

The Glycine-Glomus-Rhizobium symbiosis

I. Phosphorus effect on nitrogen fixation and mycorrhizal infection

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Soybean [*Glycine max* (L.) Merr. cv. Lancer] plants were grown in a sterile rooting medium watered daily with a nutrient solution containing 4, 20, 100, or 500 μM P. Plants were inoculated with *Rhizobium japonicum* strain 61A118 and grown in the presence or absence of the endomycorrhizal fungus *Glomus fasciculatus* Gerdemann et Trappe. Plants grown at the highest P regime had six times higher shoot dry weight than those grown in the lowest P regime. Nodulation did not occur at 4 μM P. Nodule dry weight increased 200-fold from the 20 to the 500 μM P treatment. Percentage P in shoots and nodules differed significantly among all treatment levels. Acetylene reduction by nitrogenase increased logarithmically with increasing amounts of P. Hydrogen evolution was not detectable at the 20 μM P level. The relative efficiency of nitrogen fixation increased with increasing P stress. Infection by *Glomus fasciculatus* at the 500 μM P level was negligible and did not affect the parameters measured. At all other treatment levels the mycorrhizal plants had significantly higher rates of N_2 fixation, plant and nodule mass and P content.

Key-words: Endomycorrhiza; *Glomus fasciculatus*; *Glycine max*; *Rhizobium japonicum*.

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Introduction

Endomycorrhizal fungi of the Endogonaceae (Gerdemann and Trappe 1974) enhance plant growth through increased uptake of relatively immobile mineral nutrients in deficient soils (Bielecki 1973, Gerdemann 1975, Mosse 1977a). The concentration of available P in the soil solution depends on the form and amount of bound P in the soil system (Helyar and Munns 1975, Larsen 1967, Wilson 1968) and is many times lower than that of other macronutrients (Barber *et al.* 1963). The ability of vesicular-arbuscular mycorrhizal (VAM) fungi to explore a far greater soil volume than that available to the host plant's root system alone is therefore important in the P nutrition of the host (Bielecki 1973, Hattings *et al.* 1973, Rhodes and Ger-

demann 1975). The contribution of VAM fungi to the tripartite symbiotic association is particularly significant in nodulated legumes growing under a regime of limiting combined N. This is due to the high P requirement of nodulation (Asai 1944, Carling *et al.* 1978, Daft 1978) and N_2 fixation (Bergersen 1971).

To study the response of the legume symbiosis to P availability under controlled conditions, sterilized soils in combination with various amounts of P fertilizers of differing solubility are customarily used (Mosse 1977b, Mosse *et al.* 1976, White 1972). This approach is an approximation to field conditions in that the majority of the P present in the soil is bound. However, it does not permit exact determination and control of the concentration of available P throughout the experiment be-

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cause of soil-fertilizer interactions and various rates of P fixation (Obihara and Russel 1972, Wilson 1968). This study therefore made use of an inert rooting medium and controlled amounts of soluble P replenished on a daily basis in order to establish whether meaningful differences in plant growth and N₂ fixation could be demonstrated in such a system.

The purpose of the experiment was to determine the effect of P on nodule activity, on RE in terms of C₂H₄ and H₂ evolved by nitrogenase, and on VAM infection.

Abbreviations: RE, relative efficiency of N₂ fixation, calculated as $RE = 1 - (H_2 \text{ evolved}/C_2H_2 \text{ reduced})$; VAM, vesicular-arbuscular mycorrhizal.

Materials and methods

Soybean [*Glycine max* (L.) Merr. cv. Lancer] plants were grown in 15 cm white styrofoam pots in a greenhouse at Baton Rouge, Louisiana, December 1979 to February 1980. Day and night temperatures varied from day to day, but day temperatures did not exceed 35°C and night temperatures did not fall below 21°C during the experiment. Daylength was extended to 16 h by 1000 W metal halide lamps mounted vertically in parabolic reflectors and arranged to provide photosynthetic photon flux density of 300 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at plant emergence level. The growth medium consisted of perlite and a nutrient solution equivalent to 0.25-strength Johnson's solution (Johnson *et al.* 1957) made up of 1 mM CaCl₂, 0.5 mM KH₂PO₄, 0.5 mM K₂SO₄, 0.25 mM MgSO₄, 2 mM NH₄NO₃, and micronutrients augmented by 0.5 μM CoCl₂. Three other solutions were prepared by decreasing the concentration of KH₂PO₄ and increasing that of K₂SO₄ commensurately to keep [K⁺] constant at 1.5 mM. The four resulting P treatments were 500, 100, 20, and 4 μM in P. Plants in each treatment group were watered daily with excess solution and flushed once a week with deionized water. Concentrations of P thus varied daily between a known replenishment level and a 24-h depletion level. Effluent from pots was tested and no accumulation of P was observed.

