Abstract: Phosphorus (P) deficiency is a main factor limiting legume production in most agricultural soils due to its necessity for growth and nitrogen fixation. The effect of phosphorus supply on nodule development and function in soybean (Glycine max (L.) Merr. cv. Heinong 35) was studied in nutrient solution. Plants were inoculated with Bradyrhizobium japonicum and were grown for 35 days in glasshouse at 25/15 °C day and night. While increasing P supply increased concentrations of P and N in shoots and roots, P concentration in nodules was not significantly affected by external P supply, and the amount of N fixed per unit nodule biomass decreased with increasing P supply. Nitrogen content in the shoots correlated well with P content (r = 0.92**). At inoculation level of 102 cells mL-1, P supply did not affect nodule number. At inoculation levels of 103.5 and 105 cells mL-1, increasing P supply increased both nodule number and size. Irrespective of inoculation level, increasing P supply increased nodule biomass relative to biomass of host plants. It is suggested that P deficiency specifically inhibited nodule development and thus total N2 fixation.
Nodule Formation and Development in Soybean (*Glycine max* L.) in Response to Phosphorus Supply in Solution Culture*1

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ABSTRACT

Phosphorus (P) deficiency is a main factor limiting legume production in most agricultural soils due to its necessity for growth and nitrogen fixation. The effect of phosphorus supply on nodule development and function in soybean (*Glycine max* (L.) Merr. cv. Heinong 35) was studied in nutrient solution. Plants were inoculated with *Bradyrhizobium japonicum* and were grown for 35 days in glasshouse at 25/15 °C day and night. While increasing P supply increased concentrations of P and N in shoots and roots, P concentration in nodules was not significantly affected by external P supply, and the amount of N fixed per unit nodule biomass decreased with increasing P supply. Nitrogen content in the shoots correlated well with P content (*r* = 0.92**). At inoculation level of 10² cells mL⁻¹, P supply did not affect nodule number. At inoculation levels of 10³ and 10⁵ cells mL⁻¹, increasing P supply increased both nodule number and size. Irrespective of inoculation level, increasing P supply increased nodule biomass relative to biomass of host plants. It is suggested that P deficiency specifically inhibited nodule development and thus total N₂ fixation.

**Key Words**: inoculation level, nitrogen fixation, nodulation, phosphorus, soybean

INTRODUCTION

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In many soils, phosphorus (P) is the most limiting nutrient for the production of crops (Cao et al., 2002; Chen et al., 2004; Han et al. 2005; Wang et al., 2005). Nitrogen-fixing plants of legumes require P to meet adequate growth and nodulation (Andrew and Robins, 1969; Israel, 1993; Tang et al., 2001). However, there are arguments on the direct role of P in nodulation and N2 fixation of legumes. The supply of P directly and positively stimulated nodulation in red clover (Hellsten and Huss-Danell, 2000), pea (Jakobsen, 1985) and soybean (Israel, 1987, 1993). P had specific effects on nodulation in Discaria trinervis (Israel, 1987; Hellsten and Huss-Danell, 2000), but nodule initiation was less affected by P deficiency than host plant growth (Claudio et al., 2002). There were also other reports suggesting that P indirectly induced nodulation by a positive effect on plant growth (Yang, 1995; Reddell et al., 1997). P may increase nodulation and stimulate nitrogenase activity through improving plant growth (Gentili and Huss-Danell, 2003).

Many studies have suggested that the supply of a nutrient favored nodulation and growth in legumes such as white clover (Joses et al., 2000), Lupinus (Tang et al., 1990; 1991; 2006) and Discaria trinervis (Claudio, 2002). Early studies suggested that P deficiency reduced root growth and limited the photosynthetic carbohydrate supply to nodule (Jakobsen, 1985). P deficiency limited nodulation, nodule growth and function, and thus symbiotic N2 fixation was reduced (Cadisch et al., 1989). It is accepted that P interacts with the plant demand for N to control nodule growth, and modulates symbiotic process of legume and rhizobium (Wall et al., 2000; Hellsten and Huss-Danell, 2000). However, the effect of P supply on nodule development and function in soybean are poorly understood.

The aims of this paper were to elucidate the role of P supply in solution on nodule formation and function, to investigate the interaction between nitrogen and phosphorus concentrations in plant tissues and to examine how rhizobium inoculation level affects nodulation in soybean.

