

## Effect of Phosphorus on the Effectiveness of Strains of *Rhizobium japonicum*<sup>1</sup>

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### ABSTRACT

We examined the relationship between P nutrition of soybean [*Glycine max* (L.) Merr. cv. 'Davis'] and N<sub>2</sub> fixation by five strains of *Rhizobium japonicum* ranging from an ineffective (SM-5) to a highly effective (USDA 110) symbiosis with soybean. Phosphorus at 0, 50, 125, and 400 mg P kg<sup>-1</sup> soil (P<sub>0</sub>, P<sub>50</sub>, P<sub>125</sub>, P<sub>400</sub>) as Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>·H<sub>2</sub>O was applied to pots containing 2.7 kg of a P-fixing humoxic tropohumult free of *R. japonicum*. Treatments consisting of +N and five strains of *R. japonicum* and the four P treatments were arranged in a complete factorial replicated three times. With 400 mg P kg<sup>-1</sup> soil treatments ranked +N > USDA 110 > USDA 31 > USDA 123 > USDA 33 > SM-5 for shoot dry weight and accumulation of N in the shoot. There were no significant strain effects for shoot dry weight, shoot N or nodule activity at P<sub>0</sub>. Strain rankings were the same at the intermediate levels of P but differences were smaller. Although nodule number was somewhat enhanced by P, it bore no relationship to strain effectiveness and was not a factor limiting N<sub>2</sub> fixation at low P. Nodule dry weight and nitrogenase activities significantly increased with P additions. The concentrations of N and P in the shoot also increased with P additions. The concentrations of shoot P and N were higher in plants provided mineral N at the low and intermediate P levels than in the inoculated plants. The results show that P nutrition is important in interpreting the N<sub>2</sub> fixation capability of strains of *Rhizobium*. Synergism between P and *Rhizobium* inputs requires that to obtain maximal response to the application of one input necessitates employing the other at maximum levels.

**Additional Index Words:** N<sub>2</sub> fixation; nodule; nitrogenase; acetylene reduction.

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PHOSPHORUS and N are limiting factors to crop production in many tropical soils (12). Efforts to expand soybean production and exploit the soybean [*Glycine max* (L.) Merr.] -*Rhizobium japonicum* symbiosis in tropical soils demand that both the P and rhizobial inputs be considered and managed appropriately.

The amount of symbiotically fixed N<sub>2</sub> in a legume-*Rhizobium* system will be determined by the genetic compatibility between host and microorganism and their interaction with the environment. It is reasonable to assume that the symbiosis will not fix more nitrogen than that required to meet a yield potential defined by the complement of other input factors in the system. Nutritional constraints such as low phosphorus may reduce yield and demand for fixed N or may act directly upon N<sub>2</sub> fixation in the nodule and cause simultaneous N deficiency in the shoot. Phos-

phorus nutrition which affects yield potential and dry matter distribution of soybean (6, 11) may have an indirect effect on N<sub>2</sub> fixation through the supply of phytosynthate to nodules and the strength of the sink for nodule products.

Andrew and Robins (1) reported positive correlations between the concentrations of P and N in nine species of legumes and concluded that P applications beyond those required for maximum dry matter production resulted in increasing concentrations of N in the shoot.

Cassman et al. (3, 4) demonstrated that strains of *R. japonicum* could be selected for tolerance to low concentrations of phosphate and suggested that this may have agronomic significance under conditions of low P availability. There exists substantial interaction between soil N availability and P uptake by plants (7). *Rhizobia* vary in their ability to fix N<sub>2</sub> with a given soybean genotype and thus may alter shoot-root ratios, root growth and the ability of soybean to accumulate P. By using strains of *R. japonicum* with a range of symbiotic capabilities, we were able to examine the interaction between N<sub>2</sub> fixation and P nutrition under conditions of both P and N-defined limits to yield.

