

Seedling treatments and phosphorus solution concentrations affect nodulation and nodule functions in soybean (*Glycine max* L.)

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ABSTRACT

The effect of three seedling treatments: T₀, normal germination; T₁, cotyledons removed; T₂, cotyledons removed 5 days earlier than in T₁; and two phosphorus levels (P₀ and P₃₀) on nodulation and nodule function in soybean [*Glycine max* (L.) Merr.] were investigated in nutrient solution culture. The number of nodules formed at P₀ was in the order T₂ > T₀ > T₁, but it was T₀ > T₂ > T₁ at P₃₀. Nodule dry weight per plant had the same tendency as the nodule number. Nodule size (dry weight per nodule) in seedlings ranged from 0.601 to 1.089 mg in the order T₀ > T₁ > T₂, regardless of P level. For example, nodule size in T₀ was larger by 86% and 52% than T₂ at P₀ and P₃₀, respectively. Furthermore, regardless of P level, a specific acetylene reduction activity (ARA, μM C₂H₄/h/g nodule) increased with P content in seedlings, but no significant difference was found ($P < 0.05$). Leghemoglobin (Lb) content was not significantly affected by P level; however, seedlings (T₀ and T₁) significantly affected the Lb content per unit plant biomass ($P < 0.05$). All these results suggest that seedling P content plays a key role in nodulation and nodule function of soybean.

Keywords: soybean [*Glycine max* (L.) Merr.]; seedling; phosphorus nutrition; nitrogenase activity; leghemoglobin concentration

Phosphorus is one of the most important macrolelements indispensable for plant growth and development (Marschner 1986). Although soybean P₂O₅ requirements are considerably lesser than those of N or K, P is equally important for plant growth and productivity. Because of P functions for the growth and metabolism of plants, its deficiency retarded plant growth, cell and leaf expansion (Marschner 1986). Some authors reported that P sufficiency significantly increased leaf surface of soybean (Wu 1999). It is known that P regulation of photosynthesis and carbohydrate metabolism in leaves was one of the major factors limiting the plant growth (Marschner 1986). Moreover, P has stimulating effects on nodule growth and nitrogenase activity in nodules of legumes (Jakobsen 1985, Israel 1987, Hart 1989, Tang et al. 2001); on the contrary its deficiency decreased nodule

mass (Singleton et al. 1985, Israel 1987, Ribet and Drevon 1995, Drevon and Hartwig 1997), nodule number (Jakobsen 1985, Tang et al. 2001) and nitrogenase activity (Sa and Israel 1991).

All these reports focused on how exogenous P affected nodule formation and function. There is no report on the role of P reserved in seedlings in nodulation and nodule function. Seed's P was sufficient to establish nodules; however in the case of the lack of P, plants used the stored P in the seed to grow. Otherwise, nodules can be autoregulated without effect by exogenous P (Claudio et al. 2002). Early nodulation might be related to the P concentration in seeds; high P in seeds may thus facilitate the establishment of symbiosis (Claudio et al. 2002). However, the relationship between seedling P concentration, nodulation and nodule function is unclear. In this work, we

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present the results on the role of seedling P in the nodulation and nodule functions of soybean [*Glycine max* (L.) Merr.].

MATERIAL AND METHODS

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 mmol/l CaCl₂ and 5 μmol/l H₃BO₃. Three P treatments were used on seedlings: (1) T₀, total P concentration was 1.316 mg/g, normal germination; (2) T₁, total P concentration was 1.232 mg/g, cotyledons were removed when the first true leaf partly expanded, and (3) T₂, total P concentration was 1.067 mg/g, cotyledons were removed 5 days earlier than in T₁. Seeds were germinated for 4–5 days when radicles were about 4 cm in length. Eighteen uniform seedlings were transferred into a 5-litre pot with two P nutrient solution, i.e. 0 [P₀] and 30 [P₃₀] μmol/l. Phosphorus was supplied as KH₂PO₄ in nutrient solution. The composition of the solution was as follows (in μmol/l): K₂SO₄, 600; MgSO₄·7 H₂O, 200; CaCl₂·2 H₂O, 600; H₃BO₃, 5; ZnSO₄·7 H₂O, 0.75; MnSO₄·H₂O, 1; CoSO₄·7 H₂O, 0.2; CuSO₄·5 H₂O, 0.2; Na₂MoO₄·H₂O, 0.03; Fe-NaEDTA, 10.