MATERIALS AND METHODS

For P effect on nodule development and function, uniform-sized seeds of soybeans (Glycine max L. Merr. cv. Heinong 35) were imbibed in water overnight, and then transferred onto a mesh sitting above an aerated solution (pH=6) of 1 mM CaCl2 and 5 µM H3BO3. Seeds were germinated for 4-5 days when radicles were about 4 cm in length. Eighteen uniform seedlings were transferred into a 5 L pot with 5 P treatments. Solution in the following composition (in µM): K2SO4, 600; MgSO4, 200; CaCl2, 600; H3BO3, 5; ZnSO4, 0.75; MnSO4, 1; CoSO4, 0.2; CuSO4, 0.2; Na2MoO4, 0.03; Fe-NaEDTA, 10. Phosphorus was supplied as KH2PO4 at 0, 1, 4, 16 and 50 µM. Bradyrhizobium japonicum strain Hefeng 25 was added to pots as water suspension with a final concentration about 10^8 cells mL^{-1} in the nutrient solution. This suspension was added again 4 days later when solution was renewed. The plants were grown in a glasshouse at 25/15 °C day and night. Nutrient solutions were renewed twice a week. Solution pH was adjusted to 6.5-7.0 daily or twice daily if necessary. The experimental design was a complete factorial one. The treatments were replicated three times and randomized within replicates.
For the effect of inoculation level, the experiment consisted of 3 P levels (0, 4 and 50 µM) and 3 *Bradyrhizobium japonicum* levels (R1-10⁶ cells mL⁻¹; R2-10³⁵ cells mL⁻¹ and R3-10⁵ cells mL⁻¹). Treatments were replicated three times and randomized within replicates.

Two plants were sampled from each pot at 7, 9, 12, 15, 20, 25, 30 and 35 days after transplanting (DAT) for the experiment of nodule development and function. Two plants were sampled from each pot at 10, 13, 16, 20, 25 DAT for the experiment of inoculation level. Plants were separated into shoot, root and nodule. Plant tissues were oven-dried at 80 °C until constant weight for P and N analysis. At each sampling date, biomass of shoots, roots and nodules was measured, root length and nodule number were calculated. The P digested by H₂SO₄ and H₂O₂ was analysed with ultraviolet spectrometer (UV2500, Japan) (Zhang et al., 2003) and N concentration in plant tissues was analysed by auto-titration with H₂SO₄ and H₂O₂ digested. The relative growth rate and the ratio of root length to biomass were calculated.

The t-test was used to identify statistically significant differences. The data were subject to one-way analysis of variance. The least significant difference (LSD) at P=0.05 was used to compare the means between treatments.

RESULTS

*Plant growth*

Plants in all P treatments showed pale shoot colour at 17 DAT, and were recovered 4 days later (21 DAT). The relative growth rate (RGR) from 7 DAT to 20 DAT and from 20 DAT to 35 DAT increased with the increase of P supply in the solution, and reached the maximum at 16 µM P. The RGR from 20 DAT to 35 DAT was greater than that from 7 DAT to 20 DAT (Fig. 1a). Root biomass of all P treatments was similar, while on average, plants grown at 4 µM P produced greater total root length than that at 50 µM P (Fig. 2). The ratio of root length to root biomass at 4 µM P was greater than that at 0 or 50 µM P, especially after 30 DAT.
N and P concentration in plant tissues

N concentration in shoots increased as P supply changed from 0 to 50 µM in the solution (Fig. 1b). N concentrations in roots and nodules were not clearly affected by P supply. Nodules had significantly higher N concentration than shoots and roots. The higher N concentration in nodules was observed at 1 µM and 50 µM, while the highest N concentration in roots was found at 16 µM. From 16 µM P, N concentration in both nodules and shoots increased, while the N concentration in roots decreased (Fig. 1b).

Fig. 2 Root biomass and root length, and the ratio of root length to root biomass in plants of Glycine max (L.) Merr. cv. Heinong 35 grown at 0-50 µM P during 12-35 days after treatment. Vertical bars represent the LSD values at P=0.05.

Fig. 3 Effects of P supply in solution on P concentration in shoots, roots and nodules of Glycine max (L.) Merr. cv. Heinong 35 grown at 0-50 µM P for 35 days. The vertical bar represents the LSD value at P=0.05.
P concentration in all plant tissues decreased with the days of growing. Increasing P supply increased P concentration in roots and shoots (Fig. 3). P concentration in nodules did not show any significant difference between different P supply treatments, although its concentration was higher than that in roots and shoots (Fig. 3). Interestingly, a close correlation between N content and P content in shoots was observed \( r = 0.92^{**}, n=15 \) (Fig. 4).

**Nodule formation**

P supply markedly increased both nodule number and nodule fresh weight per plant (Fig. 5). Nodule fresh weight at 0 and 4 µM P reached peak, while nodule fresh weight at 50 µM continued to increase at 25 DAT (Fig. 5). The maximum nodule fresh weight at 50 µM P treatment was about 3-fold more than that at 4 µM P treatment. The effect of P on nodule fresh weight was even greater from 25 to 35 DAT than from 15 to 25 DAT. No significant difference was found between 0 and 4 µM P treatment in nodule fresh weight during all sampling days, although relatively greater from 25 DAT at 4 µM P treatment.

![Figure 4: The relationship between N content and P content in shoots of Glycine max (L.) Murr. grown at 0-50 µM P in nutrient solution at 35 DAT.](image)

![Figure 5: Effects of P supply in solution on nodule number and fresh weight per plant, nodule number per unit root length, and nodule fresh weight per nodule of plants of Glycine max (L.) Merr. cv. Heinong 35 grown at 0-50 µM P for 35 days. Vertical bars represent the LSD values at \( P=0.05 \).](image)
From 25 DAT, nodule fresh weight per nodule increased with P supply in the solution. 50 µM P treatment significantly increased nodule fresh weight per nodule, and no difference was observed between 0 and 4 µM P treatments during all sampling days. In contrast, nodule fresh weight per nodule of 50 µM P was doubled than that of 0 and 4 µM P after 30 DAT (Fig. 5). All these resulted in a significant increase for nodule fresh weight per plant from 25 DAT onward.

