

## The *Glycine-Glomus-Bradyrhizobium* symbiosis. VIII. Phosphorus-use efficiency of CO<sub>2</sub> and N<sub>2</sub> fixation in mycorrhizal soybean

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Brown, M. S., Thamsurakul, S. and Bethlenfalvay, G. J. 1988. The *Glycine-Glomus-Bradyrhizobium* symbiosis. VIII. Phosphorus-use efficiency of CO<sub>2</sub> and N<sub>2</sub> fixation in mycorrhizal soybean. – *Physiol. Plant.* 74: 159–163.

Soybean [*Glycine max* (L.) Merr. cv. Hobbit] plants nodulated by *Bradyrhizobium japonicum* strain USDA 110 were grown in pot cultures in severely P- and N-deficient soil and either colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe or fertilized with a high (HP) or low (LP) level of KH<sub>2</sub>PO<sub>4</sub> (0.6 or 0.3 mM, respectively). After 7 weeks of growth, nodule and chloroplast activities (C<sub>2</sub>H<sub>4</sub> reduction and CO<sub>2</sub> exchange rate) were determined. Photosynthetic P-use efficiencies were calculated as mg CO<sub>2</sub> reduced per g of leaf or nodule P per plant. The P-use efficiency of CO<sub>2</sub> fixation was significantly higher in VAM than in HP plants, while that of nitrogenase activity was lower. The LP plants were intermediate in both respects. The ratio of nodule to chloroplast activity [mol C<sub>2</sub>H<sub>4</sub> reduced (mol CO<sub>2</sub> fixed)<sup>-1</sup>] was highest in HP and lowest in VAM plants. Root colonization by the VAM fungus significantly increased nodule number and dry weight and reduced nodule specific mass and activity in comparison to HP plants. In spite of lower nodule activity, VAM plants were significantly larger and had higher N concentrations than the HP plants. The results suggest nonnutritional, VAM-elicited and host-mediated effects on the symbiotic functions of the legume association.

**Key words** – *Bradyrhizobium japonicum*, *Glomus mosseae*, *Glycine max*, mycorrhiza, nitrogen fixation, nitrogen nutrition, nodule activity, phosphorus nutrition, photosynthesis, soybean, vesicular arbuscular mycorrhiza.

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### Introduction

Vesicular-arbuscular mycorrhizal (VAM) fungi enhance the activities of the chloroplast or the root-nodule bacteroid in reducing CO<sub>2</sub> or N<sub>2</sub> when P in the growth medium is limiting. This, and generally improved P nutrition, is a response by the host plant to relief from P limitation mediated by the fungus, and is an important effect of the plant-fungus symbiosis. To accentuate this effect, much of the experimentation with VAM fungi was conducted in the past utilizing non VAM controls grown under P limitation. The often dramatic effects in growth enhancement led to the widely held view that

the VAM fungus is essentially a biological P fertilizer (Aczon-Aguilar et al. 1979, Barca and Aczon-Aguilar 1983), and to an interpretation of the VAM effect in terms of P nutrition (Schönbeck 1987). More recent comparisons between VAM plants and P-sufficient non-VAM plants show nonnutritional effects of the VAM fungus on plant and nodule response to colonization (Bayne and Bethlenfalvay 1987). Since some of these differences were in the efficiency with which P is utilized (Brown and Bethlenfalvay 1988), it has become likely that the fungus elicits responses from its host in addition to those due to improved P nutrition.

We designed the present experiment to compare the

effects of P input via a VAM fungus or two levels of P fertilization on plant growth, chloroplast activity, nodule activity, and the efficiency with which the CO<sub>2</sub>- or N<sub>2</sub>-fixing processes of VAM or non-VAM plants utilize P. The objective of the experiment was to determine whether plant response to P input was modified by VAM colonization separately from, or because of P availability.

**Abbreviations** – CER, CO<sub>2</sub> exchange rate; HP, high phosphorus; LP, low phosphorus; VAM, vesicular-arbuscular mycorrhizal.

