

# The *Glycine-Glomus-Rhizobium* Symbiosis<sup>1</sup>

## VII. PHOTOSYNTHETIC NUTRIENT-USE EFFICIENCY IN NODULATED, MYCORRHIZAL SOYBEANS

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### ABSTRACT

Four consecutive trifoliolate leaves of 56-day-old symbiotic or nonsymbiotic soybean plants were evaluated individually for CO<sub>2</sub> exchange rates (CER), leaf area and dry weight, and leaf N, P, and starch concentrations. Plants had been inoculated with the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* and *Rhizobium japonicum*, with either of the endophytes alone, or with neither at time of planting. Plants lacking one or both endophytes received N and/or P fertilizers to produce plants of equal total leaf dry weight in all four treatments. Photosynthetic P-use efficiency (CER per unit leaf P) was higher in the leaves of VAM plants than in P-fertilized plants regardless of the N source (N<sub>2</sub> fixation or combined N). Photosynthetic N-use efficiency was also higher in VAM than in non-VAM plants, but it was affected by the N source, with higher CER in the nodulated plants. The greatest differences in CER, starch accumulation and leaf area were found between the nonsymbiotic plants and those with both endophytes. Statistical evaluations of leaf parameters for treatment or nutrient concentration (N and P) effects between the tripartite and the nonsymbiotic treatments showed significant changes in concentration of P, but not N, with decreasing leaf age. Both endophytes apparently enhance CO<sub>2</sub> fixation at N and/or P concentrations lower than those of the nonsymbiotic plants. The effects of the endophytes on CO<sub>2</sub> fixation were additive.

of the tripartite legume association (6), should be different in magnitude from that found in nonsymbiotic plants if the symbiotic N or P sources differ in C-sink activity from the nonsymbiotic ones.

Changes in the activity of a plant in providing a product relative to the utilization or availability of a necessary requirement affect the efficiency of its resource utilization. Efficiency has many facets. Generally, nutrient-use efficiency has been defined in terms of dry matter produced per unit of nutrient applied or absorbed (10, 11, 24). A more specialized aspect of nutrient-use efficiency relates to the activity of a specific plant process as a function of that nutrient in the tissue involved. Our use of the term photosynthetic nutrient-use efficiency *sensu* Sage and Percy (23) is defined as CER per unit of leaf N or P, and is particularly useful in comparing the nutrient-use potentials of symbiotic legumes relative to nonsymbiotic controls.

The purpose of this study was to investigate the effects of symbiotic and nonsymbiotic N and P supplies on CER in order to determine the response of CER to the presence or absence of the endophytes and to differences in leaf N and P concentrations resulting from the nature of the supply.

### MATERIALS AND METHODS

**Biological Materials.** Soybean (*Glycine max* [L.] Merr. cv Hobbit) plants were inoculated with the diazotrophic bacterium *Rhizobium japonicum* strain USDA 136 (abbreviated 'R') and/or with the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe ('G'). Plants without *R. japonicum* received N fertilizer ('N'), and those without *G. mosseae* received P fertilizer ('P'). A control set of nonsymbiotic plants received only the combined fertilizer ('PN'). Fertilizer regimes were adjusted to produce plants of the same weight when harvested after 56 to 59 d of growth. Details of soil, inocula, and growing conditions were described in a companion study (8).

**Experimental Design.** The design was a 2 × 2 × 4 factorial, with N source (R or N), P source (G or P), and leaf position (four consecutive leaves) as the three factors. From each of the 8 replicate plants of each treatment combination (GR, GN, PR, PN), the second through fifth trifoliolate leaves were used as soon as the sixth leaf was fully expanded.

**Measurements and Analyses.** Carbon dioxide exchange rate, area, fresh and dry weights, and N, P, and starch concentrations were determined for each of the leaves. By averaging measurements for leaves at each position, the effect of leaf age could be isolated from other experimental variables. To determine means, the CER of each leaf of a given position was multiplied by its leaf area, the products for all replicates were added, and the sum was divided by the total leaf area of the eight replicates. Similar calculations were made to arrive at mean N, P, and starch concentrations.

