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Glycine-Glomus-Rhizobium Symbiosis[1]

VI. PHOTOSYNTHESIS IN NODULATED, MYCORRHIZAL, OR N- AND P-FERTILIZED SOYBEAN PLANTS

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ABSTRACT

Soybean (*Glycine max* [L.] Merr. cv Hobbit) plants were grown in a growth chamber for 56 days in a phosphorus- and nitrogen-deficient soil and were colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* (Nicol. & Gerd) Gerd. and Trappe and *Rhizobium japonicum* strain USDA 136, or by either organism alone, or by neither. Non-VAM plants received supplemental phosphorus and nonnodulated plants supplemental nitrogen to achieve the same rate of growth in all treatments. Plants of all four treatments had the same ($P > 0.05$) dry weights at harvest, but VAM plants had higher rates of CO_2 exchange (CER, $P < 0.05$) and lower leaf P concentrations ($P < 0.01$). Leaf nitrogen concentrations were lower in nodulated than in nitrogen-supplemented plants ($P < 0.01$) while starch concentrations were higher ($P < 0.01$). There was a significant negative relationship between nitrogen and starch ($r = -0.989$). Statistical evaluation of the data showed that some parameters (CER, leaf area and phosphorus content) were associated with phosphorus nutrition (or the presence of the VAM fungus), others (leaf fresh weight and root dry weight) with nitrogen nutrition (or the presence of *Rhizobium*), and some (leaf nitrogen and starch content) by both factors. The development of microsymbiont structures and nodule activity were significantly lower in the tripartite association than in plants colonized by one endophyte only. The findings suggest that endophyte effects go beyond those of simple nutrition and associated source-sink relationships.

A constant and regulated supply of Pi to the chloroplast is essential for C assimilation at optimal rates (7). In the symbiotic legume association a similar demand for Pi is exerted by nitrogenase with a concomitant high demand for reducing power (16). When P in the growth medium is limiting, VAM[2] fungi are capable of alleviating P stress, an observation which has given rise to a voluminous literature comparing well nourished, large VAM plants and P-starved, small non-VAM plants. This syndrome is particularly marked in legumes, where P stress may be accompanied by N stress, due to the high P requirement of nodule activity. The metabolic consequences of severe P deficiency make P-deprived non-VAM plants unsuitable as controls in an effort to determine VAM-fungal effects on host plant development (14). Yet, differences in host plant form and function persist, even if non-VAM comparison plants are provided with VAM-equivalent amounts of P (13).

In view of the role of Pi in regulating CO_2 fixation (9, 17), and the role of VAM fungi in P uptake, the relationship between photosynthesis and the VAM condition is of particular interest. If there are links between P and C demand and supply in VAM and non-VAM, P-fertilized plants (5, 10, 12), it is important to determine whether, and if so, to what extent, non-P mediated consequences of the VAM condition exist. Thus, the purpose of our experiment was to relate N and P nutrition in leaves of symbiotic or fertilized plants to the levels of rhizobial and VAM-fungal colonization, in order to assess the individual and combined effects of VAM-fungal and rhizobial colonization and of P and N fertilization on CER and other parameters of interest in leaves.

MATERIALS AND METHODS

Experimental Design. The experiment was designed as a 2 × 2 factorial of four treatments, with eight replications of each for a total of 32 units of single plants. The factors were P or N input. Each was provided either by inoculation with an endophyte or by fertilization. The results were evaluated statistically by ANOVA and Duncan's multiple range test for multiple comparisons (host parameters) and *t* test for two-way comparisons (symbiotic parameters). Individual comparisons between host parameters were also made by *t* test, where such comparisons further clarified the effects of P input by the VAM fungus or P fertilizer, and of N input by the diazotroph or N fertilizer.

Biological Materials and Growth Conditions. Soybean (*Glycine max* [L.] Merr cv Hobbit) plants were grown in a walk-in type growth chamber for 56 d at day/night regimes of 16/8 h, 27/21°C and 40/60% RH, and photosynthetic photon flux density of 800 $\mu E/m^{-2} \cdot s^{-1}$ at the shoot tip. Only the center of the chamber platform was utilized, providing uniform light and temperature conditions. The soil (1.25 L/pot) was a Balcom series (Yolo County, California) heavy silt loam (Typic Xerorthent) of pH 7.7 (paste), NH_4HCO_3-extractable P of 3.2 $\mu g/g$, total N of 0.7 mg/g and a sand/silt/clay content of 20.5/55.6/23.9%. It was mixed with fine sand (2:1, v:v, soil:sand) and autoclaved.

