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: T_0, normal germination; T_1, cotyledons removed; T_2, cotyledons removed 5 days earlier than in T_1; and two phosphorus levels (P_0 and P_{30}) on nodulation and nodule function in soybean [*Glycine max* (L.) Merr.] were investigated in nutrient solution culture. The number of nodules formed at P_0 was in the order $T_2 > T_0 > T_1$, but it was $T_0 > T_2 > T_1$ at P_{30}. 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 $T_0 > T_1 > T_2$, regardless of P level. For example, nodule size in T_0 was larger by 86% and 52% than T_2 at P_0 and P_{30}, respectively. Furthermore, regardless of P level, a specific acetylene reduction activity (ARA, μM C_2H_4/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 (T_0 and T_1) 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 macroelements indispensable for plant growth and development (Marschner 1986). Although soybean P_2O_5 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 $CaCl_2$ and 5 μmol/l H_3BO_3. Three P treatments were used on seedlings: (1) T_0, total P concentration was 1.316 mg/g, normal germination; (2) T_1, total P concentration was 1.232 mg/g, cotyledons were removed when the first true leaf partly expanded, and (3) T_2, total P concentration was 1.067 mg/g, cotyledons were removed 5 days earlier than in T_1. 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 [P_0] and 30 [P_{30}] μmol/l. Phosphorus was supplied as KH_2PO_4 in nutrient solution. The composition of the solution was as follows (in μmol/l): K_2SO_4, 600; $MgSO_4.7\ H_2O$, 200; $CaCl_2.2\ H_2O$, 600; H_3BO_3, 5; $ZnSO_4.7\ H_2O$, 0.75; $MnSO_4.H_2O$, 1; $CoSO_4.7\ H_2O$, 0.2; $CuSO_4.5\ H_2O$, 0.2; $Na_2MoO_4.H_2O$, 0.03; Fe-NaEDTA, 10.

Bradyrhizobium japonicum strain Hefeng 25 was added into pots as water suspension to reach the final concentration of about 10^5 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 H_2SO_4 and H_2O_2 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 T_0 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 T_0 at P_0 level was by 41% and 33% higher than in T_2, respectively; for P_{30} level they were higher by 63% and 33%. For both shoots

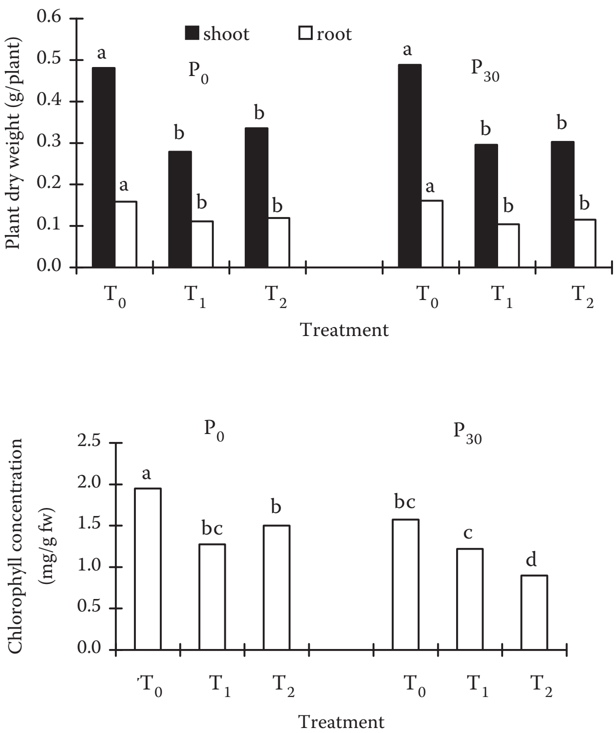

Figure 1. Dry weight of shoots and roots and chlorophyll concentration in the youngest expanded leaves of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels after 5 weeks; letters on the top of the bars indicate significant differences at 5% level

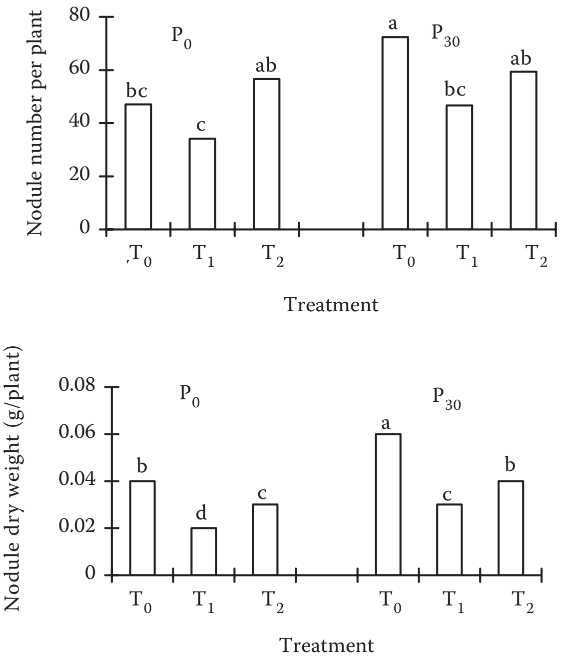

Figure 2. Number of nodules and nodule dry weight of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