A LI-COR LI-6000 Portable Photosynthesis System was used

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The microsymbionts of the legume root, the VAM<sup>2</sup> fungus and the diazotrophic bacterium *Rhizobium*, may be regarded as the primary sources of P and N to legumes growing in soils deficient in plant-available forms of those nutrients (4). The sink demand resulting from the growth and metabolic activity of these root endophytes has been associated with a compensatory increase in activity by the C source of the association, the chloroplast (21). Given these premises, endophyte effects on source activity for C and sink demand for N and P by leaves should be measurable in terms of CER and N and P concentrations, and these parameters should be different from those found in nonsymbiotic plants of equivalent development. In particular, since the effects of sinks and sources on one another (especially in young annual plants) are inversely proportional to the distances separating them (28), there should be a shift in endophyte effects on CER in individual leaves with position along the stem. This change, as influenced by the complex three-way source-sink relationships

<sup>1</sup> A contribution of the Plant-Soil Symbiosis Group, Plant Development-Productivity Research Unit.

<sup>2</sup> Abbreviations: VAM, vesicular-arbuscular mycorrhizal; CER, carbon dioxide exchange rate; PNUE, photosynthetic nitrogen-use efficiency; PPUE, photosynthetic phosphorus-use efficiency.

to measure CER. Nutrient and starch determinations were made by standard methods (9). Nodule activity and VAM colonization were reported in a previous paper (8) and are not a material part of this report.

## RESULTS

The photosynthetic nutrient-use efficiency was significantly higher in most leaves of VAM plants than in those of nonVAM plants (Fig. 1). The presence or absence of nodulation in nonVAM plants did not influence PPUE while PNUE was significantly higher in the leaves of nodulated than in non-nodulated nonVAM plants. In VAM plants, CER was not affected by the nature of the N source in most leaves. Both PPUE and PNUE were greater in the presence of the VAM fungus in almost all cases, while nodulation increased only PNUE in the nonVAM plants. Both PNUE and PPUE were significantly greater in all leaves of the nodulated VAM (GR) plants than in the nonsymbiotic (PN) plants.

The rates of CO<sub>2</sub> exchange were significantly greater in all except the youngest measured (fifth) leaves of both nodulated and non-nodulated VAM plants than in those of the nonsymbiotic plants (Table I). In nodulated non-VAM plants (PR) only the oldest measured leaf (second) had higher CER than the nonsymbiotic comparison. In the youngest measured leaf, CERs of all treatments were not significantly different. Phosphorus concentrations of all VAM-plant leaves were significantly lower than those of the leaves of nonsymbiotic plants. The same was true only for the oldest leaf of the nodulated nonVAM plants. Nitrogen concentrations of all leaves of all three symbiotic treatments were lower than those of the nonsymbiotic plants. Such differences in nutrient content and CER did not affect total leaf or plant dry weight as determined by Duncan's multiple range test ( $P > 0.05$ ). Fresh weight, however, was influenced by nodulation (GR = PR > GN = PN), and leaf area by VAM colonization (GR = GN > PR = PN) (Table II).

