

## Possible influence of *Rhizobium* on VA mycorrhiza metabolic activity in double symbiosis of alfalfa plants (*Medicago sativa* L.) grown in a pot experiment

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**Summary.** Alfalfa (*Medicago sativa*, L. cv Aragón) plants were grown under greenhouse conditions in pots of inert sand and vermiculite. The plants were inoculated with *Rhizobium meliloti* strain 102F28, with *Glomus fasciculatus* or with a mixture of both microorganisms. Plants inoculated with both *Rhizobium* and *Glomus* had the highest shoot dry weight and the lowest root-to-shoot ratio. Roots from dually inoculated plants also had a higher oxygen uptake and nodule nitrogenase activity than those from plants inoculated with either of the two microsymbionts alone. However, the dry weight of the roots from only VAM-infected plants was higher than those from *Rhizobium* or from *Rhizobium* plus *Glomus*-inoculated ones. These differences did not correlate with succinate dehydrogenase activity, which was similar between treatments. Nutrient element concentrations were increased in dually infected plants in comparison with those of plants inoculated with only *Rhizobium* or *Glomus*. These data suggest that *Rhizobium* may affect fungal metabolism and that the effect is not achieved via the tricarboxylic acid pathway.

**Key words:** *Rhizobium meliloti* – *Glomus fasciculatus* – *Medicago sativa* – Succinate dehydrogenase – Acetylene reduction assay (ARA) – VA mycorrhiza – Alfalfa

Legumes are unique among higher plants in that they can be infected by both *Rhizobium* and vesicular-arbuscular mycorrhizal (VAM) fungi. The advantages that leguminous plants can obtain from the double symbiosis have been well documented from a plant ecological viewpoint as well as from crop production aspects (Barea and Azcón-Aguilar 1983; Harley and Smith 1983).

Since the pioneer work of Asai (1944), which indicated that nodulation of some legumes was dependent on the formation of mycorrhizas, the role that

VAM fungi play in the N<sub>2</sub> fixation process carried out by *Rhizobium* has been the subject of numerous studies (Barea and Azcón-Aguilar 1983). It is well established that mycorrhizas supply phosphate to the plant and that this improved P supply gives increased *Rhizobium* activity (Asimi et al. 1980). Mycorrhizal infection also increases the yield and N content of soybean plants compared with nonmycorrhizal controls (Ganry et al. 1985), and using <sup>15</sup>N, Kucey and Paul (1982) showed that dually infected soybean plants fix more N<sub>2</sub> than nodulated but non-mycorrhizal hosts.

In studies on the effect of the dual symbiosis on legume nutrition and growth, most work has been done in different types of soils and has been focused on investigating the effect of mycorrhizal infection on the development of nodulated legumes. In the present paper we report the results of a series of experiments carried out to assess the possible effects of *Rhizobium* on VAM fungal metabolic activity.

### Materials and methods

Seeds of alfalfa (*Medicago sativa*, L. cv Aragón) were surface-sterilized with HgCl<sub>2</sub> for 10 min and then thoroughly rinsed with sterile water. After germination, seedlings were selected for uniformity, then planted in autoclaved 500-g plastic pots containing a mixture of pure sand and homoionic vermiculite (1:1 v/v) and grown under greenhouse conditions: supplementary light (Sylvania incandescent and cool-white lamps, 400 nmol m<sup>-2</sup> s<sup>-1</sup>, 400–700 nm), 16/8 h light/dark cycle, 27/17 °C day/night temperature and 50% relative humidity. All plants (two plants per pot) were fed weekly with the nutrient solution described by Hepper and O'Shea (1984) at pH 7.2. During the first 2 weeks the nutrient solution also contained 2 mM KNO<sub>3</sub>. Plants were inoculated at sowing with *R. meliloti* alone, with *G. fasciculatus* alone, or with a mixture of both microorganisms. The *R. meliloti* strain 102F28 was originally obtained from J. C. Burton (Nitragin Co., Mo, U.S.A.) and is a field isolate. Bacteria were maintained asymbiotically on yeast-mannitol slopes and resuspended in sterile water (10<sup>8</sup> cells ml<sup>-1</sup>) before using for inoculation. *G. fasciculatus* inoculum consisted of 3 g soil from maize-plant stocks which contained spores, mycelium and infected root fragments. Soil filtrate (filter paper Whatman no. 1) from the rhizosphere of mycorrhizal-infected maize plants was added to each treatment. The filtrate contained common soil microorganisms but no propagules of *Endogonaceae*. No rhizobial or mycorrhizal fungi infection was observed in control plants.

those infected with any of the two microorganisms or the control plants. Single inoculation of plants with either *R. meliloti* or *G. fasciculatus* also increased the nutrient content over that of the uninoculated plants.

## Discussion

Although earlier work established that inoculation of leguminous plants with a combined treatment of VAM fungi and *Rhizobium* increased plant growth (Harley and Smith 1983), the present study extends that finding to alfalfa plants grown in an inert rooting medium (Table 1). Our data contrast with the apparent absence of any mycorrhizal effect on soybeans inoculated with *G. fasciculatus* and grown in a similar medium for 9 weeks (Bethlenfalvay et al. 1982). It is possible that the amount of P applied to the rooting medium (0.81 mg throughout the experiment) was sufficient to avoid competition between the host plants and the fungal microsymbiont for P, thus preventing the depressive effect on plant growth described by other authors.

