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COINOCULATION OF BLACK LOCUST WITH *RHIZOBIUM* AND *GLOMUS* ON A DESURFACED SOIL

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Seedlings of black locust (*Robinia pseudoacacia* L.) were coinoculated with a local effective strain *Rhizobium* species RpI1 and with *Glomus deserticola*. The effects of both symbioses on plant growth were studied in a greenhouse experiment with four treatments: two single inoculations, one double inoculation, and no inoculation, in a nonsterilized substrate from a desurfaced soil. A synergic effect was observed because double inoculation produced significantly higher shoot biomass, nodule biomass, and N_2 fixation, as measured by the acetylene reduction assay (at least 93%, 50%, and 192%, respectively) than any single inoculation treatment and than noninoculated controls, which were nodulated by soil-born rhizobia. Mycorrhizal colonization did not improve the growth of control plants, but it did significantly improve the specific nitrogenase activity of plants inoculated also with *Rhizobium* species RpI1 by 77%. We conclude that prenodulation of plants is necessary for better growth on harsh substrates and that mycorrhizal colonization helps nodulated plants reach their P demand in P-limited soils. (Soil Science 2008;173:00–00)

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Key Words: Desurfaced soil, mycorrhiza.

GOOD agricultural quality lands in Buenos Aires province (Argentina) have traditionally been mined to extract topsoil for the manufacture of bricks and other building materials. The loss of organic matter severely limits plant growth in desurfaced soils. Afforestation could be a good use for these soils, but the physical and chemical deficiencies typical of desurfaced soils can limit forest production. Poor growth of *Eucalyptus* species was observed in a desurfaced field in Buenos Aires province because of bad physical and chemical properties of the soil, mainly low available N (Gimenez et al., 2002).

An alternative to the expensive and environmentally risky use of fertilizers is the establishment of N-fixing plants, such as legumes and actinorhiza, especially when, in addition, they can form associations with endomycorrhizal and ectomycorrhizal fungi. The establishment of multiple symbioses provides the plants with extra nutrient-uptake abilities (Wall, 2000), improving survival and growth in deficient soils. Thus, coinoculation of N-fixing plants with effective strains of *Rhizobium* or *Frankia* and with mycorrhizal fungi may help the plants to alleviate nutrient limitations.

It has been reported that dual inoculation with *Rhizobium* and arbuscular mycorrhiza produced higher biomass and nodulation in the legumenous trees *Acacia auriculiformis*, *Acacia mangium*, *Cajanus cajan*, and *Paraseriathes falcataria* (Chang et al., 1986; De la Cruz et al., 1988; De Lucena Costa et al., 1990). On the other hand, the interaction among *Gliricidia sepium*, *Rhizobium*, and arbuscular mycorrhiza did not occur or was negative (antagonist effect) because the plants inoculated with *Rhizobium* had higher biomass than plants inoculated with *Rhizobium* and *Glomus mosseae* or *Gigaspora* species (Manguiat et al., 1990). Gardezi et al. (1988) reported no significant interactions among *Acacia saligna*, *Rhizobium* species, and *Glomus* species. These negative interactions may be caused by competition between symbionts for the root infection sites. In experiments with soybean, *Bradyrhizobium* species, and *Glomus* species, the simultaneous inoculation

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did not produce competitive effects, but prior inoculation with one symbiont inhibited the establishment of the other (Bethlenfalvay et al., 1985).

In a greenhouse experiment with *Robinia pseudoacacia*, *Rhizobium*, and arbuscular and ectomycorrhiza, important synergic effects were observed. The multiple inoculation provided greater growth than any other combination of microsymbionts (Tian et al., 2003).

In actinorhizal plants, beneficial interactions were also detected. In the symbiosis *Casuarina equisetifolia–Frankia–Glomus fasciculatum*, synergistic effects on the total biomass were observed (Vasanthakrishna et al., 1994) with no evidence of competition for the infection sites (Sempavalan et al., 1995).