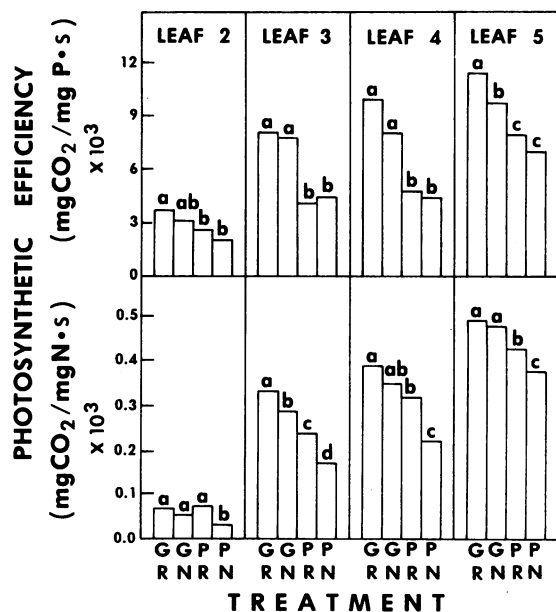


FIG. 1. Photosynthetic nutrient-use efficiency in symbiotic and nonsymbiotic plants based on leaf P and N contents. CER were calculated for trifoliolate leaves 2 through 5 (means of 8 replicates) relative to total P or N contents of the individual leaves. Differences between treatments (Glomus + Rhizobium, Glomus + N, P + Rhizobium, and P + N) were determined by Duncan's multiple range test for each leaf. Values marked by the same letter are not significantly different ( $P < 0.05$ ).

Table I. Comparisons between Leaf Parameters in Symbiotic Relative to Nonsymbiotic Plants

Numbers show the percentage of enhancement or inhibition (-) in CER and P and N concentrations in trifoliolate leaves 2 through 5 of symbiotic (Glomus + Rhizobium, Glomus + N, P + Rhizobium) versus nonsymbiotic (P + N) soybean plants. Treatment comparisons (symbiotic versus nonsymbiotic) were evaluated by *t*-test (NS, >5%; \*, <5%; \*\*, <0.1%; \*\*\*, <0.01%).

Leaf No.	Change (%)		
	GR vs. PN	GN vs. PN	PR vs. PN
	<i>CER</i>		
2	24.7**	210.7*	30.2**
3	32.6***	33.5**	-3.1 NS
4	34.4**	24.9**	10.7 NS
5	5.8 NS	9.6 NS	-0.4 NS
	<i>P concentration</i>		
2	-26.1***	-19.8**	17.1*
3	-29.7***	-26.6***	7.0 NS
4	-27.6***	-17.9**	-8.3 NS
5	-37.9***	-30.8***	-5.5 NS
	<i>N concentration</i>		
2	-38.5***	-31.4***	-33.0***
3	-34.1***	-22.8***	-33.2***
4	-26.3***	-15.0***	-26.1***
5	-22.3***	-14.5***	-15.6***

Table II. Leaf Weights and Areas of Symbiotic and Nonsymbiotic Soybean Plants

The values show means (8 replications) of total plant leaf weights and areas. Treatment (Glomus + Rhizobium, Glomus + N, P + Rhizobium, P + N) comparisons were made by Duncan's multiple range test. Numbers followed by the same letter were not significantly different ( $P < 0.05$ ).

Leaf parameter	Treatment			
	GR	GN	PR	PN
Dry weight (g)	3.0 a	3.0 a	2.8 a	2.7 a
Fresh weight (g)	6.9 a	7.5 b	6.6 a	7.5 b
Area (cm <sup>2</sup> )	314 a	305 a	292 b	272 b
Total plant dry weight (g)	8.0 a	9.0 a	7.8 a	8.3 a

Table III. Soybean Leaf N/P Ratios Supplied Symbiotically or Through Fertilization

Leaf numbers identify the 2 through 5 trifoliolate leaves in four treatments: Glomus + Rhizobium (GR), Glomus + N (GN), P + Rhizobium (PR), and P + N (PN). Numbers are calculated from percentages. Numbers followed by the same letter a, b within leaves; x, y; z within treatments are not significantly different ( $P > 0.05$ ) by Duncan's multiple range test.

