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Influence of two legume species on hyphal production and activity of two arbuscular mycorrhizal fungi

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Abstract Two arbuscular mycorrhizal (AM) fungi (*Glomus mosseae* and *G. intraradices*) were compared for abundance of intraradical and soil-borne hyphae in association with *Astragalus sinicum*, a small-seeded, and *Glycine max*, a large-seeded legume. *A. sinicum* was more responsive than *G. max* to mycorrhizal formation, especially at early growth stages. Biomass allocation was greater in roots than shoots for mycorrhizal *A. sinicum*, while the opposite was true for *G. max*. Hyphal development in root and soil compartments was estimated by trypan blue staining and after staining for succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) activity. Total fungal abundance increased steadily in roots and soil with time to a maximum 8 weeks after planting. SDH- and ALP-active AM hyphae increased in roots during plant growth but decreased in soil at later harvests. Mycorrhizal root mass in *A. sinicum* and *G. max* increased about 14-fold and 2.5-fold, respectively, but total length of soil hyphae produced per plant differed little, so that the pattern of AM soil to root abundance of the two fungi varied considerably with the host plant.

Key words Arbuscular mycorrhiza · Hyphal abundance · Legumes · Root · Soil

Introduction

One of the major effects of arbuscular mycorrhiza (AM) is to increase plant nutrient uptake through the absorbing surface created by hyphae of the symbiotic fungus spreading in the soil and the extensive symbiotic interface for transfer of nutrients across arbuscules (Cooper and Tinker 1978; Smith and Gianinazzi-Pearson 1988; Allen 1991). Thus to understand AM function both the metabolic states of intraradical and soil-borne hyphae and the relationship between them must be considered. Succinate dehydrogenase (SDH), a mitochondrial enzyme, is used as an indicator of mycorrhiza viability but does not appear to reflect mycorrhizal efficiency in plant growth enhancement (Vierheilig and Ocampo 1989; Smith and Gianinazzi-Pearson 1990; Guillemin et al. 1993). Alkaline phosphatase (ALP) activity, located within the phosphate-accumulating vacuoles of AM hyphae (Gianinazzi et al. 1979; Smith and Gianinazzi-Pearson 1988), has been proposed as a physiological marker for analysing the efficiency of mycorrhiza (Tisserant et al. 1993, 1996). Measurements of these two enzyme activities have been used to follow the development of AM fungi within plant roots (MacDonald and Lewis 1978; Gianinazzi-Pearson and Gianinazzi 1978; Smith and Gianinazzi-Pearson 1990; Tisserant et al. 1993, 1996). Such vital staining of enzyme activity make it possible to compare the total production of fungal tissue with the metabolically active proportion. In this study, we have compared simultaneously the production of hyphae within roots and in soil of two species of AM fungi. Our objectives were to determine whether (1) hyphal biomass produced in the two compartiments is interdependent, (2) the proportion of metabolically active hyphae differs with time, and (3) fungal behaviour is influenced by the host plant.

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Materials and methods

Experimental design

A clay loam soil (pH 7, 26 ppm Olsen P), collected from the Domaine d'Epoisses Experimental Station, INRA, Dijon, was sieved (2 mm), gamma-irradiated (10 Gy) and 400 g aliquots placed in pots. Seeds of *Glycine max* (var. Helong 28) and *Astragalus sinicum* were surface disinfected in a 3.5% calcium hypochlorite solution for 10 min. After washing 5 times with sterile water, the seeds

were germinated in sterile vermiculite at 22 °C for *G. max* and in Petri dishes at 26 °C for *A. sinicum*. Three uniform, germinated seeds were sown in each pot. The mycorrhizal fungi were *Glomus mosseae* (Nicol. & Gerd.) Gerdeman and Trappe (BEG12) and *G. intraradices* Schenck & Smith (LPA8). A 10-g inoculum consisting of a mixture of soil, spores and infected roots from pot cultures of leek was placed in a hole beneath the seeds. Each seed received 0.5 ml of a dense suspension of *Rhizobium huakueii* (7653-r) for *A. sinicum*, or *Bradyrizobium japonicum* (G49 USDA) for *G. max*. Control pots were not inoculated with mycorrhizal fungi but received the *Rhizobium* suspension and 20 ml of filtered (Whatman N° 1) washings of a mixture of fungal inoculants. Sixteen replicate pots per treatment were randomized in blocks in a growth chamber under controlled environmental conditions: day/night temperature 22/26 °C, 16 h photoperiod, relative humidity 70–80%, irradiance 320 $\mu E.m^{-2}.s^{-1}$. One week after seedling emergence, each pot was thinned to keep only two *A. sinicum* or one *G. max* plant. Plants were watered daily to field capacity with distilled water. Three weeks after planting, each pot of uninoculated *A. sinicum* seedlings was fed weekly 20 ml of Long Ashton nutrient solution (Hewitt 1966) containing 26.4 μM phosphorus.

