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Glycine-Glomus-Rhizobium Symbiosis

II. ANTAGONISTIC EFFECTS BETWEEN MYCORRHIZAL COLONIZATION AND NODULATION

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GABOR J. BETHLENFALVAY*, MILFORD S. BROWN, AND ALLAN E. STAFFORD
United States Department of Agriculture, Agricultural Research Service, Western Regional Research Service, Albany, California 94710

ABSTRACT

Soybean (*Glycine max* [L.] Merr.) plants grown in pot cultures were inoculated with the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe and *Rhizobium japonicum* strain 61A118 at planting (G_1R_1) or at 20 days ($G_{20}R_{20}$), or with one of the endophytes after the other has colonized the host root (G_1R_{20}, $G_{20}R_1$). Nodulated (PR_1) and VAM (G_1N) dipartite associations, or nonsymbiotic plants (PN) using nutrient solutions with N, P, or N + P concentrations providing endophyte-equivalent nutrient inputs were used as controls. The delayed tripartite associations received the appropriate N, P, or N + P amendment while one or both endophytes were absent during the first 20 days of growth. Prior inoculation with one endophyte significantly inhibited development of the other. Root hexose sugar concentrations were negatively correlated with VAM colonization ($r = -0.89$), nodule activity ($r = -0.91$), and root P content ($r = -0.93$). Nodule ($r = 0.97$) and root ($r = 0.96$) P content correlated positively with VAM colonization. Nodule weight or VAM-fungal biomass were significantly greater in associations grown with only one endophyte. Dry weights of the PN, G_1N, PR_1, and $G_{20}R_{20}$ plants were significantly greater than those of tripartite plants inoculated at planting with either or both endophytes. Interendophyte inhibition is attributed to competition for root carbohydrates, and this effect apparently also affects overall plant productivity. The objective of the study was to determine if the timing of endophyte introduction and establishment affected the development of the other symbiotic partners.

Since Asai's demonstration of the importance of VAM[1] fungi to N_2 fixation in legumes (3) most of the interest in VAM fungus-nodule interactions has centered on the role of VAM fungi as a P source to legumes under P stress (5). This role is clearly important and may influence nodule activity either through the enhanced nutritional condition of the host plant (15) or may directly and preferentially stimulate nodule function (5). Whether the energy cost of this VAM fungus-mediated P uptake is significant to the tripartite association is not yet clear (25), but recent results show that VAM fungal colonization and nodulation are affected differently by carbohydrate stress (6). In plants not under carbohydrate stress, VAM fungi may inhibit growth depending on P availability (7) in spite of increased photosynthetic output (23) in response to the additional C demand by the endophytes (9).

Little is known, however, about direct interactions between the endophytes except for those which may be deduced from source-sink effects within the association (15). When inoculated at the same time, nodulation and VAM-fungal colonization proceed simultaneously, indicating that the endophytes do not compete for infection sites (27), but subsequent to infection, VAM fungi do not usually invade nodule cortical tissues (19). Since both *Rhizobium* and VAM fungi influence hormonal levels in plants (4, 5), the resulting changes may mutually affect the development of the endophytes. Stimulation or inhibition of endophyte growth will, in turn, affect the host plant (20) and also the biota of the rhizosphere (2).

Since symbiotic associations between plants and microorganisms depend for their formation and function on interactions between their constituents (5), an elucidation of such mutual effects is needed for a more complete understanding of the symbiosis. The present study was made to determine the effect of previous establishment of one endophyte on subsequent infection by the other, using controls not limited by nutrient deficiency.

MATERIALS AND METHODS

Experimental Design. The experiment consisted of eight treatments with six replications for a total of 48 plants. Plants were inoculated with VAM fungus (G) and a diazotrophic bacterium (R) simultaneously at planting (G_1R_1), at 20 d after planting ($G_{20}R_{20}$), or with one organism at planting and the other at 20 d (G_1R_{20}, $G_{20}R_1$). At 20 d after planting each symbiont introduced at 1 d had achieved a certain level of infection, so that inoculation by the second organism was superimposed on an already established symbiosis thus forming a tripartite association. The temporary absence of the VAM fungus (P source) was compensated for by the use of an N-free nutrient solution containing P which produced a plant growth response equivalent to that caused by the fungus. Phosphorus equivalency was established by a method described elsewhere (21) in which nutrient solutions of different P concentrations were used to achieve a growth range in non-VAM plants which bracketed the size (dry weight) of the VAM plant. The 'VAM-equivalent' P concentration could then be determined from the growth curve based on the P concentration. Since P equivalency is affected by environmental conditions and the taxonomic identity of the symbionts, adjustments in P concentration must be made to achieve equivalent VAM and non-VAM plant growth when any of the factors are changed. The absence of the diazotroph (N source) was similarly accounted for by a P-free solution containing N (22). In addition, dipartite VAM and N_2-fixing associations were grown using P and N complements for the entire time (G_1N, PR_1). For comparison, a N_2-fixing association was also included without the provision of P or N (R_1), as well as nonsymbiotic plants provided with both P and N (PN). The concentrations of nutrient complements (N, P, or N and P) were so chosen that dipartite or nonsymbiotic plants had the same dry weight at harvest.