Leaf No.	N/P ratio Treatment			
	GR	GN	PR	PN
2	23.3 bx	23.4 bx	15.8 cx	27.4 ax
3	24.5 bx	25.7 ax	16.2 cx	25.8 axy
4	22.4 abx	23.2 ax	15.2 cx	21.2 by
5	22.9 ax	22.8 ax	16.3 cx	18.4 bz

Nitrogen and P concentrations (Fig. 2) increased significantly ( $P \leq 0.05$ ) with increasing leaf number in the symbiotic plants. In the nonsymbiotic plants there was an increase only in P concentration. The N/P ratios (Table III) for all leaves of all VAM

plants were at least 65% above those considered to be normal for soybean plants near optimal growth (27), while those of non-VAM plants were 12% to 100% above the norm of 13.5. Thus, in all the leaves of all treatments, P rather than N appeared to be limiting. Suboptimal P concentrations in all plants were also indicated by the increase in P with leaf number, showing the export of P from older to younger leaves which is usually associated with P deficiency (22). The same was true for N concentrations, except in the nonsymbiotic plants (Fig. 2). The N/P ratio, often used as a diagnostic tool in preference to the concentrations themselves due to its stability through time or plant part sampled (18), was stable from leaf to leaf in the symbiotic plants but decreased significantly with decreasing leaf age in the nonsymbiotic ones (Table III). The N/P ratios of all leaves of the nodulated nonVAM plants were markedly lower than those of the other treatments.

The rates of CO<sub>2</sub> exchange increased linearly with leaf number as a function of leaf P and N concentrations in all four treatments (Fig. 3). In addition to nutrient reallocation from older to younger leaves, changes in activity associated with leaf ontogeny may also have influenced CER (25). Although plants of the different treatments were at the same state of development at harvest, nutrient effects due to treatment were highly significant (Table IV, Fig. 2) at the level of each leaf number; VAM plants maintained levels of CER comparable to or higher than those in non-VAM plants at lower P concentrations. A greater demand for N by nonsymbiotic plants than by symbiotic ones to produce comparable levels of CER was indicated by the differences in their N concentrations. Comparisons between treatments showed a significant interaction (ANOVA,  $P \leq 0.05$ ) between nutrition-related changes in CER and leaf position with regard to P (but not N) concentration (Fig. 3, Table IV). This interaction suggested increased PUE of the younger leaves in the nodulated VAM plant relative to its nonsymbiotic counterpart. Similar leaf area/nutrient concentration patterns (Fig. 4) indicated a relationship between endophyte effects on CER and leaf expansion. Leaf dry

weight was affected significantly by both treatment and nutrient content (Fig. 5), but a significant interaction was observed only for P content between tripartite and nonsymbiotic plants (Table IV). Starch concentrations were lowest in all leaves of the nonsymbiotic plants (Fig. 6). Treatment, CER, and N and P concentration each had significant effects on the shift of starch concentration from leaf to leaf, but the only significant interaction occurred between the tripartite and nonsymbiotic plants with P concentration as the independent variable (Table IV).

## DISCUSSION

Alteration of photosynthetic rates (15) or photosynthate partitioning (19) in relation to the presence of root endophytes has been related to sink demand by the endophytes (7, 21). Our data not only confirm the hypothesis that the symbiotic plant can make use of otherwise underutilized photosynthetic capability to satisfy endophyte demands, but also show that utilization of this additional capacity is dependent on the proximity of the C source to the symbiotic sink. This observation suggests that changes in CER in response to endophyte sink demand may become masked in larger plants if CER is measured on the whole plant rather than on each leaf.

While the effects of endophyte presence on CER were anticipated as part of our starting hypothesis, the relationship between CER and photosynthetic nutrient-use efficiency as a function of the N and P source were unexpected. The data (Fig. 1) show that, in general, the uptake of CO<sub>2</sub> per unit of leaf N or P was significantly greater in symbiotic than in nonsymbiotic plants. Although we cannot exclude the possibility of chemical signals resulting from the symbiotic condition (5), we consider it more probable that the enhancement of CER in VAM plants is the consequence of enhanced entry of CO<sub>2</sub> into the leaves and/or its more efficient assimilation by the leaves. Increased stomatal conductance (and thus, implicitly, CO<sub>2</sub> influx) in VAM plants is well documented (2, 3, 17, 29). The role of P in the process, however, remains controversial. Koide (20) reasoned that the mycorrhizal effect of stomatal resistance is mediated by plant P status, since P amendments to P-deficient plants had similar effects. Our results do not confirm this view. In our plants, the greater CER at lower nutrient concentrations in the presence of either or both microsymbionts than in nonsymbiotic plants suggests a "symbiosis effect" rather than a "VAM-mediated P effect." That this symbiosis effect was independent of nutrient level is shown by the greater CER per unit of leaf N in nodulated nonVAM plants than in nonsymbiotic ones despite similar N concentrations (Fig. 1, Table I). Similarly, non-nodulated VAM plants had greater CER relative to nonsymbiotic plants even though they were supplied equally with fertilizer N.