Seedlings were either inoculated at planting with the VAM fungus *Glomus fasciculatus* (Thaxt. sensu Gerd.) Gerd. et Trappe or left uninoculated as controls. Four ml of inoculum (obtained from S. Woodhead, Abbott Laboratories Research Center, Long Grove, IL 60047) consisting of an unidentified number of infected root segments and approximately 50 spores mixed with soil were used per pot. Control plants were initially watered with washings (44 μm sieve) of the inoculum, which supplied a sample of the microflora without *G. fasciculatus*. Plants were inoculated with *Rhizobium japonicum* strain 61A118 (obtained originally from J. C. Burton, The Nitragin Co., Milwaukee, WI 53209) 3 weeks after planting, when differences in plant size

due to P treatment became apparent. At this time, the N content of the nutrient solution was decreased to 0.5 mM NH₄NO₃.

Plants were harvested 50 days after planting. Nodule activity was determined as described previously (Bethlenfalvai and Phillips 1977a). The RE of electron transfer to N₂ via nitrogenase was calculated from C₂H₂-reduction and H₂-evolution data [RE = 1 - (H₂ evolution/C₂H₂ reduction)] according to Schubert and Evans (1976). After removal of nodules lateral roots were excised from the taproot, aligned, and cut into 1 cm segments. The segments were randomized by dispersal in water and stained according to Phillips and Hayman (1970). Fifty stained root segments per plant were selected at random, aligned on slides, and inspected for the presence of VAM infection under a dissecting microscope. Values of 1, 5, or 10 were assigned to segments showing trace, 50%, or 100 % infection. Percentage infection per plant was calculated by adding the numerical values assigned to individual segments and dividing the sum by the maximum possible value of 500. Shoots and nodules were dried at 80°C for 2 days and weighed. Nodule and shoot P content was determined according to Kitson and Mellon (1944). Data were based on five replications.

Results

Host plant and endophyte development was affected drastically as P concentration in the nutrient solution was raised from 4 to 500 μM . Changes in plant development due to differences in P regime became apparent about 3 weeks after planting. At this time cotyledons had dropped from plants under the most severe P stress, and leaf initiation was less advanced than in plants under higher P regimes. Plants were inoculated with *R. japonicum* at this time to minimize the effect of stored P reserves on nodulation.

Plants watered with a nutrient solution containing 4

Tab. 1. Shoot and nodule weights of 'Lancer' soybeans grown at different P concentrations in the presence or absence of *Glomus fasciculatus*. Means and standard errors were calculated from five replicates. Means of mycorrhizal and non-mycorrhizal plants significantly different by t-test are indicated with * (0.01 < p < 0.05) or ** (0.001 < p < 0.01).

Part	Treatment [P], μM	Dry weight, g	
		Mycorrhizal	Non-mycorrhizal
Nodule	500	0.470 ± 0.035	0.445 ± 0.029
	100	0.030 ± 0.003	0.019 ± 0.002*
	20	0.002 ± 0.0002	0.001 ± 0.0001**
	4	—	—
Shoot	500	5.28 ± 0.31	5.02 ± 0.32
	100	3.39 ± 0.24	2.86 ± 0.17
	20	1.28 ± 0.06	0.88 ± 0.03**
	4	0.88 ± 0.06	0.74 ± 0.05**

Tab. 2. Phosphorus content of shoots and nodules in 'Lancer' soybeans grown at different P concentrations in the presence or absence of *Glomus fasciculatus*. Means and standard errors were calculated from five replications. Means of mycorrhizal and non-mycorrhizal plants significantly different by t-test are indicated with *($0.01 < p < 0.05$) or **($0.01 < p < 0.01$).

Parameter	Treatment [P], μM	Phosphorus content			
		Mycorrhizal		Non-mycorrhizal	
		Shoot	Nodule	Shoot	Nodule
P, %	500	0.32 \pm 0.01	0.49 \pm 0.01	0.34 \pm 0.001	0.52 \pm 0.01
	100	0.120 \pm 0.004	0.42 \pm 0.04	0.110 \pm 0.004	0.46 \pm 0.05
	20	0.064 \pm 0.005	0.15 \pm 0.02	0.062 \pm 0.004	0.17 \pm 0.02
	4	0.050 \pm 0.005	—	0.054 \pm 0.004	—
Total P, mg	500	17.1 \pm 1.1	2.29 \pm 0.05	17.2 \pm 0.3	2.32 \pm 0.07
	100	3.99 \pm 0.3	0.12 \pm 0.01	3.12 \pm 0.30**	0.08 \pm 0.01**
	20	0.83 \pm 0.08	0.04 \pm 0.004	0.54 \pm 0.04**	0.02 \pm 0.003**
	4	0.52 \pm 0.06	—	0.44 \pm 0.03**	—

Tab. 3. Hydrogen evolution and acetylene reduction by nodulated 'Lancer' soybeans grown at different P concentrations in the presence or absence of *Glomus fasciculatus*. Means and standard errors were calculated from five replications.