and roots, dry weight was in the order: $T_0 > T_2 > T_1$ (Figure 1). Significant differences were recorded in the case of dry weight of shoots and roots between T_0 and T_2; however, no significant differences were observed between T_0 and T_1 or T_1 and T_2. 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 P_0 level, seedlings did not significantly affect nodule number; it ranged from 36 to 50 per plant in the order: $T_2 > T_0 > T_1$; at P_{30} level it ranged from 48 to 60 per plant in the order: $T_0 > T_2 > T_1$. A significant difference was found between T_0 and T_2 (Figure 2): P_{30} level increased the nodule number per plant; T_0 and T_2 treatments showed significant differences. For both P levels, nodule dry weight per plant ranged from 0.021 to 0.061 g in the order: $T_0 > T_2 > T_1$ (Figure 2); for P_{30} treatment, nodule number and nodule dry weight per plant in T_0 were by 11% and 75% higher than in T_2. 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: $T_0 > T_1 > T_2$ (Figure 3). Dry weight per nodule in T_1 was by 86% and 52% higher than in T_2 at P_0 and P_{30} levels, respectively. A statistical analysis showed that the effect of seedlings on nodule size was significant at P_0 level; however, at P_{30} level, this effect was found only between T_0 and T_1, or between T_0 and T_2 (Figure 3).

Nodule function

At the same P level, specific ARA (μM C_2H_4/h/g nodule) in T_0 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 (μM C_2H_4/h/g plant) was minor at both P levels, but P level significantly increased total ARA in T_2 (Figure 4). The total ARA in T_2 seedlings was the largest at P_{30} level (8.21 μM C_2H_4/h/g nodule), followed by T_0 (P_{30}), T_1 (P_0), T_2 (P_0), T_0 (P_0) and T_1(P_{30}). C_2H_2-induced decline (C_2H_2-ID) of ARA was observed in all the treatments (Figure 5). When 10% C_2H_2 was introduced into the gas mixture bottle with nodulated roots, the amount of C_2H_4 produced increased, and then decreased with duration of C_2H_2 exposure time, but the time until reaching the maximum value was different. At P_0 level, the amount of C_2H_4 from nodule activity

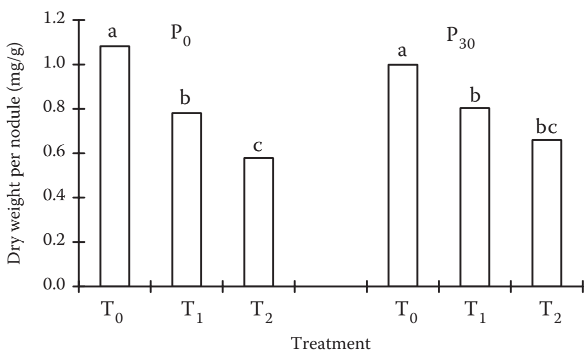

Figure 3. Nodule size (dry weight per nodule) of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

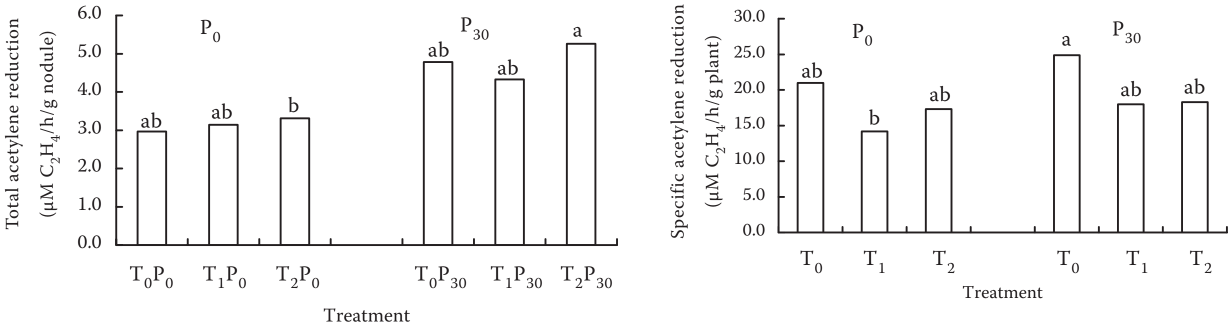

Figure 4. Total and specific acetylene reduction activity (ARA) of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week; ARA was the average ARA measured between 10 and 60 min after C_2H_2 exposure; letters on the top of the bars indicate significant differences at 5% level

increased, reaching the maximum value 40 min after initial exposure to C_2H_2. However, a double peak curve of C_2H_2-ID was produced by the nodule activity at P_{30} level (Figure 5).