The establishment of the rhizobial symbiosis has not (to our knowledge) been connected to changes in leaf conductance as is the case with VAM plants (3). DeJong and Phillips (13) have, however, observed a positive relationship between CER and leaf nitrogen content as a consequence of greater N<sub>2</sub> fixation by more effective *Rhizobium* strains. Mechanisms other than the VAM fungus-mediated changes in plant water status (3) could therefore also be involved in the symbiosis effect. As possibilities, we suggest that the establishment of either microsymbiont affects nutrient uptake, partitioning, and/or utilization by all three symbionts of the legume association. Such mechanisms may act in combination, as suggested by some additive effects of both microsymbionts on CER (Fig. 1) and on other plant parameters (Figs. 2–6, Table IV) relative to the effects of either endophyte alone. The consistency of the N/P ratios in symbiotic relative to nonsymbiotic plants (Table III) also indicates a self-regulating mechanism for nutrient utilization within the symbiotic association. Other mechanisms affecting leaf conductance, and therefore CO<sub>2</sub> fixation (16), which may be influenced by the micro-

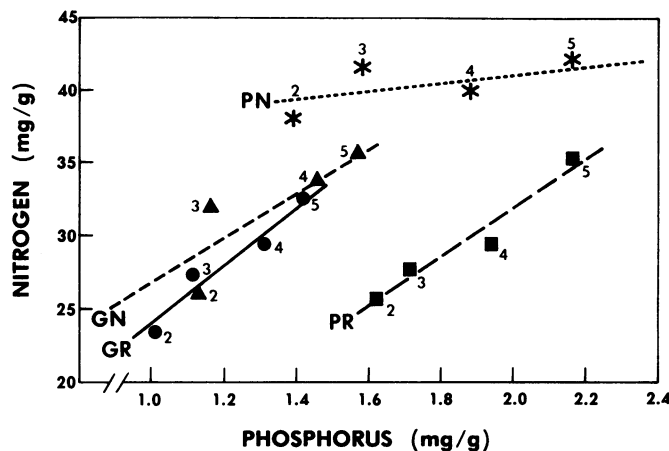


FIG. 2. Relationships between nutrient concentrations. Nitrogen concentrations were evaluated as a function of P concentration in the second through fifth trifoliolate leaves of soybean plants. Plants were grown symbiotically or with fertilizer amendments in four treatments: Glomus + Rhizobium (GR), Glomus + N (GN), P + Rhizobium (PR), P + N (PN). Points on the treatment-response lines are identified by leaf number. Slopes of the lines representing leaf-to-leaf changes in N as a function of P were significantly different ( $P < 0.05$ ) between nonsymbiotic (PN) and symbiotic (GR, GN, PR) treatments, but not among symbiotic treatments (Table IV). The significant interaction between treatment and P effects, i.e., the difference in the increase in N content with decreasing leaf age is interpreted as an improved capacity of the symbiotic plants to apportion nutrients.