Plants from 4 pots per treatment were harvested at 3, 5, 7 and 8 weeks after sowing. Eight weeks was chosen for the last harvest because mycorrhizal plants tended to become pot bound after this period. Fresh weights of shoots and roots, and dry weights of nodules were recorded at each harvest. Shoots were dried at 90 °C for 48 h, and P concentration was measured using the vanadomolybdate method. Root subsamples were taken for measurement of fungal colonization after clearing and staining with trypan blue (Phillips and Hayman 1970), for SDH (Smith and Gianinazzi-Pearson 1990) and for ALP (Tisserant et al. 1993) activities using the reaction media described below. Root colonization was estimated by the method described by Trouvelot et al. (1986), where intensity of root cortex colonization (hyphae and arbuscules) is expressed as M%. The amount of mycorrhizal root (mycorrhizal root mass) was calculated by multiplying M% by total root fresh mass.

Hyphal extraction from soil and measurement

The length of hyphae in soil was measured after extraction using a membrane-filter technique modified after Abbott et al. (1984). The activity of external hyphae was determined by staining for SDH and ALP activities.

Before harvesting plants, four soil cores (1 cm diameter and 5 cm deep) were taken randomly from each replicate pot and mixed. Three 2-g subsamples of soil were taken and assayed separately. For measurement of total mass of soil hyphae, each subsample was suspended in 250 ml distilled water and washed through a 600-μm-mesh sieve. The washings were blended at high speed for 25 s in a Turbo Blender (Moulinex, France). The blended suspensions were rapidly transferred to a flask and agitated vigorously, then left standing for 1 min. Duplicate 5-ml portions were pipetted onto a millipore filter (1.0 μm pore size) fitted to a peristaltic pump. The filter was dried and stained with trypan blue (0.05%) for 5 min before observation. For measurement of SDH-active hyphae, the second subsample was collected and covered immediately with 20 ml of an ice-cold solution of 10% sorbitol. After addition of 20 ml of Tris/HCl buffer (0.2 M, pH 7.4), containing 5 mM $MgCl_2$, 4 mg ml^{-1} Nitro Blue Tetrazolium (NBT) (Sigma) and 2.5 M sodium succinate, the soil suspension was incubated at room temperature for 3 h, and then diluted to 250 ml with distilled water, washed through a sieve and blended as described above. Hyphae collected on a millipore filter were dried and counterstained with 0.05% acid fuchsin. For estimation of ALP-active hyphae, the third subsample was covered with ice-cold 10% sorbitol solution and stained at room temperature for 3 h by addition of 20 ml Tris/citric acid buffer (0.05 M, pH 9.2) containing 1 mg ml^{-1} α-naphtyl acid phosphate (Sigma), 1 mg ml^{-1} Fast Blue salt, 0.05% $MgCl_2$ and 0.05% $MnCl_2$. The hyphae were extracted and counterstained as described for SDH staining. For each staining procedure, two filters from each original soil sample were mounted in 30% glycerol solution under a coverslip. The length of hyphae was measured using a grid intercept method (Tennant 1975) at 200 magnification and 25 random fields were viewed on each filter. Length of mycorrhizal hyphae was calculated by substracting hyphal lengths obtained from uninoculated treatments from those in *Glomus*–inoculated treatments.

Statistical analysis

Statistical analyses of data were performed using the method of two-way analysis of variance after arcsin transformation for percentages, and treatment means were compared using Student-Newman-Keuls multiple range test. Data represent the means of 4 replicates.