Robinia pseudoacacia L. (black locust), also known as "acacia blanca" or "falsa acacia" in Argentina, is a legume tree native of North America, which is very well adapted to Argentina, where it is currently used in urban afforestation and as windbreaks for agricultural protection. Its fast growth and high N-fixing capacity makes it appropriate for rehabilitation of marginal lands in different geographic and climatic conditions (Keresztesi, 1980; Hanover and Mebrahtu, 1996).

A native *Rhizobium* isolate, strain RpI1, obtained from a single nodule of *R. pseudoacacia* collected in the field showed a high effectivity to fix N_2 either in hydroponic culture or in a substrate of a desurfaced soil (Ferrari and Wall, 2007).

In experiments with desurfaced soils, it has been shown that the loss of organic matter may affect either the survival of soil-born *Rhizobium* (Habte and El-Swaify, 1986a) or the functioning of the symbiosis (Habte and El-Swaify, 1986b). Therefore, previous inoculation of seedlings (prenodulation) before being planted in the field may be necessary to assure the success of the symbiosis in soils of low fertility.

Rhizobium-mycorrhizal interactions have been broadly studied in herbaceous legumes, but only to a limited extent in woody legumes and even less on degraded soils. Thus, the objectives of this work were: (i) to select the most infective strain of arbuscular mycorrhiza fungi to colonize the roots of *R. pseudoacacia*; (ii) to study growth, nodulation, and N_2 fixation of black locust seedlings inoculated with an effective strain of *Rhizobium* and with the selected fungus on a desurfaced soil substrate; and (iii) to assess possible interactions between the different symbioses.

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MATERIALS AND METHODS

Soil

The soil was collected from a desurfaced land near Buenos Aires City. The field was used for brick manufacture 30 years ago and is now abandoned. The topsoil extraction resulted in the exposure of the B2t horizon. The soil belongs to the Molisol order and Argiacuol great group (USDA, 1998). The soil for the pot experiments was prepared as previously reported (Ferrari and Wall, 2007). This soil had 1.55% of organic C, 0.17% of organic N, 14 mg/kg of available P (Bray method), 0.16 dS/m of electric conductimetry, 14.0 cmol/kg of cation exchange capacity, and pH 5.88. The texture was silty clay loam (29.89% clay, 51.63% silt, and 18.48% sand).

Plant Growth and Inoculum

Seeds of *Robinia pseudoacacia* were scarified and germinated as previously reported (Ferrari and Wall, 2007). The plants were watered as indicated below and grown in a greenhouse with temperatures between 16 °C and 28 °C and humidity between 40% and 90%, 16 hours of light and 8 hours of darkness. Natural light was supplemented from 5.00 p.m. to 10.00 p.m. with 400-W Osram lamps that provided a light intensity of 1400 lux over plants at about 1.5 m from the lamps.

Rhizobium RpI1 inoculum was prepared as previously described (Ferrari and Wall, 2007). Briefly, cells from stationary culture were washed and resuspended in mineral Hoagland 1/4 solution (Hoagland and Arnon, 1950) at about 10^8 CFU/mL. The species of arbuscular mycorrhizal fungi available in our laboratory were *Gigaspora rosseae*, *Glomus deserticola*, and *Glomus mosseae*. The original inoculum was propagated by using trap plants of cucumber (*Cucumis sativus* L.) growing on a sterilized substrate of desurfaced soil with 20% sand. The cucumber plants were grown during 2 months in pots with regular watering. They were forced to hydric stress for 7 days before harvest. The mixture of substrate and mycorrhized roots of cucumber was used as an inoculum. The available species of ectomycorrhizal fungi were *Pisolithus tinctorius*, *Hebeloma cylindrosporum*, and *Suillus bovinus*; the former was isolated from roots of *Eucalyptus globulus* and the two latter from roots of *Pinus* species. They were cultivated in darkness, in plates with Pachlewsky 5 medium for *Pisolithus* (Martin et al., 1998) at

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28 °C, and with Moser 6 medium (Moser, 1963) at room temperature for *Suillus* and *Hebeloma*.

