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Nutrient and growth interactions in soybeans colonized with *Glomus fasciculatum* and *Rhizobium japonicum**

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Summary *Glycine max* (L. Merr. cv. Amsoy 71) plants were grown in a greenhouse in a sand/perlite medium low in plant-available N and P. Plants were either inoculated with a vesicular-arbuscular mycorrhizal (VAM) fungus alone, a strain of *Rhizobium japonicum* alone, both endophytes together or were left non-inoculated to serve as a control. All combinations received a N- and P-free nutrient solution. Nodulated plants contained 4 to 5 times the phytomass of non-inoculated controls, and plants colonized with both the VAM fungus and Rhizobium were 18% greater in dry weight than nodulated, non-VAM plants due to a positive VAM times Rhizobium interaction. Nitrogen fixation, calculated from C_2H_4 and H_2 data, was significantly higher in the tripartite symbiosis, with 80% of the increase attributable to increased nodule mass and 20% due to increases in specific nodule activity. Colonization by the VAM fungus and the development of vesicles increased significantly following nodulation. The synergistic interactions between the microsymbionts suggests that the response of the host to dual colonization is complex and depends on a balance between the three members of the symbiosis.

Introduction

In most soils N_2-fixing legumes exist as a tripartite association[3]. The host plant provides C to the Rhizobium and the vesicular-arbuscular mycorrhizal (VAM) fungus. The P input due to VAM fungi influences host growth as well as nodulation and N_2 fixation[4]. A number of studies have been performed in the field indicating that inoculation with VAM fungi can increase the N input by Rhizobium[7] or the harvest index for soybeans[16]. The effectiveness of VAM fungi is greatest in soils amended with sparingly soluble P[17], in soil low in P[18], or P-fixing soils[5]. Inoculation with VAM fungi can result in growth inhibition of the host when P is limiting, but N is not[8,13].

Parasitic growth of a VAM fungus in a sand/perlite medium amended with hydroxyapatite (HAP) has been demonstrated in a N_2-fixing

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legume[9]. The question remains: will growth inhibition following fungal colonization occur in extremely N-deficient environments? Soil N status greatly effects the external critical P requirement, and adequate growth of most soybean cultivars demands a balanced input of both minerals[11]. It is common practice to grow nodulated legumes on poor agricultural soils to increase their fertility, and effective Rhizobium strains are often used for treating seeds[7]. Endomycorrhizae have the potential for increasing the efficiency of these associations[12], but an understanding of the biotic and environmental factors that foster mycotrophic growth is required before use of VAM fungi along with Rhizobium (RHIZO) becomes an accepted agronomic practice.

The purpose of this study was to examine the effects of inoculation with either a VAM fungus alone, a strain of Rhizobium alone, or both endophytes together on the growth and mineral content of soybeans grown in a severely N-limited environment.

Materials and methods

Biological materials

Soybean (*Glycine max* L. Merr. cv. Amsoy 71) seeds (0.5 to 0.6 g) were surface sterilized and planted as before[20]. Plants were inoculated with one or both of the microsymbionts or were left non-inoculated (control). All combinations received a N- and P-free nturient solution[22] consisting of 1.5 m*M* $CaCl_2$, 0.5 m*M* K_2SO_4, 0.25 m*M* $MgSO_4$, 25 μM H_3BO_3, 20 μM FeEDDHA, 2.0 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.4 μM H_2MoO_4 and 0.6 μM $CoCl_2$. *Rhizobium japonicum* strain USDA 61A118 and the VAM fungus *Glomus fasciculatum* (Thaxter *sensu* Gerd.) Gerd. and Trappe were obtained, cultured and inoculated as described in previous research[8,19]. The sand/perlite mixture was amended with a leachate of the original VAM inoculum or 5 ml of sterile yeast-mannitol broth to equalize the microflora or N input in VAM or RHIZO-free pots. Each treatment was replicated five times.

