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Interactions between Nitrogen Fixation, Mycorrhizal Colonization, and Host-Plant Growth in the *Phaseolus-Rhizobium-Glomus* Symbiosis

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ABSTRACT

Bean (*Phaseolus vulgaris* L. cv. Dwarf) roots were inoculated with *Rhizobium phaseoli* and colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus fasciculatum* Gerd. and Trappe or left uncolonized as controls. The symbiotic associations were grown in an inert substrate using 0, 25, 50, 100, or 200 milligrams hydroxyapatite (HAP) ($Ca_{10}[PO_4]_6[OH]_2$) per pot as a P amendment. Plant and nodule dry weights and nodule activity increased for both VAM and control plants with increasing P availability, but values for VAM plants were significantly lower in all parameters than for controls. Inhibition of growth and of N_2 fixation in VAM plants was greatest at the lowest and highest P regimes. It was smallest at 50 milligrams HAP, where available P at harvest (7 weeks after planting) was 5 micrograms P per gram substrate. At this level of P availability, the association apparently benefited from increased P uptake by the fungal endophyte. Percent P values for shoots, roots, and nodules did not differ significantly ($p > 0.05$) between VAM and control plants. The extent of colonization, fungal biomass, and the fungus/association dry weight ratio increased several fold as HAP was increased from 0 to 200 milligrams. It is concluded that intersymbiont competition for P and photosynthate was the primary cause for the inhibition of growth, nodulation, and nodule activity in VAM plants. Impaired N_2 fixation resulted in N stress which contributed to inhibition of host plant growth at all levels of P availability.

Enhancement of N_2 fixation by root nodules as a result of improved P nutrition is well documented (12, 18, 20), and appears to depend on the high P requirement of the bacteriods (3). When the availability of P is low, increased P uptake in legumes colonized by VAM[2] fungi enhances host plant growth and also stimulates N_2 fixation (2, 12, 14). This has been defined as mycotrophic growth (15). Under nonmycotrophic conditions, i.e. when the plant does not benefit from enhanced nutrient uptake by the fungal endophyte, growth inhibition may occur in VAM plants as a result of fungal colonization (6, 16). The cause of this phenomenon is controversial (27). However, in associations containing a high percentage of fungal biomass the endophyte may be a significant sink for carbohydrates (6). Nodulation and N_2 fixation have also been shown to depend directly on the availability of carbohydrates (9). Competition for photosynthates by the microsymbionts of the tripartite legume/*Rhizobium*/VAM fungal association therefore appears likely.

The benefits of enhanced nutrient uptake by VAM fungi may be counteracted by the loss of carbohydrates from the host to the fungal endophyte (21). The concentration of P in the substrate appears to be crucial in determining whether VAM colonization will be beneficial, detrimental, or will occur at all. When P is extremely limiting, growth of both symbionts is inhibited (12). When P availability is low, enhanced growth of the host occurs (mycotrophy; Ref. 15). At intermediate levels of P, fungal proliferation may be at the expense of the host without enhancing P uptake (13), while at the high levels of P fungal growth is inhibited (21). In a nonsorbing medium, such as the sand-perlite culture with HAP as the P source used in the present investigation, P concentration at the absorbing root or fungal surface depends on the distance between absorbing surface and P source, since this determines the concentration gradient between them (28). The contribution of fungal hyphae thus lies in reducing the mean distance between each HAP grain and the nearest absorbing surface. Previous work with such media at high levels of HAP produced pronounced VAM fungal development and growth inhibition of the host plants (6), apparently by providing P concentrations not high enough to prevent VAM fungal colonization, but high enough for nonmycotrophic growth of the host. The objective of this work was to grow tripartite associations under extremely limiting, low and intermediate P regimes, and to investigate the effect of VAM fungal colonization on nodulation, nodule activity, and host plant response as a function of P availability.