N Fixation Estimation

Nitrogenase activity in living plants was estimated by the acetylene reduction assay (ARA) (Hardy et al., 1973). We used an Agilent model 6890 plus gas chromatograph with an Omnilab HayeSep T column (1 m in length, 1/8 in in diameter, 80/100 mesh), operated at 90 °C (isothermic), N_2 (AGA, Buenos Aires) as carrier gas at 30 mL/min, and an FID detector. Symbiotic efficiency in terms of N_2 fixation was also estimated by measuring leaf N concentration and total leaf N content at time of harvest, and comparing data from inoculated plants with those from noninoculated controls.

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Experimental Design

Experiment 1: Single Inoculation With Mycorrhiza Fungus

After germination, plants (4-5 cm mean height) were put in small pots filled with a mixture of desurfaced soil and sand (previously disinfected by autoclaving 15 min at 121 °C) in a 3:1 ratio. After transplanting, plants were inoculated with the mycorrhizal inoculum of *Glomus mosseae* (103 spores per gram), *Glomus deserticola* (143 spores per gram), and *Gigaspora rosseae* (15 spores per gram). Approximately 1 g of the arbuscular mycorrhiza inoculum was added to the pots at the moment of transfer of the plants to the pots. As an ectomycorrhiza fungus inoculum, we used 0.5 × 0.5–cm square fragments taken from the outer zone of hyphae growth in the plates, settling 5 or 6 fragments in each pot. Plants were not inoculated with *Rhizobium*. Plants not inoculated with fungi were used as controls. The number of replicates was 12.

All pots were watered only with tap water (about 40 mL per pot) for 84 days. After harvest, the root system was observed for mycorrhizal infection. For quantification of arbuscular mycorrhiza, we used the trypan blue staining method (Phillips and Hayman, 1970) and the gridline intersection method (Giovannetti and Mosse, 1980). The analysis of ectomycorrhizal colonization was done by direct observation of root under a stereomicroscope.

Experiment 2: Double Inoculation With Rhizobium *and Arbuscular Mycorrhiza Fungi*

Three days after germination, plants were transferred to 330-cm^3 pots filled with sterile perlite. Half the plants were inoculated with *Rhizobium* RpI1 by pouring 5 mL of the inoculum into each pot. The other half was not inoculated (controls). All plants were watered with mineral Hoagland 1/4 solution (Hoagland and Arnon, 1950) supplemented with 1 mg/L of N as ammonium nitrate. In a former experiment (Ferrari and Wall, 2007), it was determined that full nodulation of plants grown in hydroponic culture occurred 15 to 25 days after the inoculation.

All plants were removed from the plots 25 days after germination for examination of nodulation. No nodules were found in control plants. All inoculated plants were fully nodulated. After examination for nodulation, all the plants were transferred to new 330-cm^3 pots filled with the substrate of the desurfaced soil, not sterilized, and which had been previously sieved through a 1 × 1–cm mesh. At transfer to pots, some pots were inoculated with 3 g per pot of *Glomus deserticola* (chosen after the results of Experiment 1) inoculum, and some pots were not inoculated. Subsequently, all plants were watered twice a week with only distilled water (about 40 mL per pot), with the corresponding care to avoid cross-contamination of noninoculated plants.

Thus, four treatments were conducted: single inoculation with *Rhizobium* RpI1 (R), single inoculation with *G. deserticola* (G), double inoculation with *Rhizobium* RpI1 and *G. deserticola* (RG), and no inoculation (C). Twelve replicates constituted each treatment.

At the end of the experiment, nitrogenase activity of every intact plant was measured by the ARA. After that, all plants were harvested and separated into leaflets, root, and nodules. Plant biomass was measured as dry matter after drying for 48 hours at 60 °C. The dried leaves were milled and analyzed for total N and P. Total N was analyzed by Kjeldhal digestion (USDA, 1972) with determination of ammonia in the distillate by the Nessler method (APHA-AWWA-WEF, 1992). Total P was analyzed by digestion with sulfuric acid–hydrogen peroxide (Hach et al., 1987) with determination of phosphate by the molybdenum blue–ascorbic acid method (Murphy and Riley, 1962).