Results

Plant growth

Shoot fresh and dry mass of *G. max* plants with and without AM were not different at the first two harvests, whereas inoculation with either species of AM fungus clearly enhanced shoot growth 7 weeks after planting (Table 1). There was no significant difference in root mass between mycorrhizal and non-mycorrhizal *G. max* plants harvested at 3, 7 or 8 weeks. Formation of AM significantly enhanced both shoot and root growth of *A. sinicum* plants (Table 1). At the first harvest, plants inoculated with *G. intraradices* and *G. mosseae* already had average increases in plant biomass of 560% and 610%, respectively. Inoculation with either species of AM fungus resulted in differences in biomass allocation between *G. max* and *A. sinicum*. Mycorrhizal *A. sinicum* had a root/shoot ratio as high as non-mycorrhizal plants (greater than 1.0) throughout the experiment, whereas in *G. max* mycorrhiza formation decreased the root/shoot ratio greatly at 5 weeks after inoculation to less than 0.5 after 8 weeks.

Nodulation

Mycorrhizal inoculation significantly enhanced nodule formation in both host plants (Table 1). Mycorrhizal *G. max* had more than double the nodules of uninoculated plants from the second harvest onwards. *A. sinicum* was completely dependent on AM formation for nodulation but produced far less nodule biomass than *G. max*, even though it had a much larger root system. There was no differential influence on nodule production between the two fungi. Nodules in both legumes had a reddish colour suggesting that they were actively fixing nitrogen.

Phosphorus uptake

The increased growth of mycorrhizal plants was associated with enhanced phosphorus uptake into the

Table 1 Growth and phosphorus uptake of *Glycine max* and *Astragalus sinicum* without mycorrhiza (*NM*) or inoculated with *Glomus intraradices* (*Gi*) or *Glomus mosseae* (*Gm*). For each plant, in each column, data followed by the same letters are not significantly different ($P=0.05$)

Plant	Fungus	Week	Shoot dry mass (g)	Root dry mass (g)	Nodules dry mass (mg)	Shoot phosphorus (μg/plant)
Glycine max						
		3	0.91 e	1.14 d	0 d	32 c
	NM	5	2.10 d	2.66 a	49 c	72 b
		7	3.37 c	2.99 a	71 c	83 b
		8	3.06 cd	2.85 a	76 c	54 bc
		3	0.92 e	1.03 d	0 d	58 bc
	Gi	5	2.22 d	1.38 cd	134 b	134 a
		7	4.26 b	2.14 abc	196 a	139 a
		8	5.75 a	2.54 a	198 a	142 a
		3	0.89 e	0.71 d	0 d	55 bc
	Gm	5	2.45 d	1.59 bcd	123 b	114 a
		7	4.51 b	2.80 a	199 a	127 a
		8	5.30 a	2.55 ab	198 a	124 a
Astragalus sinicum						
		3	0.02 d	0.04 c	0 c	2 d
	NM	5	0.07 d	0.08 c	0 c	3 d
		7	0.10 d	0.15 c	0 c	5 d
		8	0.10 d	0.16 c	0 c	4 d
		3	0.19 d	0.21 c	0 c	18 d
	Gi	5	0.86 c	1.35 b	17 b	89 c
		7	1.55 b	1.82 b	65 a	153 b
		8	2.52 a	3.53 a	67 a	221 a
		3	0.17 d	0.19 c	0 c	18 d
	Gm	5	0.98 c	1.35 b	12 bc	71 c
		7	1.60 b	1.89 b	65 a	123 b
		8	2.63 a	3.61 a	67 a	193 a

shoots (Table 1). P uptake in non-mycorrhizal plants was lower, particularly in *A. sinicum* seedlings, than in mycorrhizal plants. P uptake continued to increase up to 8 weeks for AM *A. sinicum* plants, while in AM *G. max*, P uptake increased up to 5 weeks and thereafter remained constant. Based on comparisons with non-mycorrhizal plants, total P uptake was increased more than 40-fold by mycorrhiza in *A. sinicum*, and threefold in *G. max* by the end of experiment.