Bradyrhizobium japonicum strain Hefeng 25 was added into pots as water suspension to reach the final concentration of about 10⁵ cells/ml in the nutrient solution. This suspension was added again 4 days later when solution was renewed. Plants were grown in a glasshouse at 25/15°C day and night, respectively. Nutrient solutions were renewed twice a week. Solution pH was adjusted to 5.5–6.0 once or twice a day, if necessary. Treatments were replicated six times and randomized within replicates.

Plants were harvested in the 5th week after the treatments. Plants were separated into shoots, roots and nodules. Biomass of all parts was measured and nodule number was calculated. Chlorophyll was extracted with acetone and ethanol and was measured with ultraviolet spectrometer (UV2500 Japan). The total and specific acetylene reduction activity (ARA) was analyzed with gas chromatograph (GC2010 Shimadzu Japan) (Tang et al. 2001). Leghemoglobin content was measured with ultraviolet spectrometer (UV2500 Japan). Plant tissues were oven-dried at 80°C to the constant weight for P and N analysis. The total P content after digestion with H₂SO₄ and H₂O₂ was analyzed with ultraviolet spectrometer (UV2500 Japan) and

N concentration was analyzed by auto-titration (Lu 2000).

The SAS software was used to identify any statistically significant differences. Duncan's multiple test at *P* < 0.05 was used to compare the means of the treatments.

RESULTS

Plant growth

From the 2nd week, whole shoots appeared pale green, and kept the color until the later stage; this indicated a slight nitrogen deficiency. By the 5th week after the treatments, chlorophyll concentration in the youngest expanded leaves in T₀ was the highest in this experiment, irrespective of P level (Figure 1). For the same seedlings, chlorophyll concentration in the youngest expanded leaves decreased as P level increased. Dry weight of shoots and roots in T₀ at P₀ level was by 41% and 33% higher than in T₂, respectively; for P₃₀ level they were higher by 63% and 33%. For both shoots

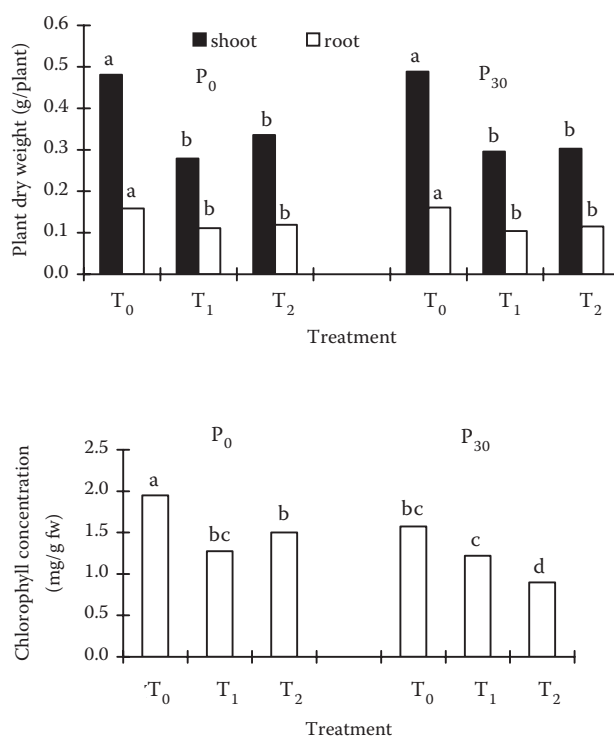


Figure 1. Dry weight of shoots and roots and chlorophyll concentration in the youngest expanded leaves of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels after 5 weeks; letters on the top of the bars indicate significant differences at 5% level