MATERIALS AND METHODS

Growth conditions. Bean (*Phaseolus vulgaris* L. cv. Dwarf) plants were grown in 1.5-L white plastic pots in a greenhouse at Albany, CA, May to June 1981. Temperature and RH varied from day to day within the day/night ranges of 30°/15°C and 45%/95%, respectively. On sunny or overcast days, PPFD averaged 500 or 300 $\mu E/m^2 \cdot s$, respectively. The majority of the days during the experimental period where overcast or partly overcast due to coastal fog. Daylength was extended to 16 h by Sylvania 1000-w metal halide lamps mounted vertically in parabolic reflectors and arranged to provide uniform supplementary PPFD of 400 $\mu E/m^2 \cdot s$ at plant emergence level. The growth medium consisted of 1.25 L of perlite/sand mixture (2:1, v/v) covered by a 2.5-cm layer of perlite. This was watered with a nutrient solution consisting of 1.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 0.25 mM $MgSO_4$. Combined N was supplied as 0.5 mM NH_4NO_3, which was optimal for N_2 fixation

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[2] Abbreviations: VAM, vesicular-arbuscular mycorrhizal; HAP, hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$); PPFD, photosynthetic photon flux density; RE, relative efficiency of nitrogen fixation, RE = 1-(H_2 evolved/acetylene reduced); SNA, specific nodule activity (μmol N_2 reduced/h·g nodule dry weight).

Table I. *Dry Weights of Symbiotic Structures of the Phaseolus-Rhizobium-Glomus Association at Different Levels of P Fertilization*

Beans roots were inoculated with *R. phaseoli* and colonized with the VAM fungus *G. fasciculatum* or left uncolonized as controls. Associations were harvested 7 weeks after planting. Weights were determined after drying for 24 hours at 80°C. Fungal biomass was determined by the chitin assay (5, 8) and represents mycelia inside and outside the host root.

Organ	Mycorrhiza	Hydroxyapatite (mg/pot)				
		0	25	50	100	200
				mg dry wt[a]		
Shoot	Control	1,610 ± 70	2,160 ± 120	2,310 ± 120	3,300 ± 350	3,840 ± 420
	VAM Plant	1,210 ± 60	1,730 ± 130	1,880 ± 150	2,190 ± 150	2,390 ± 120
Root[b]	Control	670 ± 30	780 ± 20	840 ± 40	860 ± 50	970 ± 80
	VAM Plant	680 ± 50	740 ± 20	760 ± 50	780 ± 80	860 ± 90
Nodule	Control	66 ± 1	89 ± 5	96 ± 9	201 ± 42	233 ± 29
	VAM Plant	38 ± 1	59 ± 8	72 ± 13	126 ± 8	128 ± 33
Mycelium[c]	Intraradical	7.5 ± 0.5	9.9 ± 0.4	12.1 ± 0.5	15.2 ± 1.4	21.3 ± 1.4
	Extraradical	15.1 ± 2.1	27.4 ± 0.7	43.2 ± 3.0	50.0 ± 2.6	69.6 ± 2.4

[a] Numbers are means and SE of four replications.
[b] Excluding nodules.
[c] Control plants were not colonized.

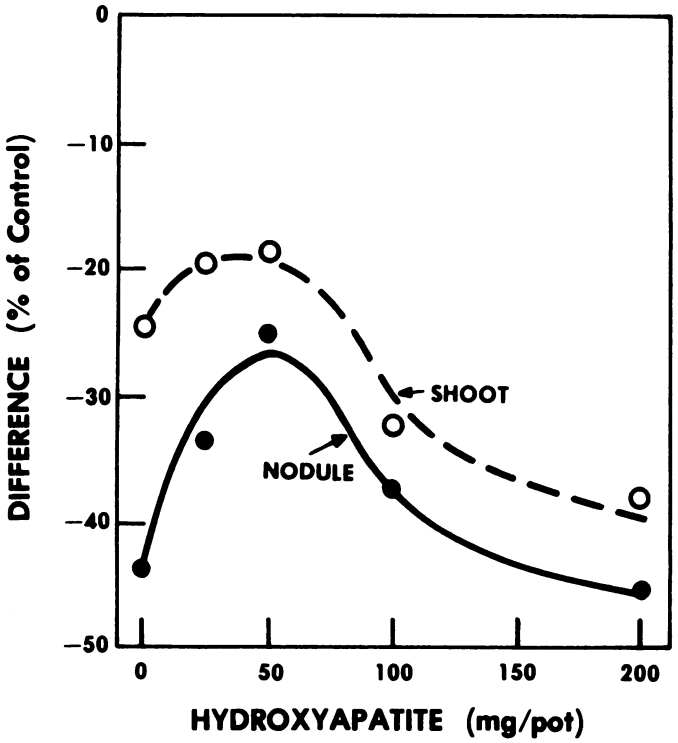

FIG. 1. Relative host plant response to mycorrhizal colonization. Bean plants were grown under different P regimes, inoculated with *Rhizobium phaseoli*, and colonized by the VAM fungus *Glomus fasciculatum* or left uncolonized as controls. Growth of VAM plants relative to controls was calculated as percent differences: ([VAM plant − control]/control) × 100.