Mycorrhiza development

Abundance of the mycorrhizal fungi within roots steadily increased with development of the root systems. Roots of *A. sinicum* and *G. max* plants were intensely colonized (55–75% of the root system cortex) by the two *Glomus* species 3 weeks after inoculation (Fig. 1). Clear differences in mycorrhiza development occurred between the two legumes. *A. sinicum* developed a higher intensity of root colonization than *G. max*. Moreover, *G. mosseae* colonized roots of *A. sinicum* more quickly than those of *G. max*. This, together with the differences in root development between the two legumes, resulted in about a 14-fold increase in mycorrhizal root mass in *A. sinicum* plants and a 2.5-fold increase in *G. max* plants during the experiment, so that the total amount of mycorrhizal root formed by the two AM fungi was significantly greater ($P = 0.001$) in *A. sinicum* than in *G. max* plants 8 weeks after inoculation (Fig. 2). No mycorrhizal formation was detected in uninoculated pots throughout the experiment. Vital staining indicated that a high proportion of the intraradical hyphae was enzymatically active. For *A. sinicum*, more than 90% hyphae produced by the two *Glomus* species in roots were SDH active at the first harvest, and about 60% of hyphae had ALP activity (Fig. 1). The amount of root mass with SDH- or ALP-active hyphae followed a similar pattern to that of the total mycorrhizal root mass until 7 weeks after planting, and tended to be slightly lower thereafter (Fig. 2). The amount of infection with active hyphae formed by the two fungi in *G. max* roots was lower at the first harvest than that in *A. sinicum* roots but increased thereafter (Fig. 1). Active hyphae of *G. intraradices* and *G. mosseae* had a similar pattern of development in *G. max* roots.

Production of soil hyphae

The abundance of total soil hyphae associated with *G. max* and *A. sinicum* increased up to the end of the experiment for both *Glomus* species, with no consistent differences between the two fungi (Table 2). Staining for SDH and ALP activities indicated that only a proportion of hyphae in soil was metabolically active. The proportion of SDH-active hyphae was constant for all fungal/plant combinations up to 7 weeks. The proportion of metabolically active hyphae subseqently decreased significantly for all treatments except *G. intra-*

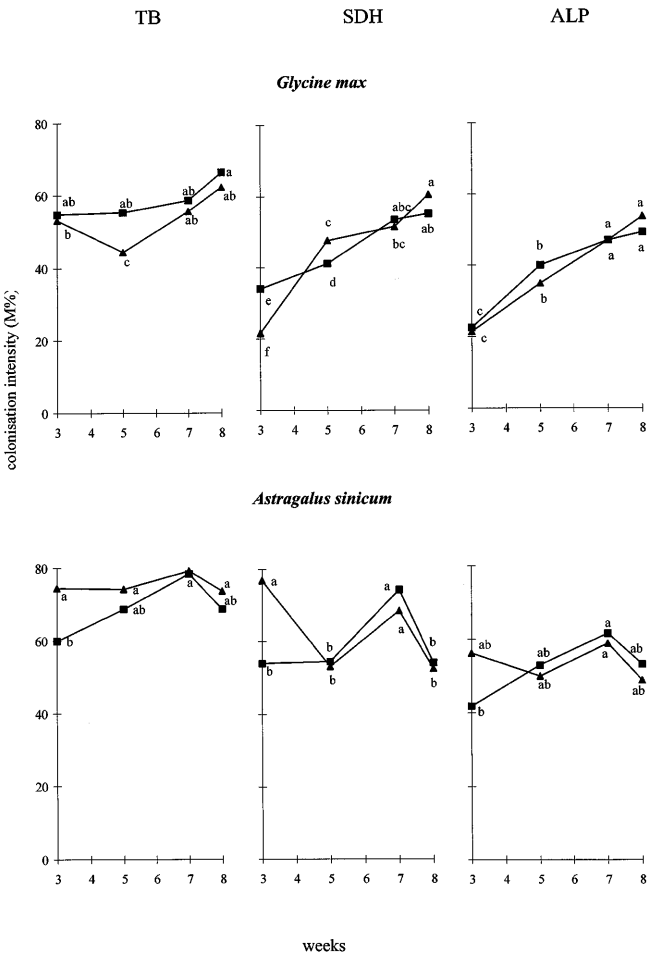

Fig. 1 Intensity of root cortex colonization (M%) by *Glomus intraradices* (-■-) and *G. mosseae* (-▲-), estimated by staining with trypan blue (*TB*), for succinate dehydrogenase (*SDH*) and for alkaline phosphatase (*ALP*) activities. In each graph, data followed by the same letter(s) are not significantly different ($P = 0.05$)