### MATERIALS AND METHODS

#### Plant culture

A greenhouse pot experiment was conducted at Maui, Hawaii during September, 1983. Plastic pots (3.0 L) were filled with 2.7 kg (oven dry basis) of Haiku clay (clayey, ferritic, isohyperthermic Humoxic Tropohumults) that had been passed through a 6.5-mm sieve. The soil was amended with oven-dried sugarcane bagasse (6.5-mm sieve) at 10 g kg<sup>-1</sup> soil to eliminate the availability of soil N (2) and limed with 3 g Ca(OH)<sub>2</sub> kg<sup>-1</sup> soil to bring the pH to 5.6. Four P treatments were instituted by mixing 0, 50, 125, and 400 mg P kg<sup>-1</sup> soil (P<sub>0</sub>, P<sub>50</sub>, P<sub>125</sub>, P<sub>400</sub>) as Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>·H<sub>2</sub>O. Other nutrients added (kg<sup>-1</sup> soil) were: 100 mg K; 25 mg Mg; 117 mg S. A liquid micronutrient concentrate (Monterrey Chemical Company)<sup>1</sup> was added at 0.5 mL kg<sup>-1</sup> soil which provided: 7.5 mg Fe, 2.5 mg Zn, 2.3 mg Mn, 1.75 mg B, 0.75 mg Cu, 0.2 mg Mo, and 0.15 mg Co per kg soil.

The soil was brought to 37% moisture (w/w) corresponding to a suction of 0.01 MPa 3 d prior to planting. Six pregerminated seedlings (36 h from imbibition) of soybean [*Glycine max* (L.) Merr. cv. Davis] were planted and inoculated with 10<sup>9</sup> cells from one of five strains of *R. japonicum*: USDA 110; USDA 123; USDA 33; USDA 31 (USDA-ARS, Beltsville, MD); and SM-5 (W.J. Brill, Univ. of Wisconsin, Madison). The inoculated treatments and a +N treatment were replicated three times in all combinations with the four P levels. Plants were thinned to three per pot 8 d after planting. The uninoculated, +N pots received a total of 600 mg N kg<sup>-1</sup> soil from NH<sub>4</sub>NO<sub>3</sub> surface applied as liquid stock in equal split applications at 17 and 23 d after planting. Strain SM-5 is completely ineffective and served as a nonfixing control. Plants were watered daily to 0.01 MPa suction and harvested 33 d after planting.

#### Harvest and Analysis

Plants were cut at soil level, dried at 65°C, weighed, and ground. Samples (0.5 g) were digested in 10 mL of H<sub>2</sub>SO<sub>4</sub>

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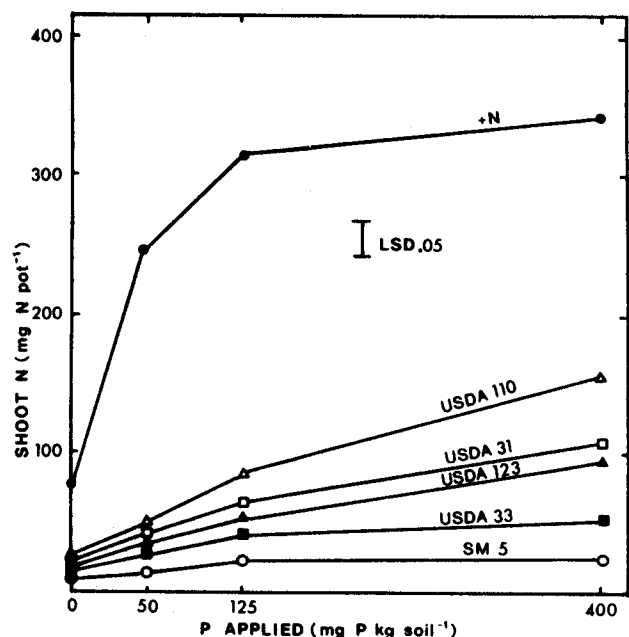
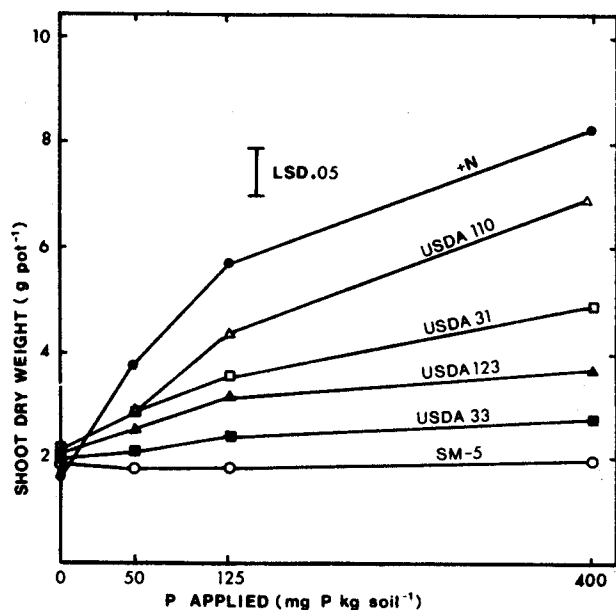
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Table 1. Extractable P in the Haiku soil at harvest.