Nodule number per unit root length increased with P supply in the solution (Fig. 5). The effect of P on nodule number per plant was similar to the effect on nodule number per root length. Nodule dry weight at 4, 16 and 50 µM was significantly greater than of 0 and 1 µM, while no difference was found among the 4, 16 and 50 µM P treatments (Fig. 6). The amount of N₂ fixed per unit nodule biomass decreased as the increase of P supply in the solution (Fig. 6).

![Graph showing N fixed per unit nodule](image)

**Fig. 6** Dry weight of individual nodules and nitrogen fixed per unit nodule biomass of *Glycine max* (L.) Merr. cv. Heinong 35 grown at 0-50 µM P for 35 days. Values followed by the same letter are not different at P=0.05.

**Rhizobia growth and N₂-fixation**

Nodule number increased as rhizobium level increase in the solution, while P supply had little effect on the nodule number at inoculation level of 10² cells mL⁻¹ (Fig. 7). Noduleumber at low rhizobium level (10² cells mL⁻¹) kept unchanged, but increased at higher rhizobium levels (10³.5 and 10⁵ cells mL⁻¹) with the growing days (Fig. 7).

![Graph showing Rhizobia growth and N₂-fixation](image)

**Fig. 7** Effects of P supply and inoculation level of *Rhizobium* in solution on nodule number and on relative nodule biomass of *Glycine max* (L.) Merr. cv. Heinong 35 grown at 0-50 µM P for 35 days. Relative nodule biomass is nodule biomass as % plant biomass.
Relative nodule biomass (as % plant biomass) increased as P supply increased in the solution (Fig. 7). At inoculation levels of $10^{3.5}$ and $10^5$ cells mL$^{-1}$, the relative nodule biomass at 0 and 4 µM P started to decline from 22 DAT, while continued to increase at 50 µM P treatment. The highest P supply and rhizobium level treatment had the highest relative nodule biomass (Fig. 7).

DISCUSSION

Effects of P deficiency on rhizobium growth

The present study demonstrated that P supply in the solution had no significant effect on nodule number at the low inoculation level ($10^2$ cells mL$^{-1}$), but increased nodule formation at higher inoculation levels before 25 DAT. The results suggest that external P was not essential for rhizobial growth or survival in nutrient solution. Alternatively, seed P might be sufficient for rhizobium infection on the roots. Seed P might play a critical role in early nodule formation in soybean, which was supported with the result by Thomson et al. (1992). In addition, increasing inoculation level had increased nodule number, indicating that a high number of rhizobia is necessary for optimal nodule formation in soybean. Furthermore, the observed interaction between rhizobial inoculation and P supply on nodulation might be explained as follows. A higher inoculation level could stimulate more nodule initials formed than a lower inoculation level. While P is essential for nodule development, more P is required for the full development of the nodule initials formed at an early stage. Under P deficiency, only a portion of these nodule initials would have developed.

Effects of P on nodulation and nodule development

In the present study, nodule formation and nodule development were evaluated as nodule number, nodule biomass and nodule density and their correlation with host plant growth. P supply in solution increased these parameters at all sampling times (Fig. 5). The decreased nodule number from 25 DAT might be due to sampling error (Fig. 5). Our results showed that nodule biomass per unit plant biomass was stimulated by increased P, whereas increasing P supply had no effect on root dry weight. This indicates that the effect of external P on nodulation is specific and independent of the host plant growth (Gentili and Huss-Danell, 2003). This study also showed that nodule weight per nodule and nodule biomass relative to the biomass of the host plants increased with increasing P supply (Figs. 6 and 7), suggesting that nodule development had a high requirement for external P supply. This was further supported by the high concentration of P in nodules than in host plants. The
results were consistent with the findings by Tang et al. (2001) in Medicago truncatula. It appeared that low P reduced shoot growth and limited water soluble carbohydrate supply to nodules (Jakobsen, 1985).

Effects of P on nodule function

The present study illustrated that P supply in solution had a significant role in nitrogen fixation in soybean. This was supported with two lines of evidence. First, increasing P supply increased N concentration in shoots and roots (Fig. 1b). Second, N content in shoots correlated highly with P content (Fig. 4). However, the amount of N fixed per unit nodule biomass was virtually decreased as P supply increased, suggesting that P does not have a specific role in nodule function. The increased N fixation by P supply had resulted from the enhanced nodule development. The results were consistent with previous findings in white clover (Hogh-Jensen et al., 2002). In other studies, P supply in solution had a specific stimulation on nodulation and nodule function (Robson et al., 1981; Jakobsen, 1985; Yang, 1995; Reddell et al., 1997; Vadez et al., 1999; Tang et al., 2001). The impaired nodule function under P deficiency was suggested to result from an inhibition of energy-dependent reactions in nodule cells, and was associated with decreased bacteroid biomass (Sa and Israel, 1991).

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REFERENCES