Inocula of the diazotrophic bacterium *Rhizobium japonicum*, strain USDA 136 (10 ml, 10^9 cells/ml) and of the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd and Trappe (60 ml soil containing 700 sporocarps with 1 to 5 spores per sporocarp) were added to each pot of soil at planting of the surface-sterilized and germinated seeds. Seedlings were selected for uniformity. Plants were inoculated with *R. japonicum*, *G. mosseae*, both, or neither. All treatments received a wash of the VAM inoculum sieved free of VAM propagules. The inoculum was grown on *Sorghum bicolor* L. as host plant, and was free of *Rhizobium* as determined

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[2] Abbreviations: VAM, vesicular-arbuscular mycorrhizal; ANOVA, analysis of variance; CER, carbon dioxide exchange rate.

previously by nodulation trials. Plants were watered with a basal nutrient solution equivalent to one-quarter strength Hoagland solution, without N and P. Nonnodulated plants received N (6.0 mM), and non-VAM plants received P (0.4 mM) in addition to the basal solution. Thus, four nutrient solutions were used, to correspond to the four treatments, which were designated GR, GN, PR, and PN, indicating the addition of *Glomus*, *Rhizobium*, P, or N.

Measurements and Analyses. Plants were harvested when the sixth trifoliate leaf became fully expanded (approximately 56 d after planting). The second, third, fourth, and fifth trifoliate leaves were used to determine CER. The first leaves could not be measured due to the configuration of the instrument (4 L leaf chamber, LI-COR LI-6000 Portable Photosynthesis System). Even though they had expanded completely, the sixth leaves had much greater within-treatment variability than the more mature leaves, and thus they are not included in the composite CER calculation described below. The remaining leaves (second through fifth) were measured in the growth chamber at approximately the height of the fifth leaves, with the plant raised to bring each leaf to the height of the measuring chamber in turn. This assured a constant level of illumination of 700 $\mu E/m^{-2} \cdot s^{-1}$ for all leaves. Carbon dioxide exchange rates are reported at an initial CO_2 concentration of 375 μl/L.

All leaves were excised following the gas exchange measurements. Leaf areas and fresh weights were determined; then the tissues were frozen in liquid N, lyophilized, weighed, and ground (40 mesh) for chemical analysis of N, P, and starch by standard methods (6). Roots were floated in water and washed over a sieve to recover fragments, resulting in negligible loss. Root, stem, and nodule dry weights were measured after drying at 70°C for 2 d. Nodule activity and VAM-fungal colonization were determined as described previously (5).

Composite values for CER and leaf N, P, and starch concentrations were derived from the second through fifth trifoliate leaves. Values were first calculated for individual leaf areas (CER) or weights (N, P, starch). These values were then summed and divided by the total area (CER) or the total weight (N, P, starch) of the four leaves.

RESULTS

Our intent to use total plant dry weight as the basis of comparability between plants of different symbiotic or fertilizer N and P inputs was achieved: plants of the four treatments varied slightly, but their dry weights were statistically ($P > 0.05$) the same (Table I.) Microsymbiont parameters were smallest in the tripartite association (GR) throughout (Table II). Fungal colonization, nodulation, and nodule activity were all reduced in the presence of the second endophyte without apparent adverse effects on host plant development. The rates of CO_2 exchange were significantly ($P < 0.05$) higher in VAM plants than in non-VAM plants (GR = GN > PR = PN), while P concentrations showed the opposite pattern (GR = GN < PR = PN, $P < 0.01$). The concentration of P in leaves of VAM plants was in the range reported for youngest fully expanded soybean leaves (8) as severely deficient, while in non-VAM plant leaves, it was low, but sufficient (8). Thus, the role of the vacuole as a sink for excess Pi was probably minimized. Concentrations of N were significantly lower in nodulated than in nonnodulated plants, while starch levels were higher in nodulated plants, with or without *Glomus* (Fig. 1).