## Materials and methods

### Experimental design and statistics

The experiment had a completely randomized design with 3 treatments and 6 replications. The treatments were P fertilization either via a VAM fungus or through application of KH<sub>2</sub>PO<sub>4</sub> at a high (HP, 0.6 mM) or low (LP, 0.3 mM) level. The parameters of interest were evaluated for statistically significant differences between the means by Duncan's multiple range test at the 5% level. Certain differences of interest were evaluated in 2-way treatment comparisons by Student's *t*-test to show significance levels close to, but above, 5%.

### Biological materials

Soybeans [*Glycine max* (L.) Merr. cv. Hobbit] were germinated for 2 days at 30°C, selected for uniformity, and planted in 1.5-l pots with or without an inoculum of the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe. The fungal inoculum consisted of 100 ml soil containing 900 ± 50 sporocarps with 1 to 5 spores per sporocarp. It was mixed into the VAM-plant growth medium uniformly before planting. An inoculum of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110, was applied to all seedlings at planting. The inoculum consisted of 10 ml of the bacterial suspension (10<sup>8</sup> cells ml<sup>-1</sup>) in yeast-mannitol broth. All plants received a wash of the VAM inoculum free of VAM-fungal propagules (43 µm screen) to equalize the microbiota of the VAM and non-VAM treatments.

### Growth conditions

Plants were grown in a greenhouse for 6 weeks, March to April, at Albany, CA, USA. Automatic control systems were operating at temperatures above 25°C and below 18°C, minimizing day-to-day variations. Day-length was extended to 16 h by General Electric 1000 W metal halide lamps mounted vertically in parabolic reflectors. Lamps provided supplementary photosynthetic photon flux density of 500 µmol m<sup>-2</sup> s<sup>-1</sup> at the soil surface level. The growth medium was a Balcom series

(Yolo County, CA, USA) heavy silt loam (Typic Xerorthent) of pH 7.7 (paste) with NH<sub>4</sub>HCO<sub>3</sub>-extractable P and total N contents of 3.3 µg and 7.0 mg (g soil)<sup>-1</sup>, respectively. The soil had a sand:silt:clay content of 20.5:55.6:23.9% (v/v), and was mixed with fine sand (2:1, v/v), sterilized by autoclaving, potted, wetted from below and allowed to stand for 2 weeks prior to planting.

Pots were watered to saturation at planting, and a nutrient solution of 1.0 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.0 mM K<sub>2</sub>SO<sub>4</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.25 mM MgSO<sub>4</sub> and micronutrients at one-quarter strength Johnson's solution (Bethlenfalvay et al. 1982) was applied at day 7 and 14 after planting. Nitrogen and P were included at low concentrations to stimulate nodulation. After 21 days of growth, when all plants were nodulated and the roots of VAM plants were 30 to 40% colonized (determined on extra plants), nutrient solutions were applied twice weekly. At this time, a solution without P was used for the VAM plants and with 0.3 or 0.6 mM P for the LP or HP plants, respectively.

### Assays

Nodule activity was measured as C<sub>2</sub>H<sub>2</sub> reduction by gas chromatography (Bethlenfalvay et al. 1982) and CO<sub>2</sub> exchange rate (CER) of the entire shoot using an infrared gas analysis apparatus described previously (Bayne et al. 1984). Percentage of VAM-fungal colonization and tissue concentrations of N and P were determined by standard methods (Bayne et al. 1984).

## Results

The general growth pattern in plants of the 3 treatments was not consistent with differences in tissue nutrient concentrations. The VAM plants had the greatest and the LP plants the smallest dry weights, while the sequence of root/leaf ratios was the opposite (Tab. 1A). Since the root/leaf ratio is often an indicator of nutrient availability (Wareing and Patrick 1975), the low ratio in VAM plants could be construed as a sign of facilitated access to P relative to the non-VAM plants. This reverse relationship between the root/leaf ratio and nutrients was clearly reflected in the HP and LP plants (Tab. 1A–D). The VAM plants, on the other hand, had the lowest root/leaf ratio, but the roots and leaves had P concentrations similar to the HP plants, and supplied the leaves with a greater amount of N than were the roots of the P-fertilized plants. Because the leaves of VAM plants were largest of the 3 treatments, the lower P concentration is the result of distribution of this P throughout a greater mass of tissue. This P concentration, as well as that of the LP plant leaves, was near the critical level of 0.15% (de Mooy et al. 1973), yet the VAM plant leaves had a much greater total carbon uptake than the LP plant leaves, as measured by both CER and dry weight.