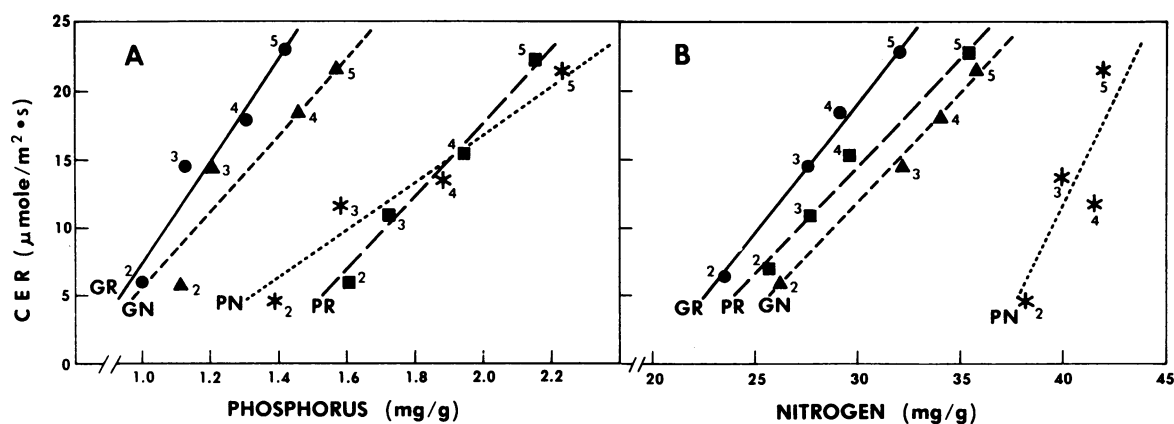


FIG. 3. Relationships between nutrient concentrations and CER. Leaf-by-leaf changes in CER as a function of P and N concentrations were evaluated in the second through fifth leaves of symbiotic and nonsymbiotic soybean plants. Annotations are as in Figure 2. A significant ( $P < 0.05$ ) interaction was found only for the CER versus P concentration relationship between the tripartite (GR) and nonsymbiotic (PN) treatments (Table IV). This interaction indicates a significantly greater response of CER to changing P concentration with decreasing leaf age in the tripartite versus nonsymbiotic plants. No such CER response was found with respect to N concentration.

Table IV. *Statistical Analysis of Nutritional Status and Nutrient Source Effects on Soybean Leaf Parameters*

Nutrients (P and N) were provided through fertilization or through colonization by the VAM fungus *G. mosseae* or Rhizobium. The resulting four treatments were: Glomus + Rhizobium, Glomus + N, P + Rhizobium, and P + N. The analysis evaluated the main effects of treatment, independent variables (leaf P or N concentrations or CER), and their interactions relative to selected leaf parameters (dependent variables). The changes in these parameters are reflected individually from leaf to leaf in Figures 2 through 6. Thus, this table represents a statistical summary for the response curves of these figures.

Variables		Significance level (%)			
Dependent	Independent	Treatment	Independent variable		Significant interaction <sup>a</sup>
N	P	0.1	0.1	0.1	PN vs. GR,GN,PR (Fig. 2)
CER	P	9.0	0.1	7.4	PN vs. GR (Fig. 3)
	N	5.8	0.1	66.1	
Dry weight	P	3.4	0.1	2.1	PN vs. GR (Fig. 5)
	N	1.9	0.1	30.7	
Area	P	10.9	0.1	4.6	PN vs. GR (Fig. 4)
	N	8.5	0.1	7.6	
Starch	P	0.1	0.1	9.0	PN vs. GR (Fig. 6)
	N	0.1	0.1	5.0	
	CER	0.1	0.1	0.1	

<sup>a</sup> Interactions between the effects of treatment and independent variable were significant (5%) only for the indicated treatment comparisons.

symbionts are K uptake (26) and abscisic acid levels (1).