Table I. *Plant Growth Parameters in Symbiotic or Nutrient-Supplemented Soybean Plants*

Numbers represent the means of 8 replications. Treatments were significantly ($P < 0.05$) different by Duncan's multiple range test when followed by different letters.

Parameter	Treatment			
	GR[a]	PR[b]	GN[c]	PN[d]
Dry wt (g)				
Total plant	8.0 x	7.8 x	9.0 x	8.3 x
Shoot	5.6 x	5.5 x	6.1 x	5.6 x
Root	2.4 x	2.3 x	2.9 y	2.7 y
Root/shoot ratio[e]	0.43 xy	0.42 x	0.45 y	0.48 z

[a] *Glomus* + *Rhizobium*. [b] Phosphorus + *Rhizobium*. [c] *Glomus* + nitrogen. [d] Phosphorus + nitrogen. [e] Roots of the GR and PR treatments include nodule dry weights.

Table II. *Symbiotic Parameters in Soybean Roots*

Plants lacking one of the two endophytes were supplemented with fertilizers. Numbers represent the means of 8 replications. Significance between treatment means was evaluated by *t* test (*, 5%; **, 1%, ***, 0.1%).

Parameter	Treatment		
	GR[a]	GN[b]	PR[c]
VAM colonization (%)	57.3*	68.7	
Nodule dry wt (g)	0.44***		0.58
Nodule activity[d] (μmol/h·plant)	9.6**		20.8
Nodule/root dry wt ratio	0.23***		0.34

[a] *Glomus* + *Rhizobium*. [b] *Glomus* + nitrogen. [c] Phosphorus + *Rhizobium*. [d] Nodule activity was estimated by acetylene reduction.

The analysis of the data by ANOVA and individual treatment comparisons (Table III) showed a curious dichotomy in the effects due to P or N nutrition. Some parameters (CER and P concentration) were significantly affected by the mode of P input (VAM fungus or fertilizer). Others (leaf fresh weight, root dry weight) were affected by N input (*Rhizobium* or fertilizer); yet others (leaf N and starch concentrations) were affected by both factors. There was a significant inverse relationship ($r = -0.989$, $P < 0.01$) between N and starch concentrations. Differences in root/shoot ratios were significant only between symbiotic (GR, GN, PR) and nonsymbiotic (PN) plants. The differences between tripartite (GR) and nonsymbiotic plants were significant throughout, except for leaf and plant dry weights. Plant dry weights were the invariant by design.

DISCUSSION

Our results showing higher CER in VAM than in non-VAM plants agree with the findings of others (1, 10–12). They are, however, in disagreement with the widely accepted view (15), which ascribes this phenomenon to improved P nutrition of VAM plants. The crucial point of the present data, high CER in VAM plants in spite of low P concentrations regardless of the source of N (Fig. 1) and in spite of low microsymbiont presence or activity in the tripartite treatment (Table I), indicated that the effect was due only to the presence of the fungal symbiont, rather than to a sink demand or source limitation brought on by its presence. An explanation of this phenomenon is offered by the positive effects of VAM colonization on leaf conductance (1, 2, 4). Higher levels of leaf conductance stimulate CO_2 fixation by improving access to the limiting substrate; rapid utilization of the substrate in turn improves the concentration gradient for more CO_2 influx (18). The relationships between CO_2 and Pi limitation of photosynthesis have been discussed by Sivak and Walker (17), who showed that CO_2 limitation is primary and when additional CO_2 is supplied, the formerly adequate level of Pi may no longer be sufficient. This is especially true in leaves with a massive capacity to produce starch (such as soybean) where Pi deficiency is buffered by recycling of organic P within the stroma (9, 17). Thus, the first impact of VAM fungi on CO_2