The effect of seedlings on leghemoglobin (Lb) concentration per nodule was greater than the effect of P level, especially in T_0 and T_1 seedlings; a significant difference was found in these

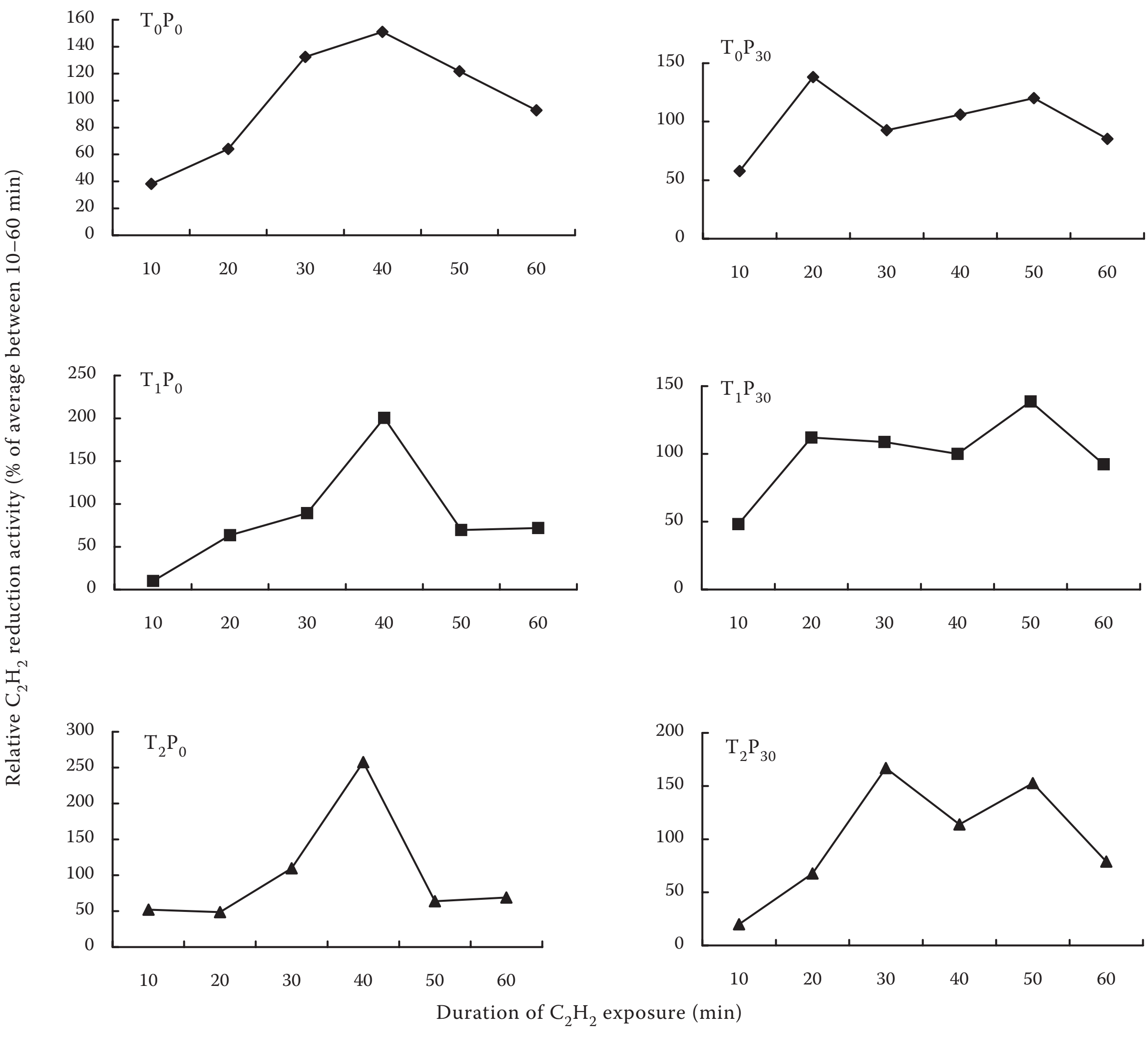

Figure 5. Time course of acetylene reduction activity (ARA) measured on soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week