Biological Materials. Soybean (*Glycine max* [L.] Merr. cv

[1] Abbreviation: VAM, vesicular-arbuscular mycorrhizal.

Hobbit] seeds were sterilized (22), germinated for 2 d at 30°C, selected for uniformity, planted in autoclaved soil in 1.5 L pots, and inoculated with the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe, and/or with *Rhizobium japonicum* strain 61A118 (originally obtained from the Nitragin Co., Milwaukee, WI[2]). This strain was previously shown to have a high rate of H_2 evolution (11). The fungus was collected at a desert site (8) and recultured under controlled conditions on sorghum (*Sorghum bicolor* L.) The inoculum consisted of 20 g soil containing approximately 300 spores and 450 root fragments partially (75%) infected with *G. mosseae*. It was mixed into the soil of those plants inoculated at planting. The plants inoculated at 20 d had six test tubes embedded in the soil at planting. The inoculum was placed in the wells formed by removal of the tubes at 20 d and the holes were capped with sterile soil. The *R. japonicum* inoculum consisted of 50 ml of nutrient medium containing 8×10^9 cells/ml which was applied to the stems of the seedlings as well as to the soil surface. Non-VAM plants received an inoculum wash free of *G. mosseae* propagules to equalize the microbiota (1).

Growth Conditions. Plants were grown in a walk-in type growth chamber at day/night regimens of 16/8 h, 27/21°C, and 60/90% RH. Photosynthetic photon flux density was 800 $\mu E \cdot m^{-2} \cdot s^{-1}$. The soil used was a Balcom series heavy silt loam (Typic Xerorthent) of pH 7.7 (paste), available ($NaHCO_3$-extractable) P of 3.3 μg (g soil)$^{-1}$ and total N of 0.069%. It was mixed with fine sand (2:1, v:v). The basal nutrient solution (used for the R_1 treatment) consisted of 1.5 mM $CaCl_2$, 0.25 mM $MgSO_4$, 1.0 mM K_2SO_4 with micronutrients (less Mn) equivalent to one-quarter strength Hoagland solution. Other solutions contained, in addition, 0.2 mM K_2PO_4 (non-VAM plants) or 2 mM NH_4NO_3 (nonrhizobial plants) or both N and P (nonsymbiotic plants). Plants were watered to saturation every 3 d initially and every 2 d during the last 20 d of the 50-d growth period. Nutrient complements (N, P, N and P) were provided starting at 10 d after planting, when initial establishment of the endophytes was first discerned. All plants were flushed with water at d 20 prior to inoculation to lower N and P concentrations in the soil. Plants were rotated periodically to avoid positional effects within the chamber. For uniform lighting, only the center portion of the chamber was used.

Assays. Nodule activity was evaluated by measuring H_2 evolution (9). The colonization of roots by VAM fungi was measured by the chitin assay (10) and expressed as total (mg) or per cent (w/w, dry matter) VAM-fungal biomass in the colonized root. Root P and N contents were determined by standard analytical methods (13) and sugar (hexoses) content by the anthrone method after extraction in 80% ethanol (18). Plant dry weights were determined after drying at 70°C for 2 d. The levels of endophyte establishment at 20 d were determined on VAM and nodulated plants (five replicates) in addition to those used in the experiment.

RESULTS

Inoculation of soybean plants with one of the endophytes at planting and with the other after establishment of the former affected all three symbiotic partners. The levels of endophyte establishment at 20 d were 33.8% ± 5.3 colonization of root length by the fungus or 86.0 ± 7.5 nodules/root (means and SE for five replicates). Although nutritional deficiencies due to the initial or permanent absence of the biological P source (*Glomus*) and/or N source (*Rhizobium*) were compensated by the addition of P and/or N in the nutrient solution, significant differences due to the timing of inoculation were noted in almost all parameters measured.