Treatment [P], μM	Specific nodule activity, $\mu mol h^{-1} (g \text{ nodule dry wt.})^{-1}$			
	Mycorrhizal		Non-mycorrhizal	
	H ₂ evolution	C ₂ H ₂ reduction	H ₂ evolution	C ₂ H ₂ reduction
500	31.2 \pm 2.0	42.1 \pm 6.5	33.0 \pm 7.1	38.7 \pm 5.5
100	0.7 \pm 0.1	17.0 \pm 1.3	0.5 \pm 0.1	17.1 \pm 0.4
20	—	7.2 \pm 0.4	—	6.9 \pm 0.3
4	—	—	—	—

μM P were not nodulated 30 days after inoculation with *R. japonicum*. When the P concentration of the solution was increased 25-fold (20 to 500 μM), an over 200-fold increase in nodule weight resulted in VAM plants (Tab. 1). The comparable change in shoot weight was only 4-fold. Differences in relative increases in shoot and nodule weights as a function of P nutrition were also

reflected by the P contents of these organs. Total nodule P increased 57-fold following a 25-fold increase in P availability, while increases in P in the shoots were smaller and approximated the increase in P of the nutrient solution (Tab. 2). Per cent P was higher in shoots than in the nodules and did not show the wide variation with P availability as did total P.

Differences in VAM and non-VAM plants were not significant ($p > 0.05$) in any of the parameters measured at the highest P regime, where fungal infection was slight (Fig. 1). Nodule and shoot weights and total P were significantly higher in VAM plants than in non-VAM plants at the lower P regimes (Tabs. 1 and 2). Fungal infection increased as P concentration decreased from 500 to 20 μM but receded at the lowest (4 μM) P concentration (Fig. 1).

Nodule activity (C₂H₂ reduction) increased logarithmically with increasing P availability (Fig. 2). Hydrogen evolution was not detectable at the lowest P concentration (20 μM) at which nodulation occurred. The limit of resolution of the instrument was 1 μM H₂. Differences in C₂H₂ reduction and H₂ evolution on a per plant basis were significant ($P < 0.01$) for VAM and non-VAM associations except at the highest P concentration. Specific nodule activity (nodule activity/nodule mass) however, did not differ significantly ($p > 0.05$) in associations infected or not infected by *G. fasciculatus* but

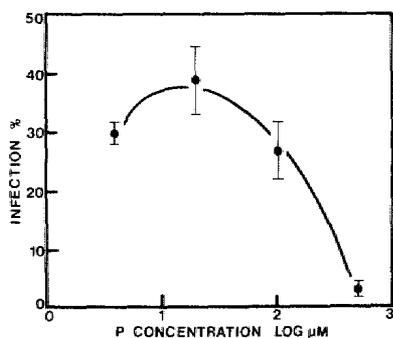


Fig. 1. Infection of 50-day-old 'Lancer' soybeans by *Glomus fasciculatus*. Plants were grown in a rooting medium consisting of perlite and nutrient solutions of different P concentrations. Data were recorded as the mean \pm SE of five replicates. Computation of percentage infection is given under Materials and methods.

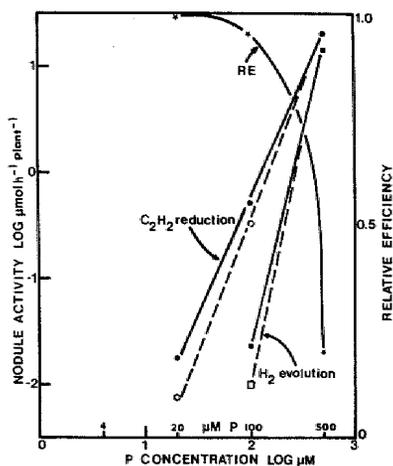


Fig. 2. Nodule activity in 50-day-old 'Lancer' soybeans. Plants were grown as in Fig. 1. Plants were inoculated or not inoculated with the VAM fungus *Glomus fasciculatus* at planting. All plants were inoculated with *Rhizobium japonicum* 20 days after planting. Data were recorded as means of five replicates. Differences in C_2H_2 reduction and H_2 evolution between VAM and non-VAM plants were not significant ($p > 0.05$) at the highest P concentration and significant ($p < 0.01$) at lower P concentrations. (●—●): C_2H_2 reduction, +VAM; (○---○): C_2H_2 reduction, -VAM; (■—■): H_2 evolution, +VAM; (□---□): H_2 evolution, -VAM; (*—*): RE, +VAM.

decreased with increasing P stress (Tab. 3). The RE increased with increasing P stress (Fig. 2). Differences in RE in VAM and non-VAM plants were not significant ($p > 0.05$). Enhancement by VAM fungi of all parameters measured was highest in plants of the 20 μM P-treatment (Tab. 4) coinciding with the highest level of VAM fungal infection (Fig. 1).