Growth conditions

Soybeans were planted in 1.25 kg of a fine sand (0.1–0.25 mm)/perlite mixture (2/1, v/v) that received 100 mg HAP (19 mg P, 8.8 $\mu g\, g^{-1}$ available[28] P), and plants were grown in a greenhouse in Albany, California, from October to December 1982. The potting mix and the nutrient solution had a pH of 6.8. Day/night temperatures varied between 30° and 21°C, and relative humidity fluctuated between 95 and 60%. Photoperiod, light levels and supplemented lighting have been described[8].

Evaluation and assays

All plants were harvested at week 9. Nitrogenase activity of excised roots (ATP-dependent H_2 evolution and C_2H_2 reduction) was determined as described previously[19]. Net nitrogen fixation (NIF) was estimated from C_2H_2-dependent C_2H_4 production and H_2-evolution data as: N_2 fixed = (C_2H_4 produced − H_2 evolved)/3. The relative efficiency (RE) of electron transfer to N_2 via nitrogenase was calculated as: RE = 1 − (H_2 evolved/C_2H_4 produced) according to Schubert and Evans[25]. Specific nodule activity (SNA) was determined as: SNA = 1/3 (C_2H_4 produced − H_2 produced) g^{-1} nodule hr^{-1}. Leaf areas, developmental stage[15], dry weight, and plant N and P were measured, and statistical analysis were evaluated as described previously[20]. Fungal infection, vesicle distribution, as well as intraradical and extraradical

VAM fungal biomass were determined[8] on VAM-infected roots shortly after harvest. The sand/perlite medium was analyzed for available ($NaHCO_3$-extractable) P[28] and total P[26].

Results

Host nutrition and development

Soybean grown in sand/perlite with HAP fertilizer and infected with Rhizobium contained 4 to 5 times the phytomass of the control (Table 1). Plants inoculated with VAM and RHIZO were 18% greater in weight than hosts infected with RHIZO only, and this increase was due to a positive VAM × RHIZO interaction. Plants colonized with the VAM fungus alone were not significantly ($P < 0.05$) different from the control. Inoculation with RHIZO significantly increased the shoot/root ratio, but VAM-fungal colonization had no effect (Table 1).

Plants inoculated with RHIZO contained twice the number of stem nodes[15] as plants that lacked nodules (Table 1). There was a five-fold increase in total leaf area for nodulated plants as compared to the control. VAM colonization did not increase leaf area significantly in the absence of RHIZO, but in the presence of RHIZO there was an 18% increase in leaf area (Table 1). The increase in total leaf area was due to increased leaf number and average leaf size. Primary (main stem) trifoliates contributed nearly 70% of the total leaf area increase, while secondary (lateral branch) leaves contributed 18%, and leaf expansion accounted for the last 12% (Table 1). Nodulated soybeans have significantly greater average leaflet area than the control, although there was no effect on the specific leaf area. The ratio of secondary to primary leaves was significantly higher in plant infected with RHIZO, and there was an even greater proportion of secondary leaves in dually-infected plants. Nodulated, mycorrhizal soybeans had more secondary leaves and twice as much secondary leaf area as plants inoculated with RHIZO only.

Leaf and root N concentrations were highest in nodulated plants, and the N concentrations of nodules was 75% greater than that of the roots (Table 2). The presence of VAM alone increased the leaf and flower N concentrations significantly over that of the control, apparently at the expense of the root N since the total plant N did not differ between the treatments. There was no VAM × RHIZO synergism for N concentration, but there was a positive interaction for total plant N in dually-infected plants. Total N in the control and VAM-only plants was essentially unchanged from seed (20 ± 5 mg). Nodulation resulted in an increase of between 110 and 140 mg N above the seed contribution.

