : causes and solutions. MSc Dissertation, University of Western Australia

Searle PL (1984) The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review. Analyst 109:549–568

Shane MW, Lambers H (2005) Manganese accumulation in leaves of *Hakea prostrata* (Proteaceae) and the significance of cluster roots for micronutrient uptake as dependent on phosphorus supply. Physiol Plant 124:441–450

Shane MW, McCully ME, Lambers H (2004) Tissue and cellular phosphorus storage during development of phosphorus toxicity in *Hakea prostrata* (Proteaceae). J Exp Bot 55:1033

Simpson RJ, Oberson A, Culvenor RA, Ryan MH, Veneklaas EJ, Lambers H, Lynch JP, Ryan PR, Delhaize E, Smith FA, Smith

SE, Harvey PR, Richardson AE (2011) Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. Plant Soil 349:89–120

Singh S, Kapoor KK (1998) Effects of inoculation of phosphate-solubilizing microorganisms and an arbuscular mycorrhizal fungus on mungbean grown under natural soil conditions. Mycorrhiza 7:249–253

Smith SE (1982) Inflow of phosphate into mycorrhizal and non-mycorrhizal plants of *Trifolium subterraneum* at different levels of soil phosphate. New Phytol 90:293–303

Smith SE, Jakobsen I, Groenlund M, Smith FA (2011) Roles of arbuscular mycorrhizas in plant phosphorus (P) nutrition: interactions between pathways of P uptake in arbuscular mycorrhizal (AM) roots have important implications for understanding and manipulating plant P acquisition. Plant Physiol 156:1050–1057

Snowball K, Robson AD (1988) Symptoms of nutrient deficiencies: subterranean clover and wheat. Department of Soil Science and Plant Nutrition, Institute of Agriculture, University of Western Australia, Nedlands, pp 73

Tibbett M, Ryan MH, Barker SJ, Chen Y, Denton MD, Edmonds-Tibbett T, Walker C (2008) The diversity of arbuscular mycorrhizas of selected Australian Fabaceae. Plant Biosyst 142:420–427

Turner BL, McKelvie ID, Haygarth PM (2002) Characterisation of water-extractable soil organic phosphorus by phosphatase hydrolysis. Soil Biol Biochem 34:27–35

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Vierheilig H, Coughlan AP, Wyss U, Piche Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64:5004–5007

Wagg C, Jansa J, Stadler M, Schmid B, Van der Heijden MGA (2011) Mycorrhizal fungal identity and diversity relaxes plant-plant competition. Ecology 92:1303–1313

Weaver DM, Reed AEG (1998) Patterns of nutrient status and fertiliser practice on soils of the south coast of Western Australia. Agric Ecosyst Environ 67:37–53