Efficient nutrient utilization by the N- and P-deficient symbiotic plants relative to the N- and P-sufficient nonsymbiotic plants is shown by higher CERs in the former. The threshold of deficiency for youngest mature soybean leaves has been defined as  $<1.5$  mg P and  $<40$  mg N per g of leaf dry mass (12). Most leaves of the symbiotic plants were below and those of the nonsymbiotic plants above these values. If one may assume that under critical P deficiency the buffering effect of Pi release from the vacuole and the recycling of organic P on Pi concentrations in the cytosol are small, then the immediate availability of Pi for influx into the chloroplast to counterbalance the efflux of reduced C (phosphate translocator) (14) should determine the rate of CO<sub>2</sub> exchange. The mechanism for the greater PPUE of the P-deficient symbiotic plants in terms of the P availability is therefore obscure, suggesting other, symbiont-related modifications of the photosynthetic apparatus. Drastic effects of symbiotic sta-

tus on starch accumulation (Fig. 7) further show that not nutrient access, but symbiont-mediated metabolic changes may determine the rates of some CER-related processes in the leaf.

We observed symbiont-related changes in several leaf parameters of young soybean plants, based on a leaf-by-leaf analysis. Symbiotic plants had both greater photosynthetic activity and greater nutrient-use efficiency than nonsymbiotic plants grown at a symbiont-equivalent nutrient regime which produced equal leaf dry weights. The effects of VAM colonization and nodulation tended to be greater than those caused by one endophyte alone. The effects cannot be explained by nutrient availability. It is suggested that the endophytes influence nutrient uptake and utilization processes beyond the traditionally accepted mycorrhizal P and root nodule-N relationships.

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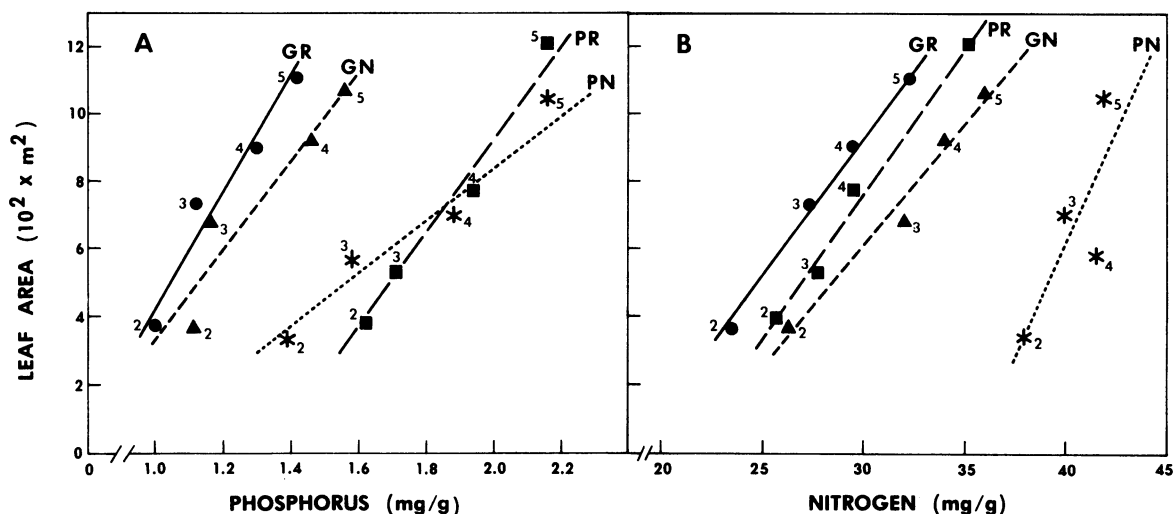


FIG. 4. Relationships between nutrient concentrations and leaf area. Annotations are as in Figure 2. Similarities in the response patterns between leaf area and CER (Fig. 3) with respect to treatments and leaf P and N concentrations indicate a close relationship between CER and leaf area responses to treatments.