Table 1. Yields and leaf area parameters for soybeans inoculated with either a VAM fungus alone, Rhizobium alone, both endophytes together, or left non-inoculated

Treatment	Plant dry wt (g)	Shoot/Root ratio	Developmental level: Stage†	Developmental level: Nodes
– VAM – Rhizobium	2.29 a	1.48 a	V5	5.1 a
+ VAM – Rhizobium	2.42 a	1.60 a	V6	5.8 b
– VAM + Rhizobium	9.55 b	2.72 b	R1	10.0 c
+ VAM + Rhizobium	11.35 c	2.76 b	R2	10.7 d

	Total leaf area (cm^2)	Average leaflet area (cm^2)	Specific leaf area, ($cm^2\ g^{-1}$)	Secondary to primary leaf area ratio
– VAM – Rhizobium	138 a	8.4 a	170 ab	0.09 a
+ VAM – Rhizobium	153 a	8.9 a	180 b	0.07 a
– VAM + Rhizobium	625 b	13.1 b	166 a	0.24 b
+ VAM + Rhizobium	737 c	12.9 b	172 ab	0.38 c

*Mean values for each parameter having common letters within a column are not significantly different at the 0.05 level by Student's T test.
†According to Fehr and Caviness[15].

Nitrogen input from N_2 fixation amounted to between 5 and 7 times seed N.

Leaf and flower P concentration were significantly higher in plants colonized by VAM only, although the root P content was not significantly different from non-VAM roots (Table 2). Nodulated plants

Table 2. Nitrogen and phosphorous contained in soybeans inoculated with either a VAM fungus alone, Rhizobium alone, both endophytes together, or left non-inoculated

Treatment	N concentration, %: Leaf	Flower†	Root	Nodule	Total N (mg $plant^{-1}$)
– VAM – Rhizobium	0.81 a	1.36 a	1.38 b	0.0 a	19 a
+ VAM – Rhizobium	0.94 b	1.61 b	1.27 a	0.0 a	20 a
– VAM + Rhizobium	1.94 c	2.38 c	1.78 c	2.88 b	130 b
+ VAM + Rhizobium	2.01 c	2.57 d	1.81 c	3.36 c	157 c

	P concentration, %: Leaf	Flower†	Root	Nodule	Total P (mg $plant^{-1}$)
– VAM – Rhizobium	0.12 b	0.24 bc	0.13 a	0.0 a	2.2 a
+ VAM – Rhizobium	0.15 c	0.26 c	0.14 a	0.0 a	2.6 a
– VAM + Rhizobium	0.09 a	0.22 ab	0.12 a	0.35 c	7.9 b
+ VAM + Rhizobium	0.10 ab	0.19 a	0.14 a	0.33 b	10.0 c

*Mean values for each parameter having common letters within a column are not significantly different at the 0.05 level by student's T test.
†For – Rhizobium treatments, bud primordia were collected and analyzed.

had the lowest leaf and flower P as a result of dilution due to growth, and dually-infected plants were as low as soybeans inoculated with RHIZO alone. This indicated the additional P provided by the VAM fungus was used to increase the biomass of the association. The P concentration in the nodule was 3 times that of the leaf or root. The higher P input in tripartite symbiosis resulted in proportionate increases in nodulation. Total P was significantly greater for VAM and RHIZO-inoculated plants (Table 2) demonstrating VAM × RHIZO synergism for total plant P.

Total P contained in the seed, HAP amendment and sand/perlite was initially 2.0, 19.0 and 14.7 mg P pot^{-1}, respectively. Available P in the rooting medium was 12 mg pot^{-1} at planting (8.8 $\mu g\, g^{-1}$) and was between 2.5 and 5 mg pot^{-1} at the 9 week harvest (2.0 to 4.0 $\mu g\, g^{-1}$). Soybeans inoculated with RHIZO assimilated between 6 and 8 mg P (Table 2) of the available P released by HAP (7 to 10 mg). Plants inoculated with both VAM and RHIZO contained 20% more P than the RHIZO-only treatment and assimilated 90% of the available P released by the system.

Endophyte development and activity

Dually-infected soybeans contained greater nodule dry weights than plants inoculated with RHIZO only (Table 3). The increase in nodule dry weight was greater than the relative increase in total plant dry weight, and this indicated that the nodule has a high P requirement.