Plant dry matter production by nonsymbiotic plants (PN), dipartite associations (G_1N, PR_1), and tripartite associations inoculated at 20 d ($G_{20}R_{20}$) was not significantly different (Table I), while dry matter produced by the tripartite associations inoculated with one or both endophytes at planting (G_1R_{20}, $G_{20}R_1$, G_1R_1) was significantly less than that by the former group.

The results show differences in the development of nodules and the intraradical VAM-fungal mycelium (Fig. 1). Thus, introduction of *Rhizobium* after the establishment of *Glomus* inhibited nodulation ($G_1R_1 > G_1R_{20}$). That this was not due to the shorter growing time available to the nodules in G_1R_{20}, or to inhibitory effects of combined N added between d 10 and 20, is shown by the ability of the R_{20} plants to produce nodules equal in weight to those of the R_1 plants when the endophytes were introduced simultaneously ($G_1R_1 = G_{20}R_{20}$). If *Rhizobium* was established first, however, the timing of *Glomus* inoculation (*i.e.* the means of P supply between d 10 and 20) had no effect on nodulation ($G_1R_1 = G_{20}R_1$). Neither did the timing of *Rhizobium* inoculation matter when *Glomus* was introduced late ($G_{20}R_1 = G_{20}R_{20}$). Thus, in all combinations, only the previous establishment of the fungus affected nodulation adversely. Nodulation was greatest in the dipartite association (PR_1).

When plants were nodulated prior to the introduction of *Glomus*, formation of the intraradical mycelium was inhibited ($G_1R_1 > G_{20}R_1$) similarly to the pattern observed for nodulation. However, unlike nodulation, total mycelium formation was significantly less when plants were inoculated simultaneously at planting than at 20 d ($G_1R_1 < G_{20}R_{20}$). This was apparently due to the larger root system of the plants growing nonsymbiotically for the first 20 d, since the percentage of fungal biomass/root dry weight in the two treatments was not significantly different ($G_1R_1 = G_{20}R_{20}$, Table I). This similarity in the intensity of infection indicates that P amendment provided the $G_{20}R_{20}$ plants between d 10 and 20 did not inhibit fungal colonization. The reaction of *Glomus* to early or late introduction of *Rhizobium* was also unlike the pattern seen in nodulation: early nodulation inhibited fungal development while late nodulation conferred an advantage on it ($G_1R_1 \ll G_1R_{20}$, $G_{20}R_1 \ll G_{20}R_{20}$, Fig. 1). That this advantage was not due to initially added N to the R_{20} treatments is seen from their lower N concentrations relative to the R_1 plants (Table I). This effect was independent of the size of the root system available for colonization, for it was true for per cent colonization as well as the weight of the fungus (Table I). As with nodulation, VAM fungal development was most pronounced in the dipartite association (G_1N).

Nodule P concentrations were approximately twice as large (Fig. 2) as root concentrations (Fig. 3), and correlated significantly with both total *Glomus* biomass and the percentage of *Glomus* biomass in the roots. Nodule P concentrations were greatest in the plants inoculated with *Rhizobium* at 20 d. Root P concentrations were lowest in the PN and highest in the G_1N plants. Plants inoculated with *Glomus* at planting had higher P concentrations than the G_{20} plants.

Significant negative correlations were observed between colonization by *Glomus* (Fig. 4) or nodule activity (Fig. 5) and the hexose sugar content of roots, respectively. Sugar content tended to decrease with increasing fungal permeation of the roots or with increasing nodule activity. The significant negative correlation between root P and sugar content reflected high P and low sugar contents in the dipartite (G_1N, PR_1) associations, low P and high sugar contents in the nonsymbiotic (PN) plants (Fig. 6). There was more sugar and less P in plants inoculated early with *Glomus* than in those inoculated late.

Root N concentrations did not correlate significantly with

[2] Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Table I. *Changes in Host Plant and Endophyte Parameters as a Result of Inoculation Timing and Nutrient Addition*

Annotation of endophyte-nutrient combinations is as in Figure 1. Numbers are the means of six replications, and are not significantly different (P > 0.05) by Duncan's multiple range test when followed by the same letter.