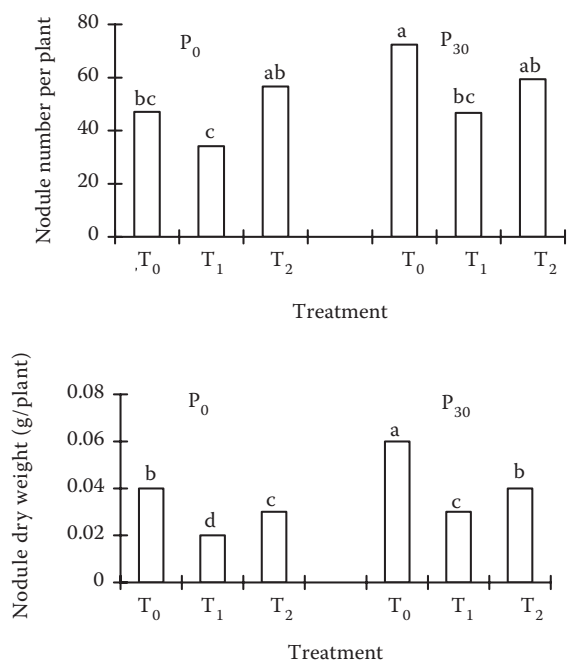


Figure 2. Number of nodules and nodule dry weight of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

and roots, dry weight was in the order: T₀ > T₂ > T₁ (Figure 1). Significant differences were recorded in the case of dry weight of shoots and roots between T₀ and T₂; however, no significant differences were observed between T₀ and T₁ or T₁ and T₂. We found that the effect of seedlings on dry weight of shoots and roots was larger than that of P level.

Nodule formation

Due to the low temperature, nodules firstly appeared during the 3rd week after the treatment. Nodule number recorded in the 5th week was affected both by seedlings and P level (Figure 2). At P₀ level, seedlings did not significantly affect nodule number; it ranged from 36 to 50 per plant in the order: T₂ > T₀ > T₁; at P₃₀ level it ranged from 48 to 60 per plant in the order: T₀ > T₂ > T₁. A significant difference was found between T₀ and T₂ (Figure 2): P₃₀ level increased the nodule number per plant; T₀ and T₂ treatments showed significant differences. For both P levels, nodule dry weight per plant ranged from 0.021 to 0.061 g in the order: T₀ > T₂ > T₁ (Figure 2); for P₃₀ treatment, nodule number and nodule dry weight per plant in T₀ were by 11% and 75% higher than in T₂. At the same

P level, seedlings significantly affected nodule dry weight. Similarly, under the same seedling treatment, the effect of P level on nodule dry weight was significantly different (Figure 2).

Irrespective of P level, nodule size (dry weight per nodule) in seedlings ranged from 0.601 to 1.089 mg in the order: T₀ > T₁ > T₂ (Figure 3). Dry weight per nodule in T₁ was by 86% and 52% higher than in T₂ at P₀ and P₃₀ levels, respectively. A statistical analysis showed that the effect of seedlings on nodule size was significant at P₀ level; however, at P₃₀ level, this effect was found only between T₀ and T₁, or between T₀ and T₂ (Figure 3).

Nodule function

At the same P level, specific ARA ($\mu\text{M C}_2\text{H}_4/\text{h/g}$ nodule) in T₀ was higher than in other treatments, but no statistically significant difference was found. Moreover, P level increased specific ARA in the same seedlings. The effect of seedlings on total ARA ($\mu\text{M C}_2\text{H}_4/\text{h/g}$ plant) was minor at both P levels, but P level significantly increased total ARA in T₂ (Figure 4). The total ARA in T₂ seedlings was the largest at P₃₀ level ($8.21 \mu\text{M C}_2\text{H}_4/\text{h/g}$ nodule), followed by T₀ (P₃₀), T₁ (P₀), T₂ (P₀), T₀ (P₀) and T₁ (P₃₀). C₂H₂-induced decline (C₂H₂-ID) of ARA was observed in all the treatments (Figure 5). When 10% C₂H₂ was introduced into the gas mixture bottle with nodulated roots, the amount of C₂H₄ produced increased, and then decreased with duration of C₂H₂ exposure time, but the time until reaching the maximum value was different. At P₀ level, the amount of C₂H₄ from nodule activity