Statistical analyses were conducted using the Student's *t* test with a Sigma Plot program.

RESULTS

Experiment 1

There were no significant differences in shoot height or in total biomass of noninoculated

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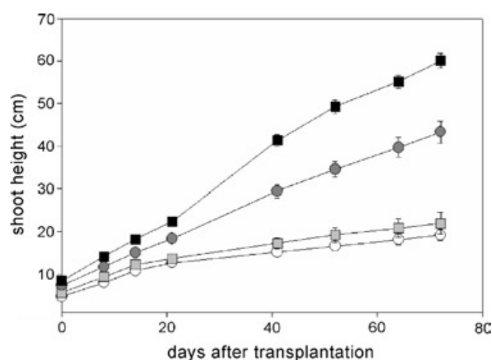

Fig. 1. Growth of black locust seedlings inoculated with *Rhizobium* Rpl1 alone (gray circle), with *Rhizobium* Rpl1 and *Glomus deserticola* (black square), with *G. deserticola* alone (gray square) and noninoculated controls (white circle) in pots filled with a desurfaced soil substrate. Bars represent standard error of the mean.

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control plants and plants inoculated with arbuscular or ectomycorrhiza fungi (data not shown). Observation of root fragments under the stereomicroscope did not reveal the typical structures of ectomicorrhizal infection in the radical apex of any plant inoculated with the ectomycorrhizal fungus. The highest infection by arbuscular mycorrhiza fungus was observed with *Glomus deserticola*, with 65% of infected roots by the gridline method. This value was significantly greater than those observed with *G. mosseae* (7%) and *G. rosseae* (8.5%).

Experiment 2

Dual-inoculated plants (treatment RG) had higher growth rate than plants with single *Rhizobium* inoculation (treatment R) because increased slope heights occurred after Day 20 after transfer to soil (Fig. 1). Plants with single *Rhizobium* RpI1 inoculation grew faster than plants inoculated with *G. deserticola* (treatment G) and than control plants (treatment C). Control and single mycorrhiza–inoculated plants had similar growth rates to the other treatments until Day 14 after transfer to pots, but subsequently they had drastically decreased growth rates (Fig. 1).

Plants with double inoculation had significantly higher dry weight for every plant component than plants with single or no inoculation (Table 1). Single inoculation with *Rhizobium* RpI1 produced higher biomass of every plant component than plants inoculated with *G. deserticola* or than noninoculated plants ($P < 0.05$). Although plants with single *G. deserticola* inoculation showed higher biomass than control plants, the difference was not significant for every plant part ($P > 0.10$). In prenodulated plants, the inoculation with *G. deserticola* resulted in a near doubling of the dry weight of every plant part (Table 1). The ratio of dry weight of every plant part and total plant dry weight gave information about which plant parts had higher relative growth. Prenodulated plants from R and RG treatments showed higher relative growth of the aerial plant portions ($P < 0.077$) and lower relative growth of the belowground part ($P < 0.05$) than plants noninoculated with *Rhizobium* RpI1, as shown by the aboveground/plant and belowground/plant ratios in Table 1.

Plants inoculated with *Rhizobium* RpI1 had significantly higher N concentrations than uninoculated plants (Table 2). There were no differences in leaf N concentration between treatments C and G and between treatments R and RG. Regarding P nutrition, there was no significant difference in leaf P concentration among treatments. The total amount of N and P in leaf biomass (N and P content) was significantly higher in treatments with *Rhizobium* inoculation (R and RG treatments) than in treatments without *Rhizobium* inoculation (C and G treatments). There were no differences between treatments C and G in the leaf N and leaf P contents. The double inoculation (RG treatment) showed higher leaf N and leaf P contents than any single inoculation (G or R treatments) (Table 2).