Parameter	Endophyte-Nutrient Combination							
	G_1		G_{20}		G_1N	PR_1	R_1	PN
	R_1	R_{20}	R_1	R_{20}				
Plant dry wt (g)	9.1 b	9.3 b	8.9 b	10.4 a	10.0 a	10.3 a	5.9 c	10.9 a
Root dry wt (g)	1.3 c	1.6 b	1.6 b	1.9 a	1.7 ab	2.0 a	1.4 c	2.1 a
VAM fungus/root (%)[a]	3.5 c	5.1 b	2.1 d	3.2 c	7.2 a			
Root N content (%)	1.62 b	1.44 c	1.46 c	1.26 d	2.06 a	1.47 c	1.38 c	1.67 b
Root/shoot ratio	0.17 a	0.21 ab	0.22 bc	0.22 bc	0.20 ab	0.24 c	0.24 c	0.24 c

[a] Percentage computed as (VAM-fungal biomass/VAM root) × 100 on a dry wt basis.

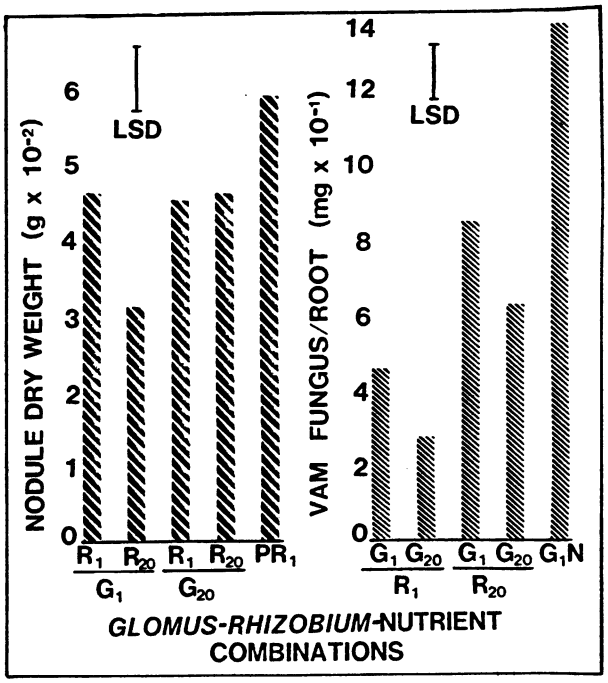

FIG. 1. Endophyte development in soybean plants inoculated with the VAM fungus *G. mosseae* and the diazotrophic bacterium *R. japonicum*. Plants were inoculated with both endophytes at planting (G_1R_1) or at 20 d after planting ($G_{20}R_{20}$) or with one endophyte following establishment of the other (G_1R_{20}, $G_{20}R_1$). When one or both endophytes were not present, the plants received a compensating P or N supplement, as did nodulated or VAM dipartite controls (PR_1, G_1N). Plants were harvested at 50 d.

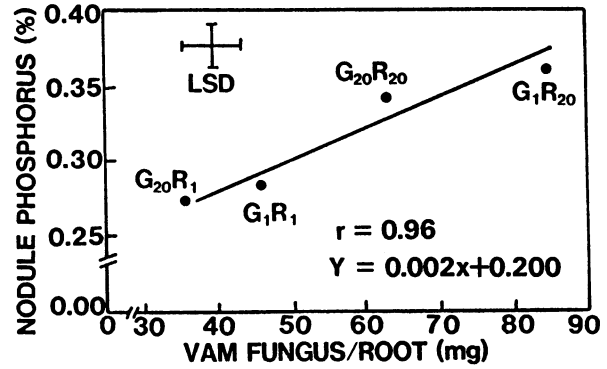

FIG. 2. Nodule phosphorus content as a function of root VAM fungal biomass. Annotations are as in Figure 1.

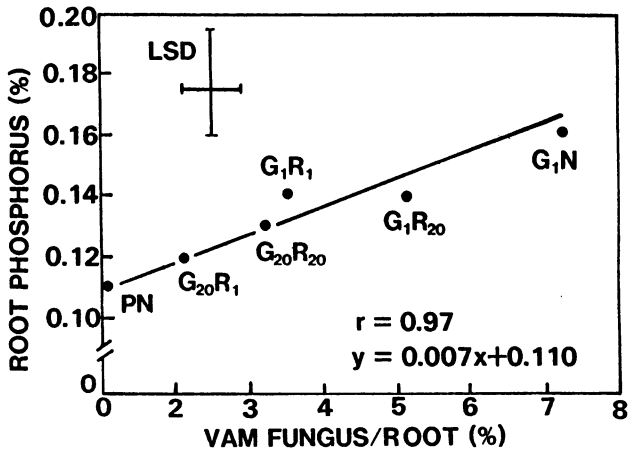

FIG. 3. Root phosphorus content as a function of per cent VAM fungal colonization determined as fungal biomass by the chitin assay. Annotations are as in Figure 1.

other parameters (Table I). However, the VAM plant supplemented with combined N (G_1N) had significantly higher N concentrations than the non-VAM, P-supplemented plants PR_1 or PN.