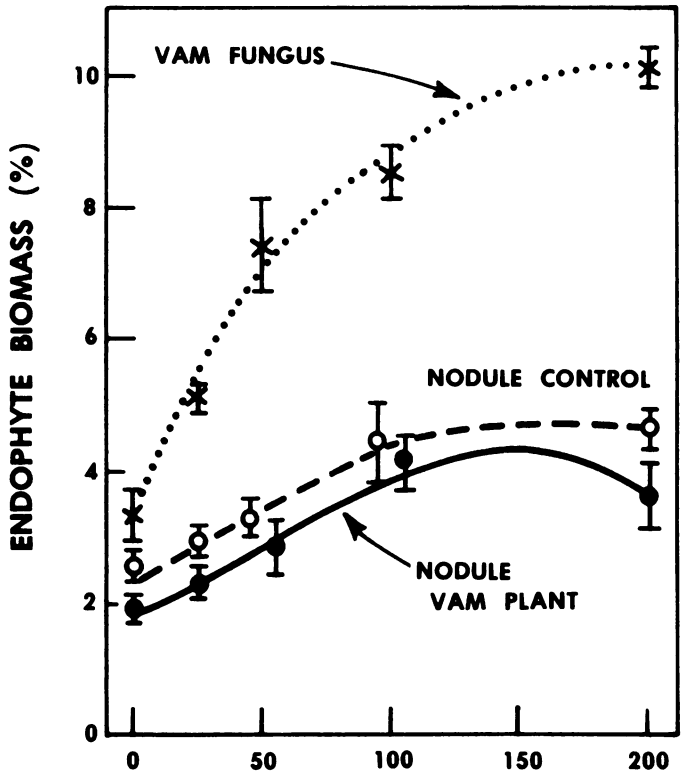

FIG. 2. Endophytic structures as percent of association biomass. Percent nodule weight was determined as a g nodule/g total association after drying for 1 d at 80°C. Total VAM fungal biomass was expressed as percent of mycorrhizal dry weight. It was measured by determining the chitin content of the mycorrhiza and of the substrate, and comparing to the chitin content of isolated mycelium. Chitin contamination by non-VAM organisms was accounted for by equivalent determinations in the controls.

(32) but limiting for host plant growth (18). The concentration of Fe was 20 μm supplied as $FeEDDA^-$ (ferric ethylenediamine di-[*o*-hydroxyphenyl] acetic acid). Micronutrients were according to Johnson *et al.* (22) at one-quarter strength and supplemented by 0.5 μM $CoCl_2$. Phosphorus was added as finely ground HAP ($Ca_{10}[PO_4]_6[OH]_2$) obtained from Sigma Chemical Company as type VI calcium phosphate, C-5287. Five P regimes were used, each consisting of 0, 25, 50, 100, or 200 mg HAP/pot.

Biological Materials. Beans were germinated for 2 d at 28°C. Seedlings were selected for uniformity and inoculated at planting with 7×10^6 cells of *Rhizobium phaseoli* strain 127K48 (obtained originally from J. C. Burton, The Nitragin Co., Milwaukee, WI). Plants were also inoculated with the Gerdemann isolate of *Glomus fasciculatum* (Thaxt. *sensu Gerd.*) Gerd. and Trappe, or left uninoculated as controls. The inoculum (obtained from S. Woodhead, Abbott Laboratories, Long Grove, IL) consisted of 10 g of soil containing approximately 30 spores and 80 root fragments partially infected by *G. fasciculatum*. Control plants were initially

Table II. *Nodule Activity and RE of R. phaseoli at Different Levels of P Fertilization*

Nodulated bean-plant roots were colonized by the VAM fungus *G. fasciculatum* or left uncolonized as controls. Acetylene-dependent ethylene production and H_2 evolution were determined by flame ionization and thermal conductivity gas chromatography.