P applied	Olsen P
mg P kg <sup>-1</sup> soil	
0	3
50	8
125	14
400	38

Fig. 1. The response of soybean shoots at 33 d after planting to phosphorus fertilization and strain of *Rhizobium japonicum*.Fig. 2. Accumulation of N in the shoot of soybean as affected by N, phosphorus fertilization, and strain of *Rhizobium japonicum*.

after pretreatment with 5 mL of H<sub>2</sub>O<sub>2</sub>. Nitrogen and P were determined in the same digests (14) by the methods of Mitchell (8) and Murphy and Riley (9) respectively.

At harvest, roots were removed from pots and incubated in 2-L plastic containers with 10% (v/v) acetylene in air for 1 h. Ethylene production was determined with a Varian aerograph 940 gas chromatograph (N<sub>2</sub> carrier; poropak T

Table 2. Effect of P and strain of *Rhizobium japonicum* on the concentration of N and P in the shoot.†

P applied	USDA											
	+N		SM-5		110		31		123		33	
mg P kg <sup>-1</sup> soil	N	P	N	P	N	P	N	P	N	P	N	P
0	42*	1.1	7	0.9	14	0.8	12	0.7	12	0.8	11	0.6
50	48	2.1	8	1.3	16	1.0	14	0.9	14	0.9	12	1.5
125	51	1.8	10	1.4	18	1.2	17	1.1	18	1.2	15	1.5
400	39	2.0	8	2.5	21	1.7	17	1.8	25	2.3	12	2.9

\* LSD 0.05 N × P: 4.1 g kg<sup>-1</sup> N, 0.40 g kg<sup>-1</sup> P.

column at temperature 70°C). Roots were then washed, nodules removed, and roots and nodules dried at 65°C.

Soil samples at harvest from each P treatment were combined, air dried, and passed through a 2-mm sieve. Plant-available P was determined according to Olsen and Sommers (10).

## RESULTS AND DISCUSSION

Results of soil P tests are displayed in Table 1. These test results cover a range of plant-available P from extremely deficient to levels at which further response is not expected (10). Figures 1 and 2 indicate the large plant response in dry matter and accumulation of N in the shoot to applications of phosphorus. All treatments except ineffective strain SM-5 responded to P. The lack of response by the SM-5 control to P indicates that P did not affect the immobilization of soil N by the addition of bagasse and the ineffective control remained N deficient. Growth responses and N accumulated by the other strains were, therefore, due to symbiotic nitrogen fixation and P nutrition. At P<sub>0</sub>, the system was completely P limited; no difference in shoot dry weight or N accumulation was observed between effective strain USDA 110 and ineffective strain SM-5 (Fig. 1 and 2). Even at moderate P applications, differences between strains were marginal. Only at the highest level of applied P was the capability for N<sub>2</sub> fixation by the superior strain-host combination USDA 110 significantly demonstrated.

The +N control remained free of nodules and produced a substantial shoot N response to P at P<sub>50</sub>. Had N been available in this treatment from planting rather than from 17 d after planting to coincide with the onset of fixation by inoculated treatments, the differences may have been even greater. This demonstrated the advantage that soybeans fertilized with mineral N have in P-deficient soils, and agrees with the conclusions of Cassman (5). Plants of treatment P<sub>400</sub> were mainly limited by N and the expression of strain effectiveness was enhanced. Even moderately effective strain USDA 123 continued to respond to P<sub>400</sub>, although not significantly.