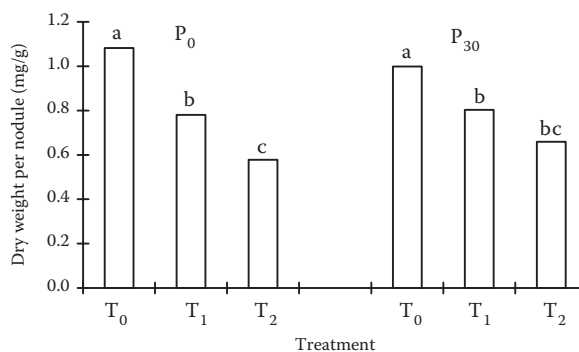


Figure 3. Nodule size (dry weight per nodule) of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

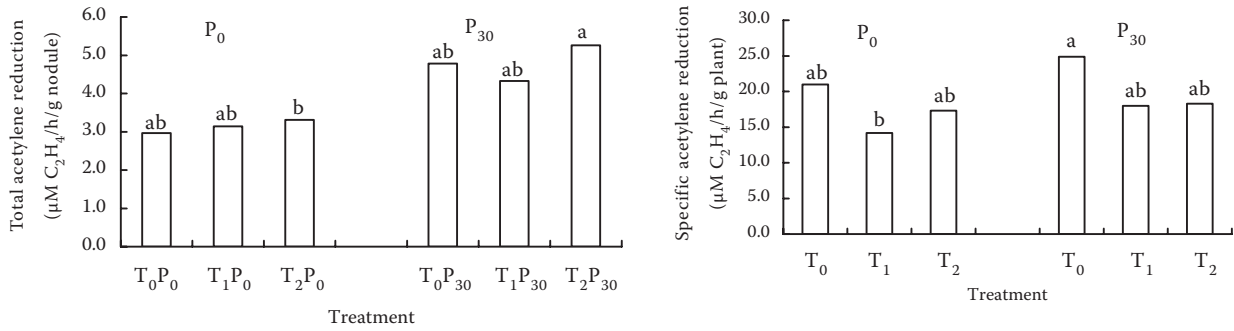


Figure 4. Total and specific acetylene reduction activity (ARA) of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week; ARA was the average ARA measured between 10 and 60 min after C₂H₂ exposure; letters on the top of the bars indicate significant differences at 5% level

increased, reaching the maximum value 40 min after initial exposure to C₂H₂. However, a double peak curve of C₂H₂-ID was produced by the nodule activity at P₃₀ level (Figure 5).

The effect of seedlings on leghemoglobin (Lb) concentration per nodule was greater than the effect of P level, especially in T₀ and T₁ seedlings; a significant difference was found in these