Assay	Mycorrhiza	Hydroxyapatite (mg/pot)				
		0	25	50	100	200
		μmol/h/plant[a]				
Acetylene reduction[b]	Control	0.18 ± 0.05	0.71 ± 0.12	0.88 ± 0.12	3.13 ± 0.75	3.72 ± 0.59
	VAM Plant	0.06 ± 0.03	0.12 ± 0.04	0.22 ± 0.05	0.49 ± 0.05	1.17 ± 0.53
H_2 evolution[b]	Control	0.10 ± 0.03	0.56 ± 0.16	0.65 ± 0.10	2.46 ± 0.50	2.65 ± 0.47
	VAM plant	0.0	0.0	0.0	0.05 ± 0.03	0.48 ± 0.24
RE	Control	0.46 ± 0.15	0.24 ± 0.10	0.26 ± 0.04	0.20 ± 0.05	0.29 ± 0.08
	VAM Plant	1.00 ± 0	1.00 ± 0	1.00 ± 0	0.88 ± 0.07	0.63 ± 0.10
N_2 fixation[c]	Control	0.027	0.050	0.077	0.223	0.357
	VAM Plant	0.020	0.040	0.073	0.147	0.230
SNA	Control	0.47	0.56	0.86	1.11	1.54
	VAM Plant	0.52	0.59	0.94	1.17	1.80

[a] Numbers are means and SE of four replications.
[b] All differences between VAM and control plants were significant at the 0.01 level.
[c] Calculated as (acetylene reduction − H_2 evolution)/3.

watered with washings (43-μm sieve) of the inoculum free of *G. fasciculatum*. Plants were harvested over a 4-d period during the seventh week after planting. All plants were stressed at harvest as indicated by yellowing of some leaves and some sloughing of nodules. Maximum development of the two endophytes does not coincide. The time of harvest was therefore selected at an intermediate point.

Assays. Nodule activity was determined as described previously (11) with the following modifications. Ethylene analyses were made with a Varian model 1400 gas chromatograph equipped with a flame ionization detector using a 0.32- × 183-cm stainless steel column filled with 80- to 100-mesh Porapak N. Hydrogen evolution was measured with a Hewlett-Packard model 5880 gas chromatograph equipped with a thermal conductivity detector using a 0.32- × 183-cm column filled with 60- to 80-mesh molecular sieve 5A. Helium served as the carrier gas for ethylene, and N_2 for H_2, both at a rate of 30 ml/min. Oven tempertures were 85°C for ethylene and 50°C for H_2. Reduction of N_2 was estimated from acetylene-dependent ethylene production and H_2 evolution data using the formula: N_2 reduced = (ethylene produced − H_2 evolved)/3. The RE of electron transfer to N_2 via nitrogenase was calculated as RE = 1 − (H_2 evolution/acetylene reduction) according to Schubert and Evans (26).

Intraradical (8) and extraradical (5) fungal biomass was determined by the chitin assay, and percent colonization of the host's root system was estimated from a large number of stained root segments (12) as described previously. Available ($NaHCO_3$-extractable) P in the substrate was determined according to Murphy and Riley (24) as modified by Watanabe and Olsen (31). Plant P content was determined according to Allen (1). Replications of plant samples were pooled for the P determination with the exception of one P regime (50 mg HAP), on which confidence intervals were determined. Dry weights of plant parts were measured after drying at 80°C for 1 d. Percent differences in dry weight between VAM and control plants were calculated as ([VAM plant − control]/control) × 100. Four replications were used, and positional differences in the greenhouse were minimized by daily rotation of pots. Significant differences were determined by Student's *t*-test.

RESULTS

Effect of VAM Fungus on Host Plant. Colonization of the nodulated host by the VAM fungus *G. fasciculatum* resulted in signficantly ($p < 0.05$) smaller shoot and nodule dry weights than in nonmycorrhizal controls, while root weights were not significantly different (Table I). Percent differences between VAM and control plants for shoots and nodules were negative, reflecting growth depression of the VAM plants relative to controls (Fig. 1). Percent differences were most pronounced for shoots and nodules at the highest and lowest levels of P fertilization, and were greater for nodules than for shoots at all levels of P. The ratio of nodule biomass to total association dry weight was lowest at 0 mg HAP (Fig. 2), and increased 2-fold as the HAP amendment was raised from 0 to 200 mg. Nodule weights between VAM and control plants were significantly ($p < 0.05$) different at 0, 25, and 200 mg HAP.