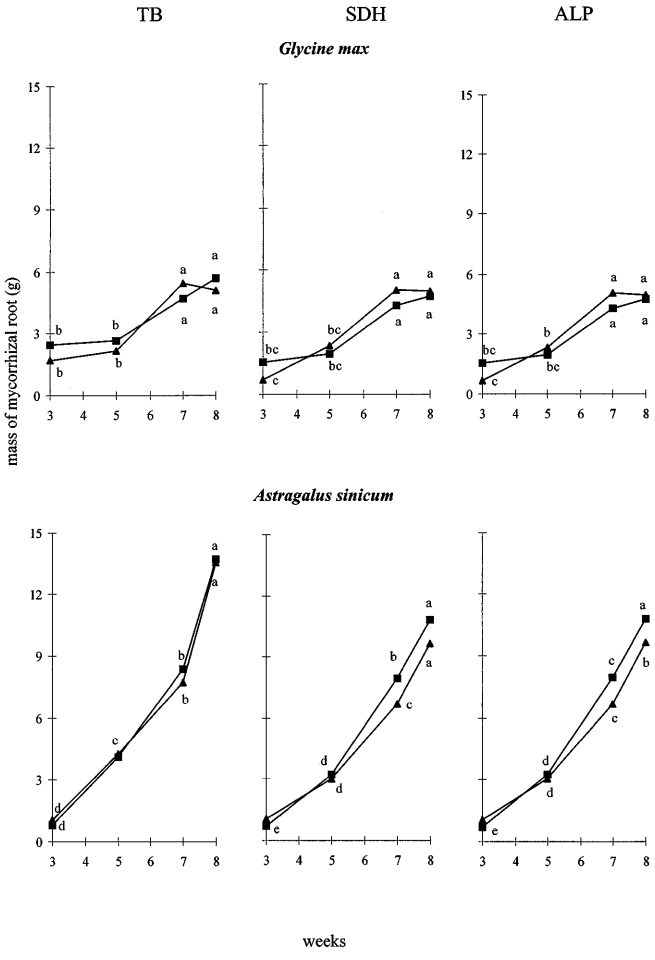

Fig. 2 Development of mycorrhizal root mass (g) inoculated with *G. intraradices* (-■-) or *G. mosseae* (-▲-), estimated by staining with trypan blue (*TB*), for succinate dehydrogenase (*SDH*) and for alkaline phosphatase (*ALP*) activities. In each graph, data followed by the same letter(s) are not significantly different ($P = 0.05$)

radices in association with *G. max*. The proportion of ALP-active hyphae was always lower than those with SDH activity (Table 2). The proportion of ALP-active soil hyphae developing from *A. sinicum* roots remained constant during early harvests at 3 and 5 weeks and decreased at 7 or 8 weeks. When the fungi were associated with *G. max*, values increased up to 5 weeks and then decreased rapidly. In uninoculated control pots, the amount of soil hyphae was low throughout the experiment, and no active hyphae were observed by SDH or ALP staining (Table 2). The ratio of soil hyphal length to fresh mycorrhizal root biomass was similar for *G. mosseae* and *G. intraradices* but varied with the host plant. For *A. sinicum*, values gradually decreased from the beginning of the experiment, while for *G. max* they increased and then decreased (Fig. 3).

Discussion

The production of AM fungal hyphae was affected by the host plant species. In particular, *A. sinicum* produced more mycorrhizal root mass than did *G. max*. The relationship between external hyphal production and intraradical fungal development also differed between the two host plants. *A. sinicum* had a higher ratio of soil hyphae to fresh mycorrhizal root biomass than *G. max* at early stages of mycorrhiza development. This then dropped to lower values with the subsequent increase in root mass of *A. sinicum* and the rapid, extensive colonization of roots by AM fungi. Growth responses resulting from mycorrhiza formation were much greater in *A. sinicum* than *G. max*, and uninoculated *A. sinicum* plants had difficulty surviving even when a nutrient solution was supplied weekly.