At harvest, the roots of plants inoculated with *G. deserticola* alone (G treatment) had 27% of mycorrhizal colonization, whereas plants with double inoculation (RG treatment) had 53% of their roots colonized, a highly significant difference ($P = 0.0013$). Under the stereomicroscope, mycorrhizal roots stained with trypan blue showed many vesicles, with a colonization density of 40 vesicles per centimeter in plants inoculated with *Glomus* alone and 99 vesicles per centimeter in plants with dual inoculation ($P = 0.00031$).

Every plant inoculated with *Rhizobium* was nodulated at harvest. About 75% of plants from C treatment and 83% of plants from treatment G, not inoculated with *Rhiziobium*, were also nodulated at the time of harvest.

Plants with double inoculation (RG treatment) had the highest nodule biomass, expressed as dry weight of nodule tissue per plant (Table 1). Nodule biomass in the single inoculation with

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TABLE 1

Mean dry weight of different plant parts of black locust seedlings growing in pots with a desurfaced soil substrate

	C	G	R	RG
Aboveground DW (mg)	659.83a (124.47)	750.00a (159.61)	2607.75b (317.65)	5025.25c (334.01)
Root DW (mg)	261.17a (40.60)	352.00a (67.31)	746.17b (136.04)	1522.92c (204.45)
Belowground DW (mg)	303.17a (48.85)	454.58a (94.84)	923.08b (148.14)	1788.08c (225.24)
Plant DW (mg)	963.00a (157.56)	1204.58a (252.40)	3530.83b (441.31)	6813.33c (516.47)
Nodule DW (mg per plant)†	56.00a (16.64)	123.10ab (35.71)	176.92b (24.70)	265.17c (30.96)
Aboveground DW/total DW	0.673a (0.033)	0.614a (0.018)	0.744b (0.020)	0.743b (0.015)
Root DW/ plant DW	0.279b (0.021)	0.318b (0.018)	0.195a (0.019)	0.217a (0.014)
Belowground DW/total DW	0.327b (0.033)	0.385b (0.018)	0.251a (0.022)	0.256a (0.015)
Nodule DW/ plant DW†	0.0635ab (0.021)	0.0807b (0.017)	0.0553ab (0.0085)	0.0391a (0.0037)
(DW nodule/DW belowground).100 (% nodulation)†	15.7ab (3.8)	20.2ab (4.1)	21.3b (2.5)	15.3a (1.4)
% Mycorrhization	—	24.5a (4.7)	—	52.9b (4.9)
% Mycorrhization/% nodulation†	—	2.61a (0.89)	—	3.40a (0.47)

Different letters indicate significant differences for $P = 0.05$ (standard error between brackets).
Belowground dry weight includes root plus nodules.
Treatments: G, plants inoculated with *Glomus deserticola*; R, plants inoculated with *Rhizobium*; RG, plants inoculated with *Rhizobium* and *G. deserticola*; C, noninoculated control plants.
Twelve replicates per treatment, except in † where number of replicates was 9 for treatment C and 10 for treatment G.
DW indicates dry weight.

Rhizobium (R treatment) was lower than the double inoculation ($P = 0.036$) but higher than nodulated plants of treatments C ($P = 0.0013$) and G, although the latter difference was not significant ($P = 0.22$) (Table 1).

Inoculation with *G. deserticola* significantly increased the nodule biomass of prenodulated plants by 50%. Mycorrhizal colonization increased nodule biomass in control plants by 120%, but this increment was only slightly significant ($P = 0.12$).

Although plants with dual inoculation showed higher total nodule biomass than plants with single *Rhizobium* inoculation, the nodule biomass–plant biomass ratio was significantly lower ($P < 0.092$) for double-inoculated plants than for single-inoculated plants.