Total P contained in the seed, sand and perlite was initially 1.0, 17.8, and 0.3 mg/pot, respectively. Available P in the sand and perlite alone was 1.6 mg P/pot, while available P due to 200, 100, 50, or 25 mg HAP was 27.3, 14.0, 6.3, and 3.2 mg P/pot, respectively. Phosphorus concentrations of shoots, roots, and nodules were not significantly different ($p > 0.05$) in VAM and control plants (data not shown). Total P in the controls was higher than in VAM plants, reflecting the greater biomass of the former. Available ($NaHCO_3$-extractable) P in the substrate of VAM plants increased linearly from 2 to 14 μg P/g substrate with increasing HAP application from 0 to 200 mg and had a value of 5 μg P/g substrate at harvest at the 50-mg HAP level, where growth inhibition of the VAM plants was smallest. Values of available P for controls were lower but not significantly ($p > 0.05$) different from P values found in the growth medium of VAM plants (data not shown).

Effect of VAM Fungus on Nitrogen Fixation. Nodule activity in terms of acetylene-dependent ethylene production and H_2 evolution was significantly ($p < 0.01$) higher in controls than in VAM plants at all levels of P fertilization (Table II). Nitrogen fixation, calculated from ethylene and H_2 data, increased linearly for VAM plants with increasing P availability but deviated from linearity in control plant nodules at the highest HAP levels.

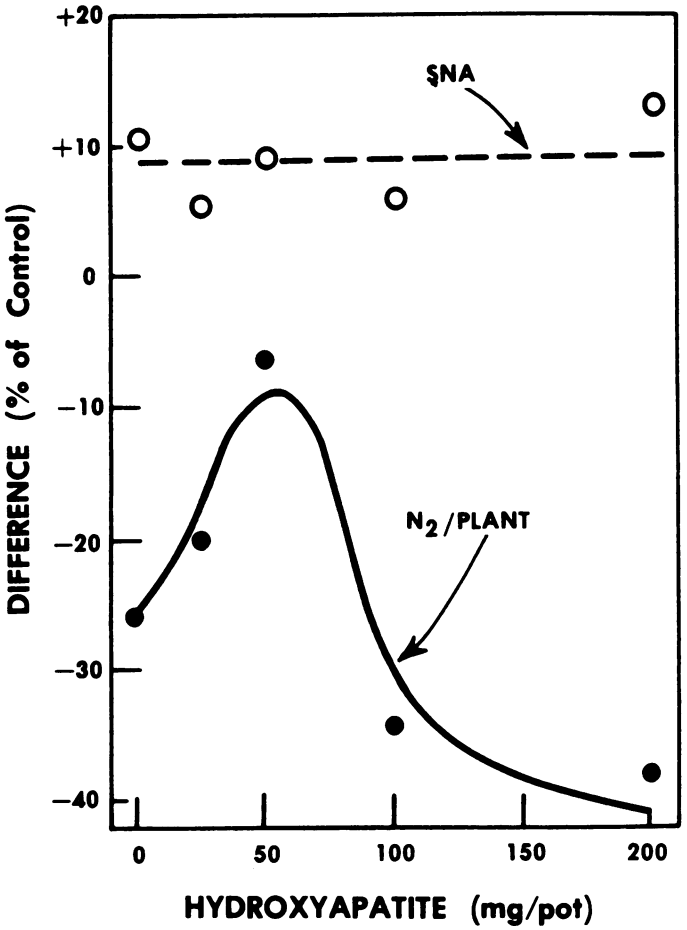

FIG. 3. Response of nodule activity to VAM fungal colonization of the host. N_2 fixation was estimated from ethylene and H_2 data as N_2 = (ethylene production − H_2 evolution)/3 and expressed as total N_2 reduced/plant or as SNA (N_2 reduced/nodule mass). N_2 fixation by VAM plants relative to controls was calculated as percent difference: ([VAM plant − control]/control × 100.