Root/shoot ratios were least in plants inoculated with *Glomus* at planting and greatest in the non-VAM plants (Table I). Plants inoculated with *Glomus* at d 20 had significantly greater root/shoot ratios than the G_1R_1 plants, but were not different from the non-VAM plants.

DISCUSSION

When nodulated legumes are P deficient. P fertilization or VAM colonization generally enhance N_2 fixation (5; also Table I). Adverse effects of VAM fungi on nodulation have not been reported previously, although a recent analysis of source-sink relationships in a legume association stressed for carbohydrates revealed competition among the symbionts for the limiting resource (6). Effects of rhizobial infection on VAM fungal establishment are not known (15), but the observation (19, 28) that fungal hyphae do not usually invade root nodules suggests the presence of an exclusion mechanism. Such a mechanism may also be operative in root segments adjacent to nodules or, to some extent, in the entire modulated root system, and needs further study. An involvement of plant hormones cannot be excluded as a possible control mechanism of the effects observed here. Another explanation of these effects is offered here by the changes in mineral nutrient and soluble carbohydrate concentra-

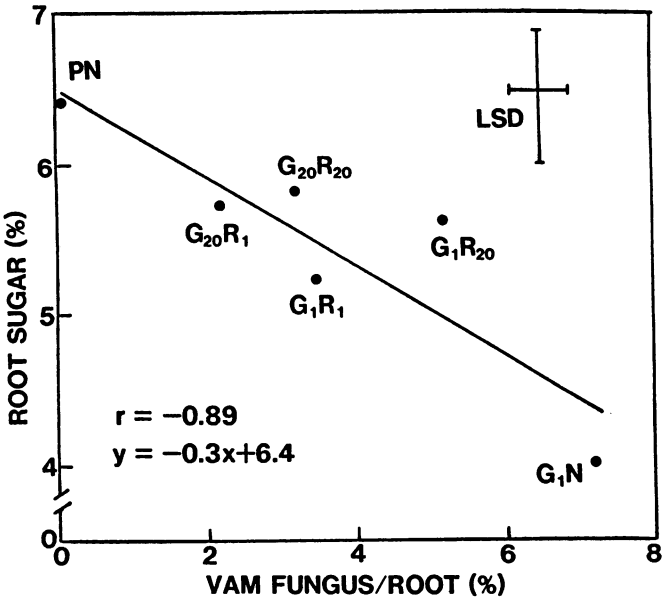

FIG. 4. Root sugar (hexose) content as a function of per cent VAM fungal colonization. Annotations are as in Figure 1.

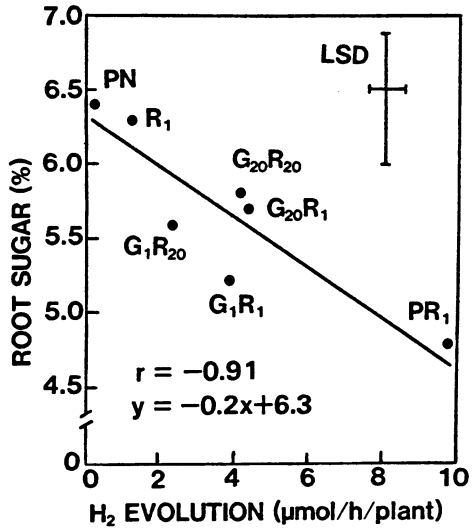

FIG. 5. Root sugar content as a function of nodule activity. Nodule activity was determined as H_2 evolution in a rhizobial strain of low relative efficiency. Annotations as in Figure 1.

tions as a result of endophyte presence or activity.

Work on endophyte interactions is hampered by the difficulties in growing appropriate, nutrient-sufficient controls (21). Addition of nutrients (N, P) to the regime of hosts lacking one or both endophytes may produce control plants similar in form but unlike in function. Furthermore, differences in nutrient regimes prior to inoculation may affect endophyte development (20) thus masking the effects of the endophytes on each other. Our evaluation of the data indicates that differences in endophyte development were due to interactions of the endophytes, rather than to nutrient effects.