Nitrogenase activity (ARA) was clearly higher in plants with double inoculation than in any other treatment (Table 3). In plants not inoculated with *Rhizobium*, ARA was detectable in only 50% of control plants and in 70% of plants with single inoculation with mycorrhiza. Plants with single inoculation of *Rhizobium* show higher values of ARA than noninoculated plants (treatment C, $P = 0.017$) and than plants inoculated only with *G. deserticola* (treatment G, $P = 0.038$). Mycorrhizal inoculation increased total ARA by 97% for control plants (compare treatment G with treatment C) and of 192% for prenodulated plants (compare treatment R with treatment RG). The specific nitrogenase activity expressed as ARA per nodule dry weight was significantly higher ($P = 0.041$) in double-inoculated plants (treatment RG) than in plants inoculated with only *Rhizobium* RpI1 (treatment R). This difference was even more pronounced ($P < 0.002$) when compared with activity of plants nodulated by endogenous rhizobia (treatments C and G). Similarly but not the same, single inoculation with *Rhizobium* RpI1 (treatment R) showed higher specific ARA than plants of treatments C and G, although the difference was not statistically significant ($P = 0.05$). Plants of treatments C and G showed no differences either in total ARA or in specific ARA ($P = 0.48$ and $P = 0.81$, respectively). Mycorrhizal colonization did

TABLE 2

Leaf N and P concentration (% dry weight) and total content (mg per plant) of black locust seedlings growing in pots filled with a desurfaced soil substrate

	C	G	R	RG
Leaf N concentration (%)	1.71a	1.87a	2.52b	2.65b
Leaf P concentration (%)	0.30a	0.28a	0.25a	0.26a
Leaf N content (mg)	15.4a	20.1a	66.0b	130.4c
Leaf P content (mg)	2.4a	2.2a	6.5b	13.1c

Different letters indicate significant differences for $P = 0.05$.
Treatments: G, plants inoculated with *Glomus deserticola*; R, plants inoculated with *Rhizobium*; RG, plants inoculated with *Rhizobium* and *G. deserticola*; C, noninoculated control plants.
Twelve replicates per treatment.

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TABLE 3

Nitrogenase activity measured by the ARA of black locust seedlings growing in pots filled with a desurfaced soil substrate, expressed as total plant ARA, specific ARA (ARA per nodule dry weight), and as ARA per plant dry weight

	C	G	R	RG
Total ARA (μmol/h plant)	0.87a (0.312)	1.356a (0.647)	3.424b (0.701)	10. 017c (0.829)
Specific ARA (μmol/h gram nodule DW)	10.178ab (4.406)	8.962a (2.735)	24.147b (6.232)	42.653c (5.795)
ARA per plant biomass (μmol/h gram plant DW)	0.900ab (0.424)	0.862a (0.289)	1.013ab (0.185)	1.533b (0.155)

Different letters indicate significant differences for $P = 0.05$ (standard error between brackets).
Treatments: G, plants inoculated with *Glomus deserticola*; R, plants inoculated with *Rhizobium*; RG, plants inoculated with *Rhizobium* and *G. deserticola*; C, noninoculated control plants.
Number of replicates: 6 for treatment C, 7 for treatment G, and 12 for treatments R and RG.

not significantly modify specific ARA in control plants (compare treatment G with treatment C); however, it resulted in a 77% increase in the prenodulated plants (compare treatment RG with treatment R). The ratio "total ARA–plant biomass" was significantly higher ($P = 0.042$) in plants with dual inoculation than in plants with single *Rhizobium* inoculation (Table 3).

DISCUSSION

The dual inoculation of black locust seedlings with an effective strain of *Rhizobium* and with *G. deserticola* was beneficial for plant growth on a desurfaced soil, producing higher growth rate, nodulation, and N_2 fixation than any single inoculation or than no inoculation. Plants not inoculated with *Rhizobium* had nodules at harvest, probably by infection with soil-borne rhizobia present in the nonsterilized desurfaced field, as previously reported (Ferrari and Wall, 2007).

Mycorrhizal colonization produced a strong stimulation of growth of previously inoculated plants, but it had no effect on noninoculated plants that were subsequently nodulated by native soil rhizobia presented in the soil sample.

The higher development of the aerial plant portion that had been inoculated with *Rhizobium* RpI1 could be explained by an active biological N_2 fixation, which provides the necessary N for protein synthesis. On the other hand, the high development of the belowground part of the plant in the treatments without *Rhizobium* inoculation would indicate a need of radical exploration of these plants for an adequate nutrient uptake. At the same time, both results suggest a lower efficiency of native rhizobia, which induced nodulation in plants of the treatments with no *Rhizobium* RpI1 inoculation.