Nitrogen fixation per plant was higher in controls than in VAM plants. This relationship was reversed for SNA (Table II). Nodules on VAM plants had a significantly higher RE ($p < 0.01$) than control plant nodules (Table II). Percent differences in SNA between VAM and control plants were positive and invariant with P availability. Percent differences in N_2 fixation per plant showed a marked maximum at the P treatment of 50 mg HAP and indicated high levels of inhibition of N_2 fixation in VAM plants at the lowest and highest levels of P amendment (Fig. 3).

Development of VAM Fungal Colonization. Percentage of colonization by *G. fasciculatum* in the host plant root system increased 5-fold (Fig. 4), and the ratio of fungal biomass to mycorrhizal dry weight increased 3-fold (Fig. 2), as HAP amendment was raised from 0 to 200 mg. Intraradical and extraradical VAM fungal mycelia increased with increasing P availability. No extraradical spores and very few vesicles were observed at any level of P.

DISCUSSION

Growth enhancement or repression of plants colonized by VAM fungi have been ascribed to the predominance of one of two opposing processes; enhancement due to increased P uptake or repression due to a drain on host carbohydrates (21). The first process is supported by overwhelming evidence (27), while the latter has been questioned on the grounds that VAM fungal biomass may be insufficient to affect the host's carbon balance (29). In the present experiment, imposition of a wide range of P

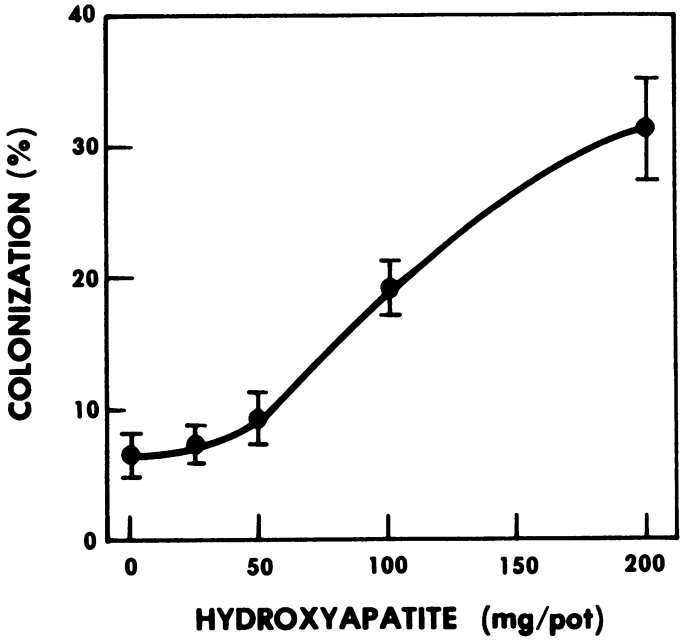

FIG. 4. Percent VAM fungal colonization. The extent of fungal colonization was determined histologically using stained root segments.

regimes resulted in growth inhibition of VAM plants relative to controls at all levels of P (Table I). However, VAM plants were least inhibited at intermediate levels of P which favored mycotrophic growth (Fig. 1). Inhibition of VAM-plant growth at all treatment levels, in spite of the positive host response to enhanced P availability at 50 mg HAP, indicated that conditions for photosynthesis were suboptimal. As a result, the increased sink demand due to the endophytes could not be fully compensated by higher production of carbohydrates. As an additional limiting condition, competition for P and reduced C between the symbionts resulted in reduced rates of nodule activity. In this tripartite association dependent on atmospheric N, concomitant N stress may have occurred which was evidenced by a slightly chlorotic appearance of the VAM plants.

Competition for P and Carbohydrates. The concentration of available ($NaHCO_3$-extractable) P was extremely limiting (2 μg P/g substrate) at the low end of the HAP gradient. This was reflected by the depressed levels of plant, nodule, and fungal dry weights (Table I). The low levels of fungal colonization (Fig. 4) and the low percentages of nodule and fungal biomass (Fig. 2) suggest that the sink capacity of the endophytes for carbohydrates was small. The high level of inhibition at the 0 mg HAP level was relieved as P availability increased to 50 mg HAP (Fig. 1). This indicated that at the lowest P regime competition for P between host and fungal endophyte was the dominant factor causing growth depression in VAM plants. Inhibition of VAM plants relative to controls deceased with increasing P availability (Fig. 1) in spite of gradually increasing fungal biomass (Table I) and percent colonization (Fig. 4). This relative increase in VAM plants can be ascribed to growth stimulation due to enhanced P uptake by the fungal endophyte at 25 and 50 mg HAP. Such growth stimulation was previously observed under similar growth conditions (6).