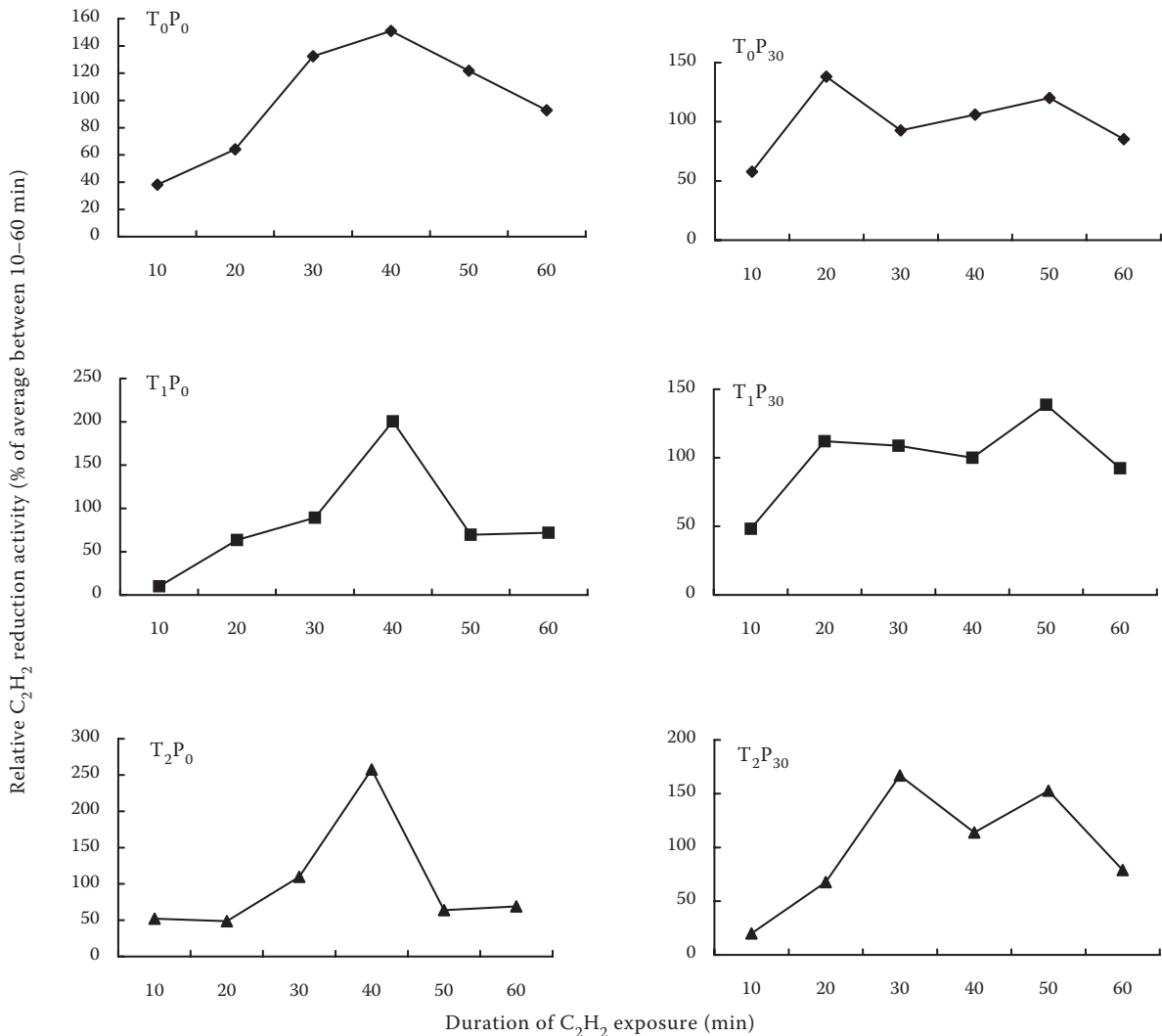


Figure 5. Time course of acetylene reduction activity (ARA) measured on soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week