Plants with double inoculation showed larger root colonization by *G. deserticola* than plants inoculated only with *G. deserticola*, demonstrating an absence of competence between *Rhizobium* RpI1 and *G. deserticola* by the infection sites in the root. Moreover, it suggests that the higher root biomass in plants with dual inoculation would have stimulated the colonization by *G. deserticola*, perhaps caused by a higher allocation of carbohydrates to the root because of an increase of N availability with respect to the other treatments. The difference in root colonization by *G. deserticola* in plants nodulated by soil-born rhizobia suggests a less efficient N_2 fixation.

Nodulation was stimulated by mycorrhizal colonization either in control or in prenodulated plants; however, this increment in nodule biomass of control plants by mycorrhiza was not statistically significant. One possible explanation of the lack of significance could be the high data dispersion because of the low number of plants that were nodulated by soil-borne rhizobia.

In prenodulated plants, mycorrhizal inoculation increased nodule biomass by 50%, total ARA by 192%, and specific ARA by 77%. In control plants, nodulation was increased 120% and ARA by 56%. The specific ARA remained unmodified. These results suggest that mycorrhizal colonization had a stronger stimulating effect in the nodule activity than in nodulation. In this way, the higher growth of prenodulated mycorrhized plants would be the result of an improvement of the efficiency of N_2 fixation in the nodules.

Mycorrhizal colonization did not change N leaf concentration, but it did increase the total amount of N in foliar tissue. It would indicate that the higher N inputs by symbiotic fixation in dual-inoculated plants produced an increase in leaf biomass, suggesting that the speed of N

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accumulation was compensated by the speed of biomass growth.

Mycorrhizal inoculation of plants not inoculated with *Rhizobium* did not improve growth, N and P nutrition status nodulation, and N_2 fixation, suggesting that mycorrhiza benefit depends on the efficiency of N_2 fixation of the nodulating *Rhizobium* strain. The benefits of double inoculation could be associated with P nutrition as follows: N-fixing plants have a higher P demand than plants dependant exclusively on soil-combined N (Zahran, 1999). Thus, mycorrhizal infection could simply help the plant match that high P demand.

There is no common agreement about the effect of P in the function of symbiotic N_2 fixation. Some authors (Yang, 1995; Reddell et al., 1997) suggest an indirect effect through a better nutrition of the host plant (that generally has higher P requirements than the N_2 fixation process per se). In contrast, a specific and direct effect of P in the regulation of nodule growth in the actinorhizal plants *Discaria trinervis* (Valverde et al., 2002) and *Alnus incana* (Wall et al., 2000) has been demonstrated.

Therefore, the higher beneficial effects of mycorrhizal colonization in prenodulated plants suggest that the levels of P in the substrate were limiting for plants dependant on symbiotic fixation, as it is known that P deficiency affects the N_2 fixation process. In contrast, P levels in the soil seem not to be limiting because control plants did not respond to mycorrhizal inoculation.

In a similar pot experiment with black locust, Tian et al. (2003) found synergic effects of the double inoculation *Rhizobium*–*G. deserticola* in plant growth and nodulation. However, they did not find stimulation of specific nitrogenase activity (measured as ARA). Apparently, the higher plant growth would have been reached because of a highly effective nodulation induced by an increase in the plant N and P demand as a consequence of the total biomass increase. The main difference between the soil of Tian et al. and ours may be the level of available P. Our soil had almost four-fold less P. This could be the cause of a higher stimulation of specific nitrogenase activity by mycorrhiza in our experiment.

Finally, double inoculation with effective strains of *Rhizobium* and mycorrhiza as a prior step to the transfer to the field would allow black locust to perform better in soils deficient in N and P (a common situation in desurfaced and other kinds of degraded soils).

ABBREVIATIONS

DW, dry weight;
ARA, acetylene reduction assay;
AM, arbuscular mycorrhiza.

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