The proportion of SDH- or ALP-active intraradical hyphae associated with *G. max* or *A. sinicum* AM roots remained high during mycorrhizal interactions up to

Table 2 Length of soil hyphae (cm g^{-1}) detected by trypan blue (*TB*) staining in association with *A. sinicum* and *G. max*, and the proportion of succinate dehydrogenase- (*SDH/TB*) and alkaline phosphatase- (*ALP/TB*) active hyphae. For each plant, in each column, data followed by the same letters are not significantly different ($P=0.05$) (*NM*, non-mycorrhizal; *Gi*, *G. intraradices; Gm, G. mosseae*)

Plant	AM fungus	Week	Total hyphae			AM hyphae
			TB	SDH/TB %	ALP/TB %	TB
Glycine max						
	NM	3	51 f	0 c	0 d	
	NM	5	122 ef	0 c	0 d	
	NM	7	236 e	0 c	0 d	
	NM	8	349 d	0 c	0 d	
	Gi	3	196 ef	73 ab	35 c	145 ef
	Gi	5	767 c	84 a	76 a	646 c
	Gi	7	1048 b	71 ab	58 b	813 b
	Gi	8	1172 b	68 ab	39 c	823 b
	Gm	3	177 ef	71 ab	58 b	126 f
	Gm	5	812 c	84 a	71 a	691 c
	Gm	7	1165 b	82 a	54 a	930 b
	Gm	8	1440 a	61 b	41 c	1091 a
Astragalus sinicum						
	NM	3	58 d	0 c	0 e	
	NM	5	153 cd	0 c	0 e	
	NM	7	232 cd	0 c	0 e	
	NM	8	290 c	0 c	0 e	
	Gi	3	233 cd	83 a	55 bc	175 c
	Gi	5	819 b	84 a	55 bc	666 b
	Gi	7	969 ab	79 a	51 bc	738 ab
	Gi	8	1114 a	61 b	26 d	824 ab
	Gm	3	311 c	85 a	71 a	253 c
	Gm	5	1005 a	79 a	64 ab	852 ab
	Gm	7	1165 a	80 a	48 c	934 a
	Gm	8	1130 a	59 b	27 d	840 ab

the last harvest, when it tended to decrease slightly in *A. sinicum*. Tisserant et al. (1993, 1996) found that the percentage of SDH- and ALP-active hyphae in *Platanus acerifolia* roots colonized by *G. fasciculatum* was initially high then decreased 6 weeks after inoculation. External hyphae developing from *A. sinicum* and *G. max* AM roots showed highest ALP activity at 3 and 5 weeks, respectively, preceding the initial plant growth responses, after which the proportion of ALP-active soil hyphae decreased. In a study using ^{32}P to detect phosphorus transport, Jakobsen et al. (1992) showed that hyphae of AM fungi had a strong affinity for ^{32}P uptake during early stages of proliferation in soil, after which hyphal transport decreased, suggesting a reduced activity of older hyphae in absorbing phosphorus. However, Hamel et al. (1990) reported that active hyphae stayed at a constant level in bromegrass roots and that the percentage of SDH-active hyphae in soil increased over 12 weeks. In the latter study, *G. intraradices* colonized roots of bromegrass slowly and only about 10% of roots were colonized 6 weeks after transplanting, so that the majority of the hyphae of *G. intraradices* may still have been young at a later stage.

The membrane-filter technique (Hansell et al. 1974) has been used to measure AM external hyphae in several studies (Abbott et al. 1984; Abbott and Robson 1985; Jakobsen et al. 1992; Kabir et al. 1997). Although this technique is efficient for extracting hyphae from soil, it is often difficult to distinguish the hyphae of AM fungi from those of non-mycorrhizal fungi. The amount of AM hyphae can only by estimated by subtracting that of fungi present in uninoculated soil (Abbott and Robson 1984; Sylvia 1992). In the present study, metabolically active hyphae were easily detected in AM-inoculated soil by their SDH and ALP activities, whereas none were present in uninoculated soil, as previously reported by Schubert et al. (1987) using FDA staining. However, care should be taken when using the described procedure: blending in excess of 40 s at high speed can lead to a loss of the purple- (for SDH) or black- (for ALP) stained contents of hyphal fragments.

Soil hyphae formed by *G. mosseae* and *G. intraradices* reached a total of over 8 and 11 m per gram dry soil, respectively, which is within the range reported by other investigators (Abbott et al. 1984; Abbott and Robson 1985; Li et al. 1991; Jakobsen et al. 1992). There was no constant relationship between intraradical fungal development and external hyphal production, supporting the conclusions of Graham et al.