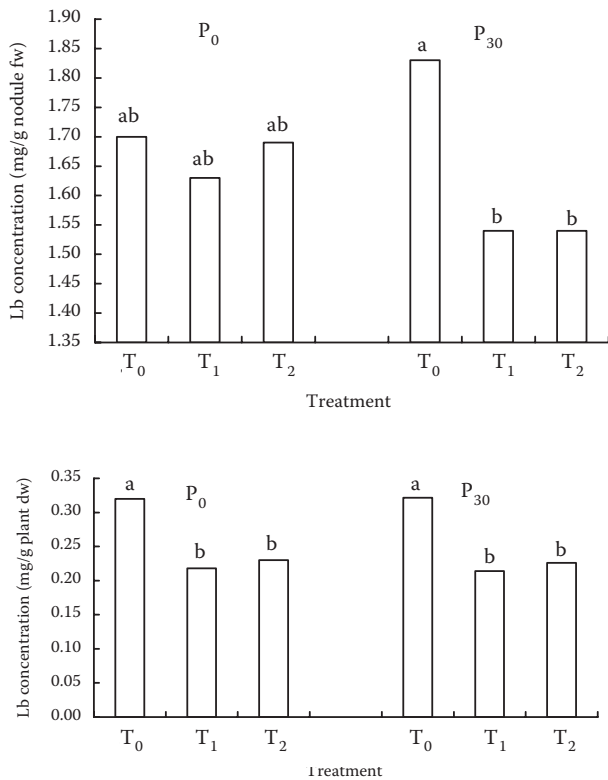


Figure 6. Leghemoglobin (Lb) concentration of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

seedlings at both P levels (Figure 6). For the same seedlings, P level had no significant effect on Lb concentration. The effect of P level on Lb concentration per plant was the same as that on specific ARA, especially at P₃₀. All these data suggest that Lb concentration in nodules can be considered as a characteristic of nitrogen fixation efficiency (Shleev et al. 2001).

Chemical composition

Nitrogen concentration (per plant and per unit weight) in seedlings ranged from 14.75 to 26.18 mg/plant and from 33.28 to 40.22 mg/g in the order: T₀ > T₂ > T₁, except for nitrogen concentration per unit weight at P₀ level (Figure 7). Regardless of P level, a significant difference of nitrogen concentration per plant was found between T₀ and T₁. For nitrogen concentration per unit weight, a significant difference was observed between T₀ and T₂ at both P levels (Figure 7). Seedling effect on P concentration per plant or per unit weight was low at P₀ level. However, P level significantly

affected P concentration, and a significant difference was observed among seedling treatments. For example, P concentration per plant and per unit weight in T₀ increased from 0.74 to 2.62 mg/plant and from 1.16 to 4.04 mg/g, respectively.

DISCUSSION

Nodule formation

In the present study, before inoculating *Bradyrhizobium japonicum* strains, P concentration of T₀ seedlings was 1.316 mg/g, for T₁ it was 1.232 mg/g, and for T₂ it was 1.067 mg/g. Nodule number was significantly affected by P levels in T₀ and T₂, not in T₁ (Figure 2). This is because T₂ seedlings had a longer total root length, which provided more chances for bacteria infection than T₁. Thus, when enough nodule bacteria were present, seedling P concentration became a key factor for optimal nodulation if the P concentration was low. P concentrations in the seedlings

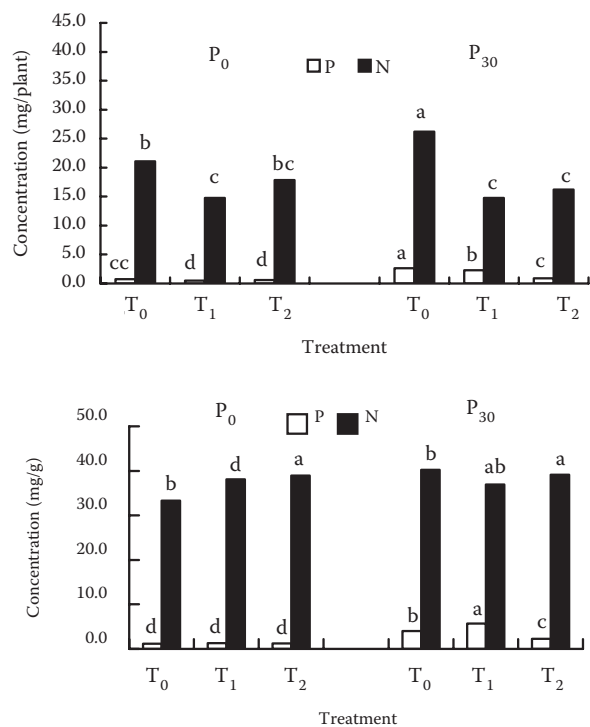


Figure 7. Nitrogen and P concentrations of soybean grown at T₀, T₁ and T₂ seedlings and P₀ and P₃₀ levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