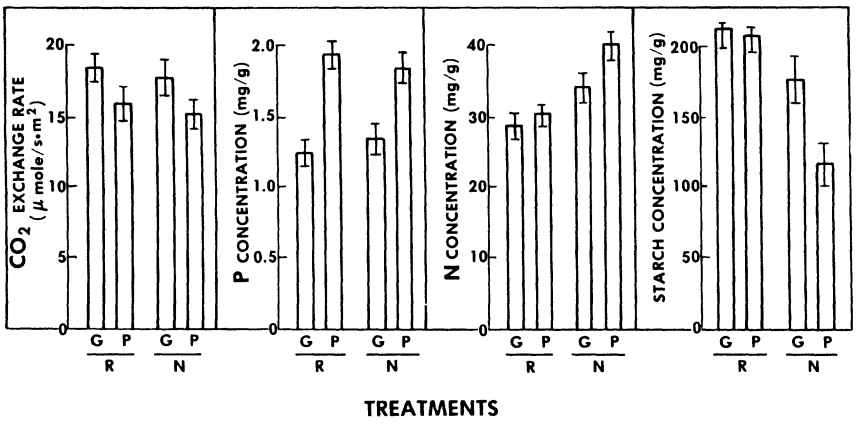

FIG. 1. Carbon dioxide exchange rates in symbiotic or nutrient-supplemented soybean plants relative to leaf N and P nutrition and starch content. Data are arranged to compare P input by *Glomus* (G) or P fertilizer (P) within *Rhizobium* (R) or N fertilizer (N) treatments. Vertical lines denote confidence intervals ($P < 0.05$).

Table III. *Evaluation of Plant Growth Parameters in Symbiotic or Nutrient-Supplemented Soybean Plants*

The four treatments (GR, *Glomus* + *Rhizobium*; GN, *Glomus* + Nitrogen; PR, Phosphorus + *Rhizobium*; PN, Phosphorus + Nitrogen) were evaluated by ANOVA or by *t* test. *t* Test comparisons were arranged to reflect G *versus* P, R *versus* N, and tripartite *versus* nonsymbiotic effects. Significance levels were: NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Inequality signs indicate which of the two treatments within each comparison is larger.

Parameter	ANOVA Treatment Effects			*t* Test				
				P effects		N effects		Symbiotic effects
	P	N	P × N	GR *versus* PR	GN *versus* PN	GR *versus* GN	PR *versus* PN	GR *versus* PN
Leaf parameter								
CER	***	NS	NS	> **	> *	NS	NS	> **
P concentration	***	NS	NS	< ***	< **	NS	NS	< **
N concentration	***	***	*	NS	< **	< **	< ***	< ***
Starch concentration	***	***	**	NS	> **	> *	> ***	> ***
Fresh wt	NS	***	NS	NS	NS	< *	< ***	< **
Dry wt	NS	NS	NS	NS	NS	NS	NS	NS
Root dry wt	NS	**	NS	NS	NS	< *	< *	< **
Plant dry wt	NS	NS	NS	NS	NS	NS	NS	NS
Root/shoot ratio	NS	**	NS	NS	< *	NS	< **	< **

fixation may be through gas exchange effects. This view is also supported by the data of Johnson (11) showing an insensitivity of photosynthesis to P concentrations in the leaves of VAM plants.

The implications of microsymbiont effects on various leaf parameters, such as the ones shown in Table III, have been discussed in detail by Harris *et al.* (10). We agree with their conclusions that a number of microsymbiont interactions are mediated by the host plant. However, our data on leaf nutrient concentrations and total plant biomass do not confirm their interpretation that sink limitation played a role in the photosynthetic responses of symbiotic or nonsymbiotic plants. The evaluation of our data indicates further that certain of these parameters are influenced by the form of input P, or of N, or of both, and that it is the presence or absence of one or the other, or of both endophytes, that is associated with these differences. Thus, changes in CER and leaf P concentration were influenced by the VAM condition regardless of the source of N (Table III). The effect of both endophytes on leaf N concentration, apparently influenced by root mass, root/shoot ratios, and the associated N uptake and allocation, is indicated by the significance of both P and N main effects (Table III). The significant interaction between these factors shows that the extent of nitrogen uptake from different sources is conditioned by the P source. Starch concentration was apparently also affected by both endophytes (Table III). The inverse correlation between starch and N concentration indicates expedited formation and export of C as amino compounds in the presence of high levels of N (3).

The comparative study of symbiotic and nonsymbiotic legumes of equal development provides insights into their formation and functions which are not always consistent with previous findings that may have been made in the absence of the endophytes. It is becoming apparent that the effects of the endophytes go beyond those of simple nutrition and the associated source-sink relationships. We suggest that the presence or absence of the microsymbionts in the legume association accounts for the apparent inconsistencies that sometimes appear when studies of plant development and function are compared. Inclusion of the microsymbionts in experimentation will make inferences drawn from such work more applicable to field conditions.

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