Applying NH<sub>4</sub>NO<sub>3</sub> significantly increased the concentration of P in the shoot compared to the highly effective symbiotic treatments USDA 110 and 31 at most levels of P employed (Table 2). With P<sub>50</sub>, the +N treatment had double the concentration of P compared to the best symbiotic treatments (USDA 110 and USDA 31). The effective symbiotic treatments remained P deficient except at the highest level of P applied. Plants inoculated with the relatively in-

**Table 3. Effect of P and strain of *Rhizobium japonicum* on nodule number and nodule dry weight.**

P applied	Number of nodules*								Weight of nodules**							
	USDA								USDA							
	+N	SM-5	110	31	123	33	+N	SM-5	110	31	123	33				
	no pot <sup>-1</sup>								mg pot <sup>-1</sup>							
mg P kg <sup>-1</sup> soil																
0	2	217	89	134	126	17	5	163	80	133	124	75				
50	0	218	105	132	124	28	0	191	159	216	192	144				
125	2	223	120	159	146	43	4	204	203	332	356	237				
400	1	228	153	179	204	32	3	246	646	607	627	199				

\* LSD 0.05, N × P: 34 pot<sup>-1</sup>.\*\* LSD 0.05, N × P: 78 mg pot<sup>-1</sup>.

effective (USDA 33) and ineffective (SM-5) symbionts had greater tissue P levels than those with highly effective symbionts at P<sub>50</sub>, P<sub>125</sub>, and P<sub>400</sub>. This relationship indicated that plants with low N<sub>2</sub>-fixing capacity were relatively more N limited than P limited at moderate levels of applied P whereas the more effective symbioses remained P deficient due to greater demand for P.

Increasing P fertility enhanced the concentration of N in the shoot (Table 2) with every N source except ineffective strain SM-5 and the +N treatment. As P became less limiting, the more effective strains (USDA 110, 31, 123) generally showed greater increases in shoot N concentration compared to less effective treatments (USDA 33, SM-5).

Measurements of nodule number and nodule dry weight (Table 3) and nitrogenase activity (Table 4) help to explain the effects of P fertility on N<sub>2</sub> fixation. Although nodule number was enhanced by increasing P, it did not explain the increased yield of symbiotic N. There was almost a twofold increase in USDA 110 nodule number with added P, but fixed N increased fivefold (Fig. 2). Singleton and Stockinger (13) demonstrated that substantial reductions in nodule number in soybean did not result in a commensurate reduction in nodule mass and nitrogen fixation. Therefore, the number of nodules present did not limit N<sub>2</sub> fixation under low-P conditions. Nodule number is a poor indicator of N<sub>2</sub> fixation under P stress. Strain USDA 110 produced substantially fewer nodules than strain USDA 123 and SM-5 at every level of P yet USDA 110 fixed significantly more nitrogen. Screening rhizobia for tolerance to low P using nodule number as a criterion would not be fruitful.

Nodule mass (Table 3) and total nitrogenase activity (Table 4) tended to be more related to shoot N and dry matter accumulation across P treatments within a strain. Nodule mass increased in all strain treatments, including SM-5, with the addition of phosphorus. Differences in nodule mass between strains at P<sub>0</sub> are large but not related to the N<sub>2</sub> fixation of the strain. At maximum P the ineffective strains had less nodule mass than the effective treatments but strains with intermediate N<sub>2</sub> fixation had a similar nodule mass to the superior strain, USDA 110.

Examining total and specific nitrogenase activities of the treatments (Table 4) indicated that total activity, like nodule mass, responded to P fertilization. No activity was observed in the +N or SM-5 treatments. Specific nodule activity also increased with P level.

**Table 4. Effect of P and strain of *Rhizobium japonicum* on total nitrogenase activity (TNA) and specific nodule activity (SNA) of soybean.**

P applied	mg P kg <sup>-1</sup> soil	USDA							
		110		31		123		33	
		TNA*	SNA**	TNA	SNA	TNA	SNA	TNA	SNA
0	0.6	22.1	0.8	18.4	0.6	14.3	1.0	40.8	
50	2.7	50.5	3.7	51.3	1.7	26.3	3.2	69.0	
125	5.2	90.0	5.8	53.4	3.3	26.7	5.7	72.9	
400	14.4	65.5	12.4	61.2	9.5	46.0	6.6	99.7	

\* Total nitrogenase activity; LSD 0.05, N × P: 2.9 μmol C<sub>2</sub>H<sub>4</sub> plant<sup>-1</sup> h<sup>-1</sup>.\*\* Specific nitrogenase activity; LSD 0.05, N × P: 24.7 μmol C<sub>2</sub>H<sub>4</sub> (g nodule<sup>-1</sup> h<sup>-1</sup>).