Note: P and N concentrations in plants were statistically analyzed separately

were higher than required P level for rhizobia growth and survival (0.5 μM P) (O'Hara et al. 1988). This showed that seedlings contained enough P to establish nodules, and were thus comparable with the seeds that reserved P as reported previously (Valverde and Wall 1999). Although the plants of T_2 seedlings had the lowest P_0 concentration, nodule number was bigger than in T_1 , which might result from the fact that the plants in T_2 had higher biomass than T_1 , and so more photoassimilates were transferred to nodule for nodule formation and growth (Voisin et al. 2003). Chlorophyll concentration in the youngest expanded leaves at P_{30} level was in the order: $T_0 > T_1 > T_2$ in the 5th week, indicating that photosynthesis could not explain the variability in growth of roots and nodules (Voisin et al. 2003). Hence, in this experiment, plant dry weight was $T_0 > T_2 > T_1$, and this trend was the same in the case of nodule number and nodule dry weight. Nevertheless, the effect of seedlings on plant growth was weaker than on nodule formation and development, suggesting thus that P had a direct and positive stimulation of nodulation in legumes (Jakobsen 1985, Israel 1987, 1993, Sanginga et al. 1989, Hellsten and Huss-Danell 2001). However, an elevated P level appeared to increase nodule development, which is in agreement with our previous reports (Miao et al. 2007) and other reports on soybean (Singleton et al. 1985, Drevon and Hartwig 1997).

Nodule function

This study suggested that P content in seedlings played a specific role in nodule function of soybean; its effect on nitrogenase activity per unit nodule mass was bigger than on total nitrogenase activity (Figure 4). In soybean, P deficiency-decreased nitrogenase activity was suggested to result from inhibited energy-dependent reactions in nodules (Tang et al. 2001), decreased Lb concentration in nodules or decreased bacteroid biomass (Sa and Israel 1991). In our experiment, low P in seedlings decreased Lb concentration per plant irrespective of P level, which may be caused by the fact that Lb is an oxygen-carrying heme protein in nodules, capable of binding oxygen to produce an oxygenated form only in active reduced state (Shleev et al. 2001). Thus, nodule was kept under a low free O_2 condition to prevent nitrogenase from irreversible inactivation by O_2 (Takashi et al. 2001). In mature nodules, Lb protein was detected at the onset of

nitrogen fixation (Katerina et al. 2000), and the higher nitrogenase activity plants had a relatively higher Lb concentration per plant in our experiment (Figure 6). Lb also played an essential role in nitrogen fixation in nodules (Appleby 1984, Becana and Sprent 1989).

Regardless of P level, the C_2H_2 -ID in nitrogenase activity under low P in seedlings was larger than that under high concentrations, which is consistent with previous findings on soybean (Ribet and Drevon 1995, Drevon and Hartwig 1997) and *Medicago truncatula* L. (Tang et al. 2001). At 0 $\mu\text{mol/l}$ P level, the C_2H_2 -ID reached the maximum value 40 min after C_2H_2 introduction into the gas mixture; however, the two peaks appeared after 20 and 50 min (Figure 5). The effect of seedlings and P level on nitrogen concentration per plant or per unit weight was much like that on ARA. These results show that P-increased ARA is associated with increased N concentration and N accumulation (Tang et al. 2001).