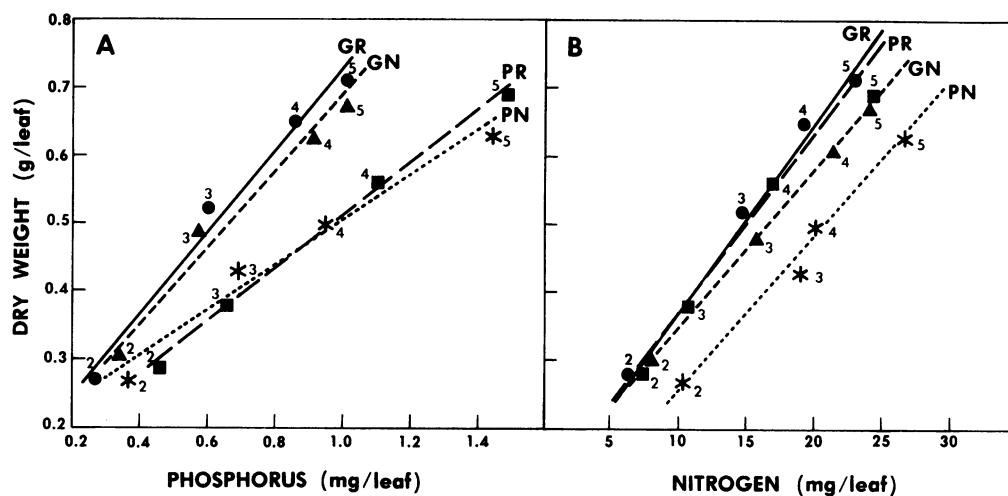


FIG. 5. Relationships between nutrient concentrations and leaf dry weight. Annotations are as in Figure 2. Leaf response to P concentrations was very similar in VAM plants (GR, GN) and in P fertilized plants (PR, PN). The interactions between P concentration and treatment was significant only for the GR versus PN comparison. The response pattern to N concentrations was the same for all treatments.

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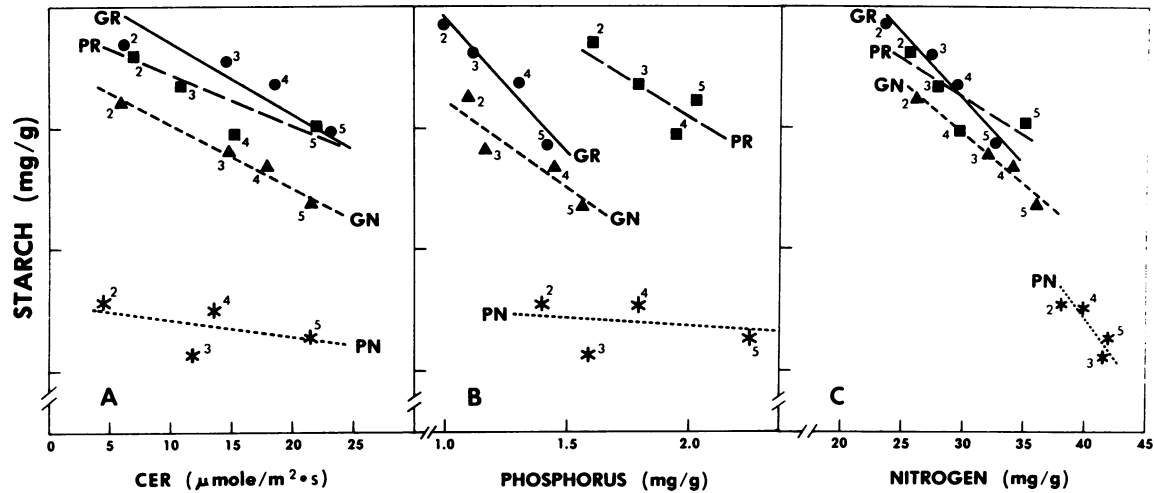


FIG. 6. Relationships between leaf starch and nutrient concentrations and CER. Annotations are as in Figure 2. Starch concentrations of four leaves of symbiotic and nonsymbiotic soybean plants were evaluated as a function of N and P concentrations and CER. The leaf-by-leaf response of starch accumulation to P concentration was significantly different ( $P < 0.05$ ) in symbiotic versus nonsymbiotic plants, as shown by the significant treatment versus P concentration interactions (Table IV).

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