Some strains such as USDA 33 exhibited relatively high specific activity at P<sub>0</sub>. Given the poor relationship between nodule number and nodule mass, however, it cannot be determined from this experiment whether changes in specific nodule activity were merely a function of bacteroid volume - nodule volume relationships or an actual effect of P on the nitrogenase system. The high specific activity of USDA 33 for example may be a result of bacteroid to nodule volume relationships since at least a part of the strain's relative ineffectiveness in this experiment is due to an inability to form large numbers of nodules. As a consequence the weight of individual USDA 33 nodules was large.

In summary, symbiotic N<sub>2</sub> fixation is subject to the "law of the minimum" for other input factors. Greater responses to inoculation of soybean will be obtained under conditions of adequate P fertility. Conversely, maximum responses to P as an input will be observed only when adequate mineral N or a superior symbiotic system is available. Testing of rhizobial strains under conditions of low fertility risks misinterpretation of the primary selection criterion; the genetic capacity of host-strain combinations to fix N<sub>2</sub>. Soybeans supplied with mineral N had an early yield advantage in low P environments compared to plants relying only on the symbiosis for N. This was true even when the application of mineral N was delayed to coincide with the onset of N<sub>2</sub> fixation by inoculated plants.

Adequate numbers of nodules were present to support plant growth under P limited conditions. Inherent strain differences for nodule number indicate that this characteristic is not a valid criterion for selecting strains of *Rhizobium* tolerant of P-deficient soils. Small direct effects of low P on nodules were observed in the form of reduced nitrogenase activity per unit nodule weight and reduced N concentration in the shoot.

Plants inoculated with any strain except ineffective SM-5 responded to P application. The magnitude of the response at intermediate levels of applied P was a function of strain effectiveness at maximum P applied and the effect of P on plant growth. Since higher yields of dry matter and N at intermediate levels of P were related to strain effectiveness strain selection for tolerance to low P would probably be of limited value.

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## REFERENCES

1. Andrew, C.S., and M.F. Robins. 1969. The effect of P on the growth and chemical composition of some tropical legumes: II. N, Ca, Mg, K, and Na contents. *Aust. J. Agric. Res.* 20:675-685.
2. Cassman, K.G. 1979. Phosphorus requirements of two grain legumes as affected by mode of N nutrition. Ph.D. thesis. Univ. of Hawaii, Manoa. (Diss. Abstr. 40.)
3. Cassman, K.G., D.N. Munns, and D.P. Beck. 1981a. Phosphorus nutrition of *Rhizobium japonicum*: strain difference in phosphate storage and utilization. *Soil Sci. Soc. Am. J.* 45:517-520.
4. Cassman, K.G., D.N. Munns, and D.P. Beck. 1981b. Growth of *Rhizobium* strains at low concentrations of phosphate. *Soil Sci. Soc. Am. J.* 45:520-523.
5. Cassman, K.G., A.S. Whitney, and R.L. Fox. 1981. Phosphorus requirements of soybean and cowpea as affected by mode of N nutrition. *Agron. J.* 73:17-22.
6. Cassman, K.G., A.S. Whitney, and K.R. Stockinger. 1980. Root growth and dry matter distribution of soybean as affected by phosphorus stress, nodulation, and nitrogen source. *Crop Sci.* 20:239-243.
7. Grunes, D.L. 1959. Effect of nitrogen on the availability of soil and fertilizer phosphorus to plants. *Adv. Agron.* 11:369-396.
8. Mitchell, H.L. 1972. Microdetermination of nitrogen in plant tissues. *J. Assoc. Off. Anal. Chem.* 55:1-3.
9. Murphy, J., and J.P. Riley. 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chem. Acta.* 27:31-36.
10. Olsen, S.R., and L.E. Sommers. 1982. Phosphorus. In A.L. Page et al. (ed.) *Methods of soil analysis*, Part 2, 2nd ed. Agronomy 9:421-422.
11. Ozanne, P.G., J. Keay, and E.F. Biddiscombe. 1969. The comparative applied phosphate requirements of eight annual pasture species. *Aust. J. Agric. Res.* 20:809-818.
12. Sanchez, P.A. 1976. *Properties and management of soils in the tropics*. John Wiley & Sons, Inc., New York.
13. Singleton, P.W., and K.R. Stockinger. 1983. Compensation against ineffective nodulation in soybean. *Crop Sci.* 23:69-72.
14. Throneberry, G.O. 1974. Phosphorus and zinc measurements in Kjeldahl digests. *Anal. Biochem.* 60:358-362.