Tab. 4. Enhancement (%) of host plant and nodule parameters by *Glomus fasciculatus* in 'Lancer' soybeans. Enhancement of parameters was computed as $[1 - (\text{non-VAM value}/\text{VAM value})] \times 100$.

Parameter	Treatment, μM P			
	500	100	20	4
Shoot: weight	4	16	31	16
P content	1	22	34	15
Nodule: weight	5	37	50	—
P content	1	38	52	—
C_2H_2 reduction	3	31	50	—

Discussion

The main contribution of VAM fungi to the host plant in P-deficient soils is to reach and translocate through their extracortical hyphae relatively immobile nutrient ions which would otherwise not be available to the host (Mosse 1973). The complex disequilibrium (Helyar and Munns 1975) between available P in the soil solution and bound P compounds in the soil-fertilizer system makes precise predictions of P availability in pot cultures unlikely over a prolonged period of time. In the present experiment the soil solution was therefore replaced by nutrient solutions of known concentrations. This system contained P concentrations which fluctuated daily between a known replenishment level and a 24-h depletion level and provided day-to-day control over P availability to the VAM association. It was demonstrated that infection of the host plant by the fungal symbiont was dependent on and could be controlled by the amount of mobile, soluble P in the rooting medium, and that functions of the association's plant and bacterial components were affected by the extent of infection. Thus, an artificial growth medium containing no bound P and soluble P in suitable concentrations could be substituted for sterilized soil-fertilizer systems. Such an artificial growth medium is advantageous in experimentation where repeatability is of interest, as it eliminates variables introduced by the use of different soil types and their interactions (Bielecki 1973, Wilson 1968) with solid P fertilizers.

Response of soybeans in pot cultures to P differs with cultivar (Howell and Bernard 1961), but a P concentration equivalent to 0.25-strength Johnson's solution (0.5 mM P) appears to be optimal for growth (Howell 1954) under conditions of P supply defined by daily replenishment-depletion regimes. Only traces of VAM fungal infection were noted at such a high P regime (Fig. 1). Infection increased to a maximum with a 25-fold decrease (500 to 20 μM) in P concentration. Further P stress (4 μM P) apparently exceeded the host plant's capability to effectively support endophytes, as VAM fungal infection declined from the level attained at 20 μM P (Fig. 1) and as nodulation did not occur (Tab. 1).

Nodulation was more severely inhibited by P stress than host-plant development (Tab. 1). At least three causes may be suggested to explain this effect. One is a proportionately lower allocation of P to nodules than to other host tissue with increasing P stress. Another is a decreased supply of photosynthate from leaves stunted due to lack of P. Lower rates of photosynthesis have been shown to have an adverse effect on N_2 fixation (Bethlenfalvay and Phillips 1977b). Finally, lack of P may induce N stress in associations relying on atmospheric N by impairing N_2 fixation (De Mooy *et al.* 1973). The adverse effect of P stress on nodulation may be due to impaired development of nodule (host plant) tissue or to increased rhizobial activity. The extremely high P requirement of rhizobia (Bergersen 1971, Daft

1978, De Mooy *et al.* 1973) suggests the latter. The present findings, which show a marked decrease in specific nodule activity with decreasing P availability (Tab. 3), support this view: the decline in C_2H_2 reduction, a property of the bacteroids, was six times greater than the reduction in nodule mass over the P gradient used. Increase in RE (Fig. 2) with decreasing nodule P content (Tab. 2) may indicate a shift in electron allocation by nitrogenase to reduce N_2 to NH_4^+ rather than H^+ to H_2 . Alternatively, an uptake hydrogenase may be activated at low levels of P availability. Such increases in RE and hydrogenase activity have been found in N_2 -fixing associations under conditions when the availability of photosynthate was limiting N_2 fixation (Bethlenfalvai and Phillips 1979).

It is apparent from this study that a more thorough permeation of the available rooting medium by VAM fungi, as compared to non-VAM roots, resulted in enhanced P uptake by the association at low levels of P availability. Thus the advantage of greater positional availability of P to freely ramifying VAM roots under field conditions appears to be valid also under confined conditions in pot cultures when P is limiting. Availability of P affected N_2 fixation more severely than host plant development. Absolute nodule activity was more sensitive to a lack of P than nodule mass, thus indicating that the primary effect of P stress was on the bacteroids.

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