Table 3. Symbiotic N_2 fixation parameters for soybean inoculated with *Rhizobium japonicum* alone or in the combination with a VAM fungus

Treatment	Nodule dry weight*, (g $plant^{-1}$)		C_2H_4 produced (μmol $plant^{-1}$ h^{-1})	H_2 evolved,
− VAM + Rhizobium	0.24 a		8.9 a	6.6 a
+ VAM + Rhizobium	0.31 b		8.3 a	4.7 a
	NIF[a] (μmol N_2 g^{-1} h^{-1})	SNA,[b]	RE,[c]	N_2 fixed[d] (mg $plant^{-1}$)
− VAM + Rhizobium	0.8 a	3.2 a	0.27 a	110.4 a
+ VAM + Rhizobium	1.2 b	3.9 a	0.44 b	137.5 b

*Means for each parameter having common letters within a column are not significantly different at the 0.05 level by Student's T test.
[a]NIF = 1/3 (C_2H_4 produced-H_2 evolved).
[b]SNA = NIF/g nodule wt.
[c]RE = [1 − (H_2 evolved/C_2H_4 produced)].
[d]N_2 fixed = Total plant N − (seed N + N from soil).

Acetylene-dependent ethylene production was similar in both nodulated treatments, but H_2 evolution was 40% larger in the RHIZO only association (Table 3). The significantly higher NIF in the tripartite symbiosis was attributable to decreases in H_2 production and increases in nodule weight. Nodules on VAM plants had a significantly higher RE than nodules from plants inoculated only with RHIZO.

Nodulated soybeans inoculated with Glomus had more infection and contained more than twice as many vesicles than plants colonized by VAM only (Table 4). Dually-infected soybeans contained proportionately more extra- to intraradical mycelium. Mycorrhizal biomass amounted to less than 4% of the root weight in plants inoculated with VAM only, but increased to more than 5% in the soybeans colonized by both microsymbionts (Table 4).

Table 4. Fungal colonization parameters for soybean inoculated with a VAM fungus alone or in combination with Rhizobium

Treatment		Fungal infection* (%)	Vesicles (%)
+ VAM – Rhizobium		36.4 a	22 a
+ VAM + Rhizobium		44.8 b	57 b
	Intraradical VAM biomass (mg)	Extraradical VAM biomass (mg)	% Biomass (VAM/Root) X 100, %
+ VAM – Rhizobium	40.1 a	51.1 a	3.7 a
+ VAM + Rhizobium	188 b	414 b	5.3 b

*Means for each parameter having common letters within a column are not significantly different at the 0.05 level by student's T test.

Discussion

Only nodulated plants responded positively to inoculation with Glomus in this N-deficient environment. Plants colonized by the VAM fungus only grew no better than the control. This serves to emphasize that the response of the host plant to the two endophytes was strongly determined by the N and P status of the substrate. Comparatively greater growth of the host following VAM colonization has been observed in a soil low in available P but adequate in N[20]. In our experiment, P was supplied as HAP, a sparingly-soluble P source, and for dually-inoculated roots the extensive extradical VAM mycelium assimilated more P than non-VAM roots by exploiting a relatively greater soil volume. Similarly, the parasitic growth of Glomus in sand/perlite occurred at high soil-available P concentrations[8]. N so limited plant growth in this medium that the added P could not be used unless the plants were nodulated. Conversely, in a P-limited soil, nodulation of *Medicago sativa* occurred only in the presence of a VAM fungus[6]. There was reasonable growth of nodulated, VAM plants in a soil low in available N and P[9] suggesting that the host response to VAM was lacking only in the absence of N.

Low P availability resulted in low levels of nodulation and N_2 fixation in legumes[18] that can lead to concomitant N stress if atmospheric N_2 is the only source of N for the tripartite association. The data indicated that N_2 fixation was essential to soybean growth and dependent on the supply of P from the root. Inoculation with a VAM fungus was capable of supplying the critical requirements of the host plant and nodules. The differences between the critical P requirements of the host root and nodules[18] can be linked to the efficient allocation of P to the nodules where it is needed by the N_2-fixing bacteroids.