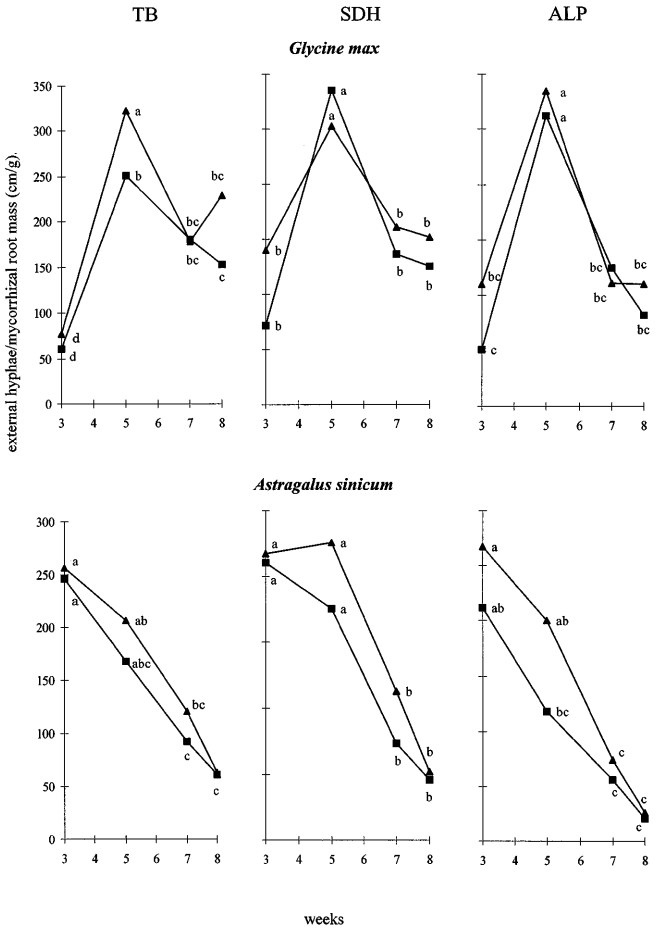

Fig. 3 Ratio of external to internal fungal abundance (cm hyphae per g mycorrhizal root) associated with *G. intraradices* (-■-) or *G. mosseae* (-▲-), estimated by staining with trypan blue (*TB*), for succinate dehydrogenase (*SDH*) and for alkaline phosphatase (*ALP*) activities. In each graph, data followed by the same letter(s) are not significantly different ($P = 0.05$)

(1982), Abbott and Robson (1985) and Zhao and Ma (1994) that the ability of AM fungi to form external hyphae in soil is not related to the amount of mycorrhizal root. Abbott and Robson (1985), using the membrane extraction technique, observed that *G. fasciculatum* produced less external hyphae in soil than *G. calospora*, even though the former fungus consistently colonized a greater portion of the root cortex of subterranean clover.

The estimates obtained in this study for the length of ALP-active soil hyphae per unit of mycorrhizal biomass showed different tendencies according to whether the fungi were in association with *A. sinicum* or *G. max* roots. It is likely that this reflected differences in physiological interactions between the AM fungi and the two host plants. Moreover, the fact that hyphae inside plant roots remained active longer than hyphae in soil suggests that internal and external hyphae of AM fungi have different life-spans. Carbon supply to the external mycelium will be a limiting factor to AM hyphal survival in the soil, and also the enzymatic activity of the fungi may be affected by soil factors. Production of external hyphae has already been found to be affected by phosphorus (Abbott et al. 1984; Schüepp et al. 1987) and soil pH (Sylvia 1990; Zhao and Liu 1992). Mycelium developing within roots will be less nutrient-limited and more protected by host tissue against direct influences of variations in soil factors.

In conclusion, the present observations show that in AM (1) intraradical fungal biomass stays active longer than soil hyphae and follows root development, (2) there is no correlation between intraradical and soil hypha development, and (3) the ratio of soil to intraradical fungal abundance is dependent on the host plant. Evaluation of the importance of AM fungi for plant growth needs to take into account the physiological interactions between fungus and host plant, but to fully understand these, measurements of fungal viability are necessary since total fungal biomass may or may not reflect the symbiotic efficiency of a mycorrhizal association in terms of enhancement of host growth.

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