Dually inoculated alfalfa plants showed the lowest root-to-shoot ratio (Table 1) and the highest nitrogenase activity (Table 3) and nutrient content (Table 4). These results agree with those reported in *Trifolium subterraneum* (Smith 1982), which indicated that the double symbiosis increased N<sub>2</sub> fixation and P uptake and that it also provided a well balanced nutrient supply to the plants.

Alfalfa plants inoculated with either *G. fasciculatus* alone or with *G. fasciculatus* plus *R. meliloti* 102F28 showed no significant differences in the percentage root length infected (Table 2), but the total mass of the infected roots was much higher in the plants inoculated with *G. fasciculatus* alone (Table 2). Nonetheless, P concentration and oxygen uptake were higher in the plants infected with both microorganisms (Tables 3 and 4). These observations suggest that *Rhizobium* may affect VAM fungal metabolism so that the fungus provides an increased supply of P which, in turn, improves the symbiotic performance of *Rhizobium* (Bergersen 1971).

The succinate dehydrogenase assay can indicate the metabolically active sites of the fungal infection (MacDonald and Lewis 1978). Since succinate dehydrogenase activity in the present study was nearly equal with both treatments (Table 2), the observed effect of *Rhizobium* on the VAM fungus metabolism could not have been reached via the tricarboxylic acid pathway. Other biochemical pathways, such as the pentose phosphate cycle, which is present in the VAM fungi (MacDonald and Lewis 1978), could be involved in this phenomenon.

Despite the relatively low levels of VAM infection found in the inert medium used in this study (Table 2)

in comparison with those obtained for alfalfa grown in soil (68%–81%; Ocampo and Barea 1985), the method is suitable for study of the basic physiology and ecology of tripartite associations between leguminous plants and *Rhizobium* and *Glomus*.

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## References

- Asai T (1944) Über die Mycorrhizenbildung der Leguminosenpflanzen. *Jpn J Bot* 13:462–485
- Asimi S, Gianinazzi-Pearson V, Gianinazzi S (1980) Influence of increasing soil phosphorus levels on interaction between vesicular-arbuscular mycorrhizae and *Rhizobium* in soybeans. *Can J Bot* 58:2200–2205
- Azcón R, Ocampo JA (1981) Factors affecting the vesicular-arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. *N Phytol* 87:677–685
- Barea JM, Azcón-Aguilar C (1983) Mycorrhizas and their significance in nodulating nitrogen-fixing plants. *Adv Agron* 36:1–54
- Bedmar EJ, Olivares J (1980) Effect of chemical inhibitors of photorespiration on nitrogenase activity in nodulated alfalfa plants. *Planta* 150:299–302
- Bergersen FJ (1971) Biochemistry of nitrogen fixation in legumes. *Annu Rev Plant Physiol* 22:121–140
- Bethlenfalvay GJ, Brown MS, Pacovsky RS (1982) Parasitic and mutualistic association between a mycorrhizal fungus and soybean: Development of the host plant. *Phytopathology* 72:889–893
- Del Rio LA, Gómez-Ortega M, Leal A, López-Gorgé J (1977) A more sensitive modification of the catalase assay with the Clark oxygen electrode. *J Anal Biochem* 80:409–415
- Ganry F, Diem HG, Wey J, Dommergues YR (1985) Inoculation with *Glomus mosseae* improves N<sub>2</sub> fixation by field-grown peas. *Biol Fertil Soils* 1:15–23
- Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *N Phytol* 84:489–500
- Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London
- Hepper CM, O'Shea J (1984) Vesicular-arbuscular mycorrhizal infection in lettuce (*Lactuca sativa*) in relation to calcium supply. *Plant Soil* 82:61–68
- Kucey RMM, Paul EA (1982) Carbon flow, photosynthesis and N<sub>2</sub> fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). *Soil Biol Biochem* 14:407–412
- MacDonald RM, Lewis M (1978) The occurrence of some acid phosphatases and dehydrogenases in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *N Phytol* 80:135–141
- Ocampo JA, Barea JM (1985) Effect of carbamate herbicides on VAM mycorrhizal infection and plant growth. *Plant Soil* 85:375–383
- Phillips JM, Hayman DS (1970) Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans Br Mycol Soc* 55:158–161
- Smith SE (1982) Inflow of phosphate into mycorrhizal and non-mycorrhizal *Trifolium subterraneum* at different levels of soil phosphate. *N Phytol* 90:293–303

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Nitrogenase activity (acetylene reduction assay) was determined on detached root systems excised at the cotyledonary node as previously described (Bedmar and Olivares 1980). These root systems were incubated with 4 mM of 10% C<sub>2</sub>H<sub>2</sub> in air before sampling for C<sub>2</sub>H<sub>4</sub> at 5 and 10 min. Oxygen uptake by detached root nodules was determined amperometrically on a Gibson oxygraph equipped as described by Del Rio et al. (1977). The final reaction volume was 3 ml of 50 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid) buffer at pH 7.0, containing 0.2 g nodule fresh weight.