At higher levels of P availability, corresponding to 100 and 200 mg HAP, VAM plant shoot and nodule dry weights became smaller relative to controls. This was accompanied by large increases in fungal biomass and nodule weight (Table I). When the fungal endophyte proliferates to such an extent, it may become a significant carbohydrate sink (6) because of its high rate of specific respiration (19) and the infection respiration of associated host tissue (30). Thus, within the concept of the two opposing processes

(21) stimulation due to enhanced P uptake no longer occurred, whereas competition for carbohydrates determined host growth response. Although compensatory CO_2 fixation by the host plant in response to increased carbohydrate requirements by microsymbionts have been noted in the past (4, 23), the conclusion that the host will meet higher demand by increased production (25) has not been verified by the results of this experiment, perhaps due to limitations on photosynthesis caused by suboptimal light conditions. Low levels of light intensity have been shown to depress nodulation (10) and VAM fungal development (17). Thus, interactions between the symbionts of the tripartite association are expected to vary with changes in photosynthesis.

Nodulation and Nitrogen Fixation. The increase in nodule dry weight was almost twice that of shoot growth over the HAP gradient in both VAM and control plants (Table I), verifying the high P requirement of these symbiotic structures (3, 18). Significantly lower ($p < 0.05$) nodule to association dry weight ratios in VAM plants than in controls at the high and low levels of P availability (Fig. 2) indicated that impairment of the host's ability to support these structures was most intense at these extremes. At the 200 mg HAP treatment, where P was most available, competition by the fungal symbiont for carbohydrates was the most likely cause for the severe inhibition of nodulation (Fig. 1). The dependence of nodulation and N_2 fixation on the products of photosynthesis is well documented (7). Development of VAM plants relative to controls showed the same pattern of inhibition for nodules as for shoots (Fig. 1). The greater inhibition of the nodules indicated that the factors causing inhibition in the host plant and the bacterial endophyte are the same but affect the microsymbiont more severely.

Percent differences in SNA were positive and invariant over the HAP gradient, while the differences in N_2 fixed per plant (Fig. 3) followed the inhibition pattern observed in shoots and nodules (Fig. 1). As the absolute values of both SNA and N_2 fixation increased almost linearly with increasing P availability (Table II), it is concluded that nodule formation, rather than nitrogenase activity, responded to the inhibitory effects of P and carbohydrate deficiency. The enhancement of SNA in VAM plants at all levels of P availability (Fig. 3) appears to be related to decreased production of H_2 (Table II) and the resulting high values of RE (Table II). The RE was affected more severely by carbohydate than by P deficiency (Table II) indicating differential sensitivity of the H_2-evolution mechanism of nitrogenase to the availability of these nutrients. A decrease in H_2 evolution with increasing carbohydrate (4, 10, 11) or P (12) stress has been noted previously. This effect was ascribed to a shift in electron allocation by nitrogenase from H^+ to N_2 reduction and to higher levels of uptake hydrogenase activity under carbohydrate stress (9). The similar effects of P and carbohydrate deficiency on H_2 evolution suggests that the availability of ATP influences RE.

Conclusions. Growth responses of the host plant to VAM fungal colonization over a range of P availability and under suboptimal light conditions were affected directly by competition of the three symbionts for P and carbohydrates, and also by the effects of such competition on N_2 fixation. The data suggest that the overriding factors are competition for P when P is severely limiting, and competition for carbohydrates when VAM fungal proliferation is pronounced. At levels of P between these extremes mycotrophic growth of the association is favored, and increased P uptake by VAM fungi has a favorable effect on the other symbionts. However, when the tripartite association is dependent on atmospheric N, reduced allocation of P and carbohydrates to the nodules due to VAM fungal competition may result in N stress. This is due to a decrease in N_2 fixation which has a high requirement for these nutrients. Further work to elucidate the effect of photosynthesis and carbohydrate availability on inter-symbiont relationships is needed and is in progress.

The growth medium utilized appears to be well suited for the manipulation of host endophyte growth relationships under controlled conditions. However, such results may not be generalized to field conditions where sorption of P and the equilibrium concentration of P in the soil solution will influence fertilizer effects.

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