Tab. 1. Whole plant parameters of nodulated soybean under symbiotic or nonsymbiotic P regimes. Symbiotic plants were colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae*. Nonsymbiotic plants received 0.3 mM P (LP) or 0.6 mM P (HP) in a complete (but N-free) nutrient solution. Numbers are means of 6 replications and are significantly different ( $P < 0.05$ , Duncan's multiple range test) when followed by different letters. \*Two-way comparisons of CER (by Student's *t* test) between VAM and LP, VAM and HP, LP and HP plants were at the 8, 41, and 6% significance levels, respectively.

Parameter	Treatment		
	VAM	LP	HP
<b>A. Total plant</b>			
Dry weight (g)	7.6 a	4.5 c	5.7 b
Root/leaf ratio	0.60 c	0.96 a	0.80 b
<b>B. Leaf</b>			
Dry weight (g)	3.4 a	1.7 c	2.3 b
Area (cm <sup>2</sup> )	815 a	397 c	590 b
P concentration (mg g <sup>-1</sup> )	1.7 b	1.7 b	2.6 a
P content (mg plant <sup>-1</sup> )	5.8 a	2.9 b	6.0 a
N concentration (mg g <sup>-1</sup> )	36.8 a	31.0 b	31.8 b
N content (mg plant <sup>-1</sup> )	125 a	52 c	73 b
Specific CER* ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	6.7	5.7	6.8
Total CER ( $\mu\text{mol CO}_2 \text{ plant}^{-1} \text{ s}^{-1}$ )	0.55 a	0.22 c	0.40 b
<b>C. Root</b>			
Dry weight (g)	2.0 a	1.7 a	1.9 a
P concentration (mg g <sup>-1</sup> )	1.8 a	1.4 b	2.1 a
P content (mg plant <sup>-1</sup> )	3.6 a	2.4 b	4.0 a
N concentration (mg g <sup>-1</sup> )	23.1 a	17.3 b	17.5 b
N content (mg plant <sup>-1</sup> )	46.2 a	29.4 b	33.2 b
VAM colonization (%)	77	-	-
<b>D. Nodule</b>			
Dry weight (g)	0.34 a	0.09 c	0.21 b
Number	97 a	33 b	30 b
Specific mass (mg nodule <sup>-1</sup> )	3.5 b	2.7 c	7.0 a
P concentration (mg g <sup>-1</sup> )	0.32 c	0.49 b	0.58 a
P content (mg)	0.11 a	0.04 b	0.12 a
Activity ( $\mu\text{mol C}_2\text{H}_2 \text{ plant}^{-1} \text{ h}^{-1}$ )	9.0 b	4.6 c	22.8 a

Leaf area and dry weight were largest in the VAM and smallest in the LP plants while their P concentrations were not significantly different ( $P > 0.05$ ). The intermediate-sized HP plants, however, contained appreciably more P (Tab. 1B). The same differences as in leaf development were evident also in total plant CER, but not in CER per unit leaf area. The CERs of VAM and HP plants were not significantly different ( $P > 0.41$ ) although the P concentration of the latter was significantly higher. These CER vs. P-concentration relationships are summarized in terms of photosynthetic P-use efficiency (sensu Brown and Bethlenfalvai 1988) which was highest in the VAM and lowest in the HP plants (Fig. 1). Phosphorus concentrations in the leaves of all

plants were in a range generally considered to be less than sufficient (0.16 to 0.26%; de Mooy et al. 1973).