Enhancement of N_2 fixation in VAM plants appeared related to decreased H_2 production relative to C_2H_2 reduction (increased RE). This effect, also noted previously in an a sand/perlite mixture[9], has not been reproduced in a sterilized soil[20]. Increased RE of N_2 fixation has been associated with decreases in photosynthate supply to the nodules[10], and decreased RE in sand/perlite (compared to soil[20]) indicated roots grown hydroponically in an inert potting mix contained surplus carbohydrates. The increased RE following VAM colonization suggests that rapid proliferation of fungal mycelium depletes excess carbohdrates in the root and influences the RE of the nodule. Thus, an artificial growth medium such as HAP-amended sand/perlite, does not appear to be well suited for the manipulation of host-endophyte growth relationships under controlled conditions, as was previously maintained[9].

Increased leaf area, leaf N and P in the tripartite association can be linked to increased levels of photosynthesis that could be capable of supplying the increased C requirements of the microsymbionts. For Glomus, there was pronounced vesicle development in the presence of Rhizobium, indicating the greater availability of photosynthate allocated to the formation of these storage organs[27] by the endophyte.

Growth increases due to dual inoculation reported here are in line with Ross and Harper[23] and Schenck and Hinson[24]. The synergistic nature of the VAM fungus × Rhizobium interaction suggests that the influence these endophytes have on the host was not linked to N and P nutrition alone. Legume inoculation with Rhizobium in this N-deficient substrate was successful since it not only improved host plant growth and nutrition, but also increased the spread and biomass of *G. fasciculatum.* Nodulation and N_2 fixation is highly dependent on P uptake[14], and the presence of the VAM fungus increased P acquisition. Dual inoculation in the sorghum-*Glomus-Azospirillum* tripartite association increased N or P input additively[21], not synergistically as in the case with legumes. Endomycorrhizae may increase rhizosphere microflora[2] or affect the balance of plant hormones[1]; contributions that might increase the productivity of the symbioses beyond the impact of the endophyte on mineral nutrition alone.

Relationships involving physiological compatibility in the tripartite association are unknown and may match in importance the ability of all three symbionts to tolerate independently a common set of environmental and edaphic factors. More study of versatile tripartite associations is required before their combined use is practical.

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References

1 Allen M F, Moore T S and Christensen M 1980 Phytohormone changes in *Boteloua gracilis* infected with vesicular-arbuscular mycorrhizae I. Cytokinin increases in the host plant. Can. J. Bot. 58, 371–374.

2 Ames R N, Reid C P P and Ingham E R 1984 Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. New Phytol. 96, 555–563.

3 Asai T 1944 Uber die Mykorrhizenbildung der Leguminosenpflanzen. Jap. J. Bot. 13, 463–485.

4 Asimi S, Gianinazzi-Pearson V and Gianinazzi S 1980 Influence of increasing soil phosphorus levels on interactions between vesicular-arbuscular mycorrhizae and Rhizobium in soybeans. Can. J. Bot. 58, 2200–2205.

5 Azcon-Aguilar C and Barea J M 1981 Field inoculation of *Medicago sativa* with vesicular-arbuscular mycorrhiza and *Rhizobium meliloti* in phosphate-fixing agricultural soils. Soil Biol. Biochem. 13, 19–22.

6 Azcon-G. de Aguilar C, Azcon R and Barea J M 1979 Endomycorrhizal fungi and Rhizobium as biological fertilizers for *Medicago sativa* in normal cultivation. Nature 279, 325–327.

7 Bagyaraj D J, Manjunath A and Patil P B 1979 Interactions between a vesicular-arbuscular mycorrhiza and Rhizobium and their effects on soybeans in the field. New Phytol. 82, 141–145.