Mycorrhizal infection was estimated by clearing and staining the roots (Phillips and Hayman 1970) and the percentage root length infected was measured by the grid-line intersect method (Giovannetti and Mosse 1980). Succinate dehydrogenase activity in the fungal mycelium was detected by the reduction of tetrazolium salt at the expense of added succinate (MacDonald and Lewis 1978), and measured according to Ocampo and Barea (1985). All plants were harvested after 7 weeks of growth. Plant dry weight was determined on plants that had been heated at 70°C for 48 h. Reduced N and P, K, Ca and Mg contents of the alfalfa plants were estimated as described by Azcón and Ocampo (1981). The experiments were repeated twice under the experimental conditions described and mean values of six replicates are reported. Data were examined by one-way analysis of variance in order to assess significant treatment effects.

## Results

Table 1 shows that inoculation of alfalfa plants with either *R. meliloti* 102F28 alone or *G. fasciculatus* alone significantly increased the root and shoot dry weights in comparison with the uninoculated ones, and that plants dually inoculated with both microorganisms averaged the highest dry weight. On the other hand, the dry weight of only VAM-infected roots was statistically higher than that of the roots inoculated with the combined addition of *R. meliloti* 102F28 and *G. fasciculatus*. Finally, although plants inoculated with either of the two microorganisms alone showed a lower root-to-shoot ratio than the control plants, the lowest root-to-shoot ratio for all treatments was found in dually inoculated plants.

Table 2 shows that the percentage of VAM-infected roots and the percentage of VAM mycelium with metabolic activity, as indicated by succinate dehydrogenase assay, were similar between plants inoculated with *G. fasciculatus* alone or with *R. meliloti* plus *G. fasciculatus*. Nevertheless, the dry weight of the roots from the former treatment was higher than that of the dually inoculated roots.

Table 3 shows that the oxygen consumption rates of roots from dually inoculated plants were higher than those of roots from plants inoculated with either *R. meliloti* alone or with *G. fasciculatus* alone. Similarly, acetylene-dependent ethylene production by *Rhizobium*-nodulated plus *Glomus*-infected roots was twice as high as that from roots inoculated with *R. meliloti* alone.

Table 4 shows that the nutrient content (N, P, K, Ca, and Mg) of alfalfa plants inoculated with both microsymbionts under study was always higher than in

**Table 1.** Root-to-shoot (R/S) ratio and shoot and root dry weights of alfalfa plants inoculated with *G. fasciculatus* alone (M), with *R. meliloti* 102F28 alone (R), with *G. fasciculatus* plus *R. meliloti* 102F28 (M + R), or uninoculated (C)

Treatments	Dry weight (mg plant <sup>-1</sup> )		R/S
	Shoot	Root	
C	63	105	1.67
M	170	296	1.74
R	113	194	1.72
M + R	185	199	1.07
LSD (0.05)	12	27	0.25

LSD, least significant difference

**Table 2.** Mycorrhizal infection of alfalfa plants inoculated with *G. fasciculatus* alone (M), with *R. meliloti* 102F28 alone (R), with *G. fasciculatus* plus *R. meliloti* (M + R), or not at all (C)

Treatments	Infected roots (mg plant <sup>-1</sup> )	Root length infected (%)	Mycelium with SDH activity (%)
C	0	0	0
M	108	40	23
R	0	0	0
M + R	74	37	27
LSD (0.05)	9	5	7

SDH, succinate dehydrogenase

**Table 3.** Acetylene reduction and oxygen uptake of alfalfa roots inoculated with *G. fasciculatus* alone (M), with *R. meliloti* 102F28 alone (R), with *G. fasciculatus* plus *R. meliloti* 102F28 (M + R), or not at all (C)

Treatments	ARA (μmol C <sub>2</sub> H <sub>4</sub> plant <sup>-1</sup> h <sup>-1</sup> )	Oxygen uptake [μmol O <sub>2</sub> (mg nod. fresh wt.) <sup>-1</sup> m <sup>-1</sup> ]
C	0	0.70
M	0	1.19
R	0.51	0.99
M + R	1.17	1.95
LSD (0.05)	0.27	0.22

ARA, acetylene reduction assay; nod fresh wt., fresh weight of nodules

**Table 4.** Nutrient content (expressed as percentage dry weight) of alfalfa plants inoculated with *G. fasciculatus* alone (M), with *R. meliloti* 102F28 alone (R), with *G. fasciculatus* plus *R. meliloti* 102F28 (M + R), or not at all (C)

Treatments	Nutrient				
	N	P	K	Ca	Mg
C	0.94	0.04	2.45	1.14	0.58
M	1.42	0.12	4.28	2.79	1.05
R	2.90	0.10	3.26	1.90	0.95
M + R	4.62	0.19	5.39	3.55	1.47
LSD (0.05)	0.28	0.03	0.41	0.36	0.27