Although root masses did not differ significantly among treatments, P concentration was significantly lower in the LP plant roots and N concentration higher in the VAM plant roots than in the other 2 treatments (Tab. 1C). Interestingly, root P concentrations were not reflected in nodule P concentrations, which were highest in the HP and lowest in the VAM plants (Tab. 1D). Nodule development and nodule activity, both sensitive to P availability, were related to root and nodule P concentrations in the HP and LP plants but not in the VAM plants, where nodule activity was intermediate and nodule mass largest even though P concentration was least. The P-use efficiency of nodule activity [ $\text{mg C}_2\text{H}_2 \text{ reduced (g nodule P)}^{-1}$ ], and the ratio of nodule to chloroplast activity [ $\text{mmol of C}_2\text{H}_2 \text{ reduced (mol of CO}_2 \text{ fixed)}^{-1}$ ] were significantly lower in VAM than in HP plants but not significantly different ( $P > 0.05$ ) from the values in LP plants (Fig. 2). These observations suggested that the processes of nodule development and activity in nonVAM plants are closely related to P availability, while in VAM plants P dependence is modified by other factors. This conclusion is supported by the finding that the number of nodules in VAM plants was 3 times that in HP plants.

Photosynthetic N-use efficiency (sensu Sage and Pearcy 1987) did not differ with treatment, although N concentration in the VAM leaves was significantly higher than in the non-VAM treatments (Tab. 1B; Fig.

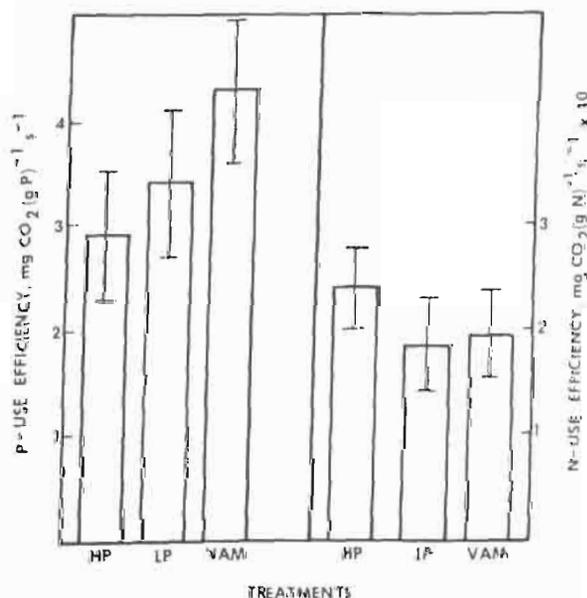


Fig. 1. Photosynthetic nutrient-use efficiency. Soybean plants were either colonized by the VAM fungus *Glomus mosseae* or fertilized with a high (HP) or low (LP) level of  $\text{KH}_2\text{PO}_4$  (0.6 or 0.3 mM, respectively). Rates of net  $\text{CO}_2$  exchange and leaf nutrient concentrations were determined on a whole-plant basis. Data are expressed as the means of 6 replications with 95% confidence intervals.

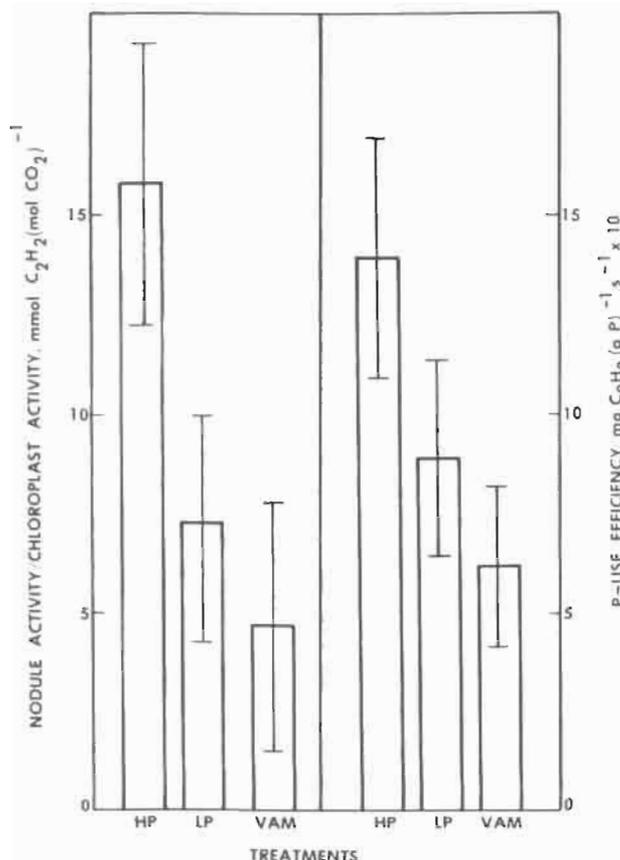


Fig. 2. Efficiency of nodule activity. Reduction of  $C_2H_2$  relative to net  $CO_2$  uptake or the amount of nodule P was calculated on a whole plant basis. Notations and statistics as in Fig. 1.