Nodulation (24, 30) and VAM colonization (17) depend on the availability of soluble carbohydrates in the host root. In the absence of photosynthetic limitation, the level of available sugars depends on endophyte activity and on the metabolic activity of the host cells. Both are influenced by P availability. Sugars accumulate in roots when energy metabolism is impaired due to

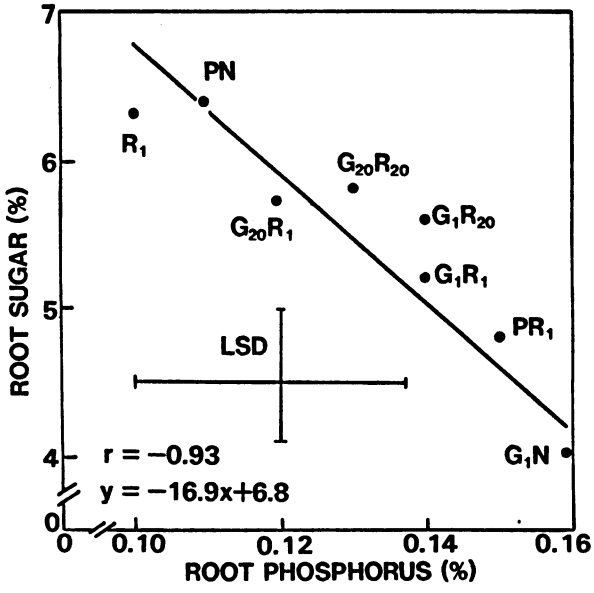

FIG. 6. Correlation of root sugar and P contents. Annotations as in Figure 1.

insufficient P, resulting in an inverse relationship between soluble carbohydrates and P (14, 16). Thus, the higher levels of root P (Fig. 3) associated with more intense VAM-fungal development correlated with a depletion of sugar concentrations (Fig. 6), presumably as a consequence of fungal sink demand and of increased P-dependent metabolic activity of the host. Similarly, higher levels of nodule P (Fig. 2) resulted in greater nodule activity and sugar depletion (Fig. 5). Phosphorus uptake by VAM fungi in nodulated legumes therefore appears to initiate a chain of cross-reactions between the symbionts, with activity determined by the level of soluble carbohydrates. In the non-VAM plants (PR_1, PN), P uptake may have been controlled by demand rather than supply limitation (29) as shown by the significantly higher P concentration in the nodulated (high P demand) than in the nonsymbiotic (low P demand) root, given an equal external P supply (Fig. 6). The significantly greater sugar depletion in the PR, compared to the PN root is consistent with a role for nodules as a competitive, P-sensitive sink for photosynthates (12) and supports the thesis of competitive nodule effects on VAM fungi in the tripartite root.

Root N content did not correlate significantly ($P > 0.05$) with any of the other parameters measured. It was, however, significantly higher (Table 1) in the dipartite VAM plant than in the non-VAM plants receiving P ($G_1N > PN > PR_1$) indicating that the presence of the fungus, rather than the form of the N supply was decisive for N uptake. Among the tripartite associations root N contents did not correlate with fungal or nodule development, but were greatest in plants inoculated early with both endophytes and lower in plants inoculated late ($G_1R_1 > G_1R_{20} = G_{20}R_1 > G_{20}R_{20}$), suggesting a positive interaction between the activities of the endophytes in N acquisition by the association.

The significantly lower dry weights of plants inoculated with one or both endophytes at planting compared to those of the other combinations ($G_1R_1 = G_1R_{20} = G_{20}R_1 < G_{20}R_{20} = G_1N = PR_1 = PN$) was not related to root sugar concentrations (Table I; Fig. 6) and suggests, therefore, a nonparasitic mechanism for host-plant growth depression. Such a mechanism may be related to the reduction of root growth without shoot limitation, and is generally observed in response to VAM colonization or greater availability of mineral nutrients (12, 26). Neither of these conditions is parasitic, but is due to a reallocation of resources, when greater root growth is unnecessary to sustain shoot development.

Such changes in root/shoot ratios were most pronounced here in plants colonized by *Glomus* at planting (Table I).

Differences in the development of the three symbionts as a function of the timing of inoculation with the endophytes suggest the presence of control mechanisms whose relative importance is little understood. Depletion of root sugar concentrations implied competitive interactions among the endophytes but did not result in a comparable growth depression of the host. Other stimuli, such as the production of inhibitory secondary metabolites, may also be present and need further study. The timing of inoculation may be an important aspect in experimentation with the developmental sequences of endophyte establishment and in agricultural practice.

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