8 Bethlenfalvay G J, Brown M S and Pacovsky R S 1982 Relationships between host and endophyte development in mycorrhizal soybeans. New Phytol. 90, 537–543.

9 Bethlenfalvay G J, Pacovsky R S, Bayne H G and Stafford, A E 1982 Interactions between nitrogen fixation, mycorrhizal colonization and host plant growth in the *Phaseolus-Rhizobium-Glomus* symbiosis. Plant Physiol. 70, 446–450.

10 Bethlenfalvay G J and Phillips D A 1977 Ontogenic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. Plant Physiol. 60, 419–421.

11 Cassman K G, Whitney, A S and Fox R L 1981 Phosphorus requirements of soybean and cowpea as affected by mode of N nutrition. Agron. J. 73, 17–22.

12 Crush J R 1974 Plant growth responses to vesicular-arbuscular mycorrhiza VIII. Growth and nodulation of some herbage legumes. New Phytol. 73, 743–749.

13 Crush J R 1973 The effect of *Rhizophagus tenuis* mycorrhizas on ryegrass cocksfoot, and sweet vernal. New Phytol. 72, 965–973.

14 Demeterio J L, Ellis R Jr. and Paulsen G M 1972 Nodulation and nitrogen fixation by 2 soybean varieties affected by phosphorus and zinc nutrition. Agron. J. 64, 566–569.
15 Fehr W R and Caviness C F 1977 Stages of soybean development. Special Report 80, Cooperative Extension Service, Iowa, pp. 1–11.
16 Ganry F, Diem H G and Dommergues Y R 1982 Effect of inoculation with *Glomus mosseae* on nitrogen fixation by field-grown soybeans. Plant and Soil 68, 321–329.
17 Gianinazzi-Pearson V, Fardeau J C, Asimi S and Gianinazzi S 1981 Source of additional phosphorus absorbed by vesicular arbuscular mycorrhizal soybeans. Physiol. Veg. 19, 33–44.
18 Mosse B, Powell C L and Hayman D S 1976 Plant growth responses to vesicular-arbuscular mycorrhiza. Part 9 Interactions between vesicular-arbuscular mycorrhiza, rock phosphate and symbiotic nitrogen fixation. New Phytol. 76, 331–342.
19 Pacovsky R S, Bayne H G and Bethlenfalvay G J 1984 Symbiotic interactions between strains of *Rhizobium phaseoli* and cultivars of *Phaseolus vulgaris* L. Crop Sci. 23, 101–105.
20 Pacovsky R S, Bethlenfalvay G J and Paul E A 1986 Comparisons between phosphorus-fertilized and mycorrhizal plants. Crop Sci., *In press.*
21 Pacovsky, R S, Fuller, G and Paul E A 1985 Influence of soil on the interactions between endomycorrhizae and Azospirillum in sorghum. Soil Biol. Biochem. 17, 525–531.
22 Pacovsky R S, Paul E A and Bethlenfalvay G J 1986 Response of mycorrhizal and phosphorus-fertilized soybeans to nodulation by Bradyrhizobium or ammonium nitrate. Crop Sci. 26, 145–150.
23 Ross J P and Harper J A 1970 Effect of Endogone mycorrhizae on soybean yields. Phytopathol. 60, 1552–1556.
24 Schenk N C and Hinson K 1973 Response of nodulating and non-nodulating soybeans to a species of Endogone mycorrhiza. Agron. J. 65, 849–850.
25 Schubert K R and Evans H J 1976 Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Nat. Acad. Sci., USA 73, 1207–1211.
26 Shelton W R and Harper H J 1941 A rapid method for the determination of total phosphorus in soil and plant material. Iowa State Coll. J. Sci. 15, 403–413.
27 Smith S S E 1980 Mycorrhizas of auxotrophic higher plants. Biol. Rev. Camb. Philios. Soc. 55, 475–510.
28 Watanabe F S and Olsen S R 1965 Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil. Soil Sci. Soc. Proc. 29, 677–678.