2). Apparently, the high rate of nodule activity of the HP plants (Tab. 1D) did not translate into commensurately high root or leaf N concentrations. However, the relationship between nodule and chloroplast activities, in terms of  $C_2H_2$  reduced vs net  $CO_2$  fixed per plant, was least efficient in VAM plants and most efficient in the HP plants.

## Discussion

Nonnutritional VAM effects can be demonstrated by comparing form and function in VAM plants with non-VAM plants of differing nutritional status (Abbott and Robson 1984, Pacovsky et al. 1986). Physiological functions of particular interest for such comparisons are  $CO_2$  and  $N_2$  fixation, because of their acute dependence on P and because both development and activity of leaves and nodules are modified by VAM colonization, even in comparison with nutritionally or developmentally 'equivalent' non-VAM plants (Brown and Bethlenfalvy 1987).

One good criterion for equivalence may be the efficiency with which symbiotic or non-symbiotic plants utilize nutrients to achieve a comparable level of activity. To provide valid comparisons for P- and N-use efficiency, leaf nutrient concentrations must be low, since at high levels much of the P and N may be stored in inactive forms in the vacuole or as storage protein without affecting the activity to be observed. The condition of low concentrations was met in the present experiment (Tab. 1B). The significantly higher P-use efficiency of our VAM plants relative to the HP plants (Figs 1 and 2) confirms our previous results (Brown and Bethlenfalvy 1988) obtained from individual leaf measurements. The influence of the VAM fungus on P utilization is indicated by the lower P-use efficiency and much lower CER of the LP plants in comparison to the VAM plants, although both had the same leaf P concentration (Tab. 1B). This relationship does not become evident in comparisons of VAM plants with severely P-deficient non-VAM plants, because of the higher P concentrations in the larger VAM plants (Stribley et al. 1980). The P concentration in tissues of non-VAM plants relative to that in VAM plants differs with the timing, kind and amount of P input by fertilization or by the endophyte. This relationship may differ with time as determined by the activities of the tissues involved and by the export of P from older to younger organs under P deficiency. The lower leaf P concentration of our VAM plants vs the HP plants cannot be explained by dilution due to size, since it was identical to the P concentration of the much smaller LP plants. Nitrogen-use efficiency, which was shown to be affected by the form of N input (Brown and Bethlenfalvy 1988), did not differ significantly between treatments ( $P > 0.05$ ), probably because of the subcritical (de Mooy et al. 1973) levels of N in all plants (Tab. 1, Fig. 2).

Unlike CER, nodule P-use efficiency (Fig. 1) was proportional to nodule P concentration, and was lowest in the VAM plants. This observation is not in consonance with reports of beneficial VAM effects on nodule activity under severe P limitation, and is reminiscent of antagonistic effects between VAM fungi and rhizobia in nutritionally comparable hosts (Bethlenfalvy et al. 1985). The observation of divergence in the VAM effect on the P-use efficiencies of the  $CO_2$ - and  $N_2$ -fixing processes is new and the molecular mechanisms are not known. We may speculate at this time that products of plant response elicited by VAM fungal colonization affect structure or function (or both) of chloroplast and bacteroid differently. This would be analogous to the synthesis and storage of such products after exposure to other microorganisms (Darvill and Albersheim 1984). Photosynthesis is also enhanced by other endophytic fungi, the Clavicipitaceae (Siegel et al. 1987), while a direct example of secondary metabolite effects on symbiotic activity was recently reported by Kapulnik et al. (1987) in *Medicago* root nodules. The P-independent differences in our nodule parameters (Tab. 1D) also

suggest non-nutritional, host-mediated interactions between the root endophytes.

**Acknowledgement** – The experiment was conducted under CRIS-WU 5325-12230-001-00D, in collaboration between ARS and USAID.

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