REFERENCES

- Appleby C.A. (1984): Leghemoglobin and Rhizobium respiration. *Ann. Rev. Plant Physiol.*, 35: 443–478.
- Becana M., Sprent J.I. (1989): Effect of nitrate on components of nodule leghemoglobins. *J. Exp. Bot.*, 40: 725–731.
- Claudio V., Alejandro F., Luis G.W. (2002): Phosphorus and the regulation of nodulation in the actinorhizal symbiosis between *Discaria trinervis* (Rhamnaceae) and Frankia BCU110501. *New Phytol.*, 153: 43–51.
- Drevon J.J., Hartwig U.A. (1997): Phosphorus deficiency increased the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta*, 201: 463–469.
- Hart A.L. (1989): Distribution of phosphorus in nodulated white clover plants. *J. Plant Nutr.*, 12: 159–171.
- Hellsten A., Huss-Danell K. (2001): Interaction effects of nitrogen and phosphorus on nodulation in red clover (*Trifolium pratense* L.). *Acta Agr. Scand.*, 49: 135–142.
- Israel D.W. (1987): Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiol.*, 84: 835–840.
- Israel D.W. (1993): Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. *Physiol. Plant.*, 88: 294–300.
- Jakobsen I. (1985): The role of phosphorus in nitrogen fixation by young pea plants (*Pisum sativum*). *Physiol. Plant.*, 64: 190–196.

- Katerina L., Gautam S., Robert V.K., Raularredondo-Peter (2000): Characterization of leghemoglobin from a mimosoid legume, *Leucaena esculenta*, root nodules. *R. Bras. Fisiol. Veg.*, 12: 37–44.
- Lu R.K. (2000): The Analysis Method of Soil Agro-Chemistry. Chinese Agricultural Academic Press. (In Chinese)
- Marschner H. (1986): Mineral nutrition in higher plants. Wd Ltd. The Greystone Press, Antrim, Northern Ireland.
- Miao S.J., Qiao Y.F., Han X.Z., AN M. (2007): Phosphorus deficiency decreases early nodule development in soybean (*Glycine max* L.). *Pedosphere*, 17: 36–43.
- O'Hara G.W., Boonkerd N., Dilworth M.J. (1988): Mineral constraints to nitrogen fixation. *Plant Soil*, 108: 93–110.
- Ribet J., Drevon J.J. (1995): Phosphorus deficiency increases the acetylene-induced decline in nitrogenase activity in soybean [*Glycine max* (L.) Merr.]. *J. Exp. Bot.*, 46: 1479–1486.
- Sa T.M., Israel D.W. (1991): Energy status and functioning of phosphorus-deficient soybean nodules. *Plant Physiol.*, 97: 928–935.
- Sanginga N., Danso S.A., Bowen G.D. (1989): Nodulation and growth response of *Allocasuarina* and *Casuarina* species to phosphorus fertilization. *Plant Soil*, 118: 125–132.
- Shleev S.V., Rozov F.N., Topunov A.F. (2001): A method for producing multiple forms of metleghemoglobin reductase and leghemoglobin components from Lupine nodules. *Appl. Biochem. Microbiol.*, 37: 195–200.
- Singleton P.W., AbdelMagid H.M, Tavares J.W. (1985): Effect of phosphorus on the effectiveness of strains of *Rhizobium japonicum*. *Soil Sci. Soc. Am. J.*, 49: 613–616.
- Takashi S., Noriyasu O., Hiroyuki F., Norikuni O., Kuni S., Takuji O. (2001): Changes in four leghemoglobin components in nodules of hypernodulating soybean (*Glycine max* [L.] Merr.) mutant and its parent in the early nodule developmental stage. *Plant Soil*, 237: 129–135.
- Tang C., Hinsinger P.J., Drevon J., Jaillard B. (2001): Phosphorus deficiency impairs early nodule functioning and enhances proton release in roots of *Medicago truncatula* L. *Ann. Bot.*, 88: 131–138.
- Valverde C., Wall L.G. (1999): Regulation of nodulation in *Discaria trinervis* (Rhamnaceae) – *Frankia* symbiosis. *Can. J. Bot.*, 77: 1302–1310.
- Voisin A.S., Salon C., Jeudy C., Warembourg F.R. (2003): Root and nodule growth in *Pisum sativum* L. in relation to photosynthesis: analysis using ¹³C-labelling. *Ann. Bot.*, 92: 557–563.
- Wu M.C., Xiao C.Z, Zheng P.Y. (1999): Study on the physiological function of phosphorus to soybean. *Sci. Agr. Sin.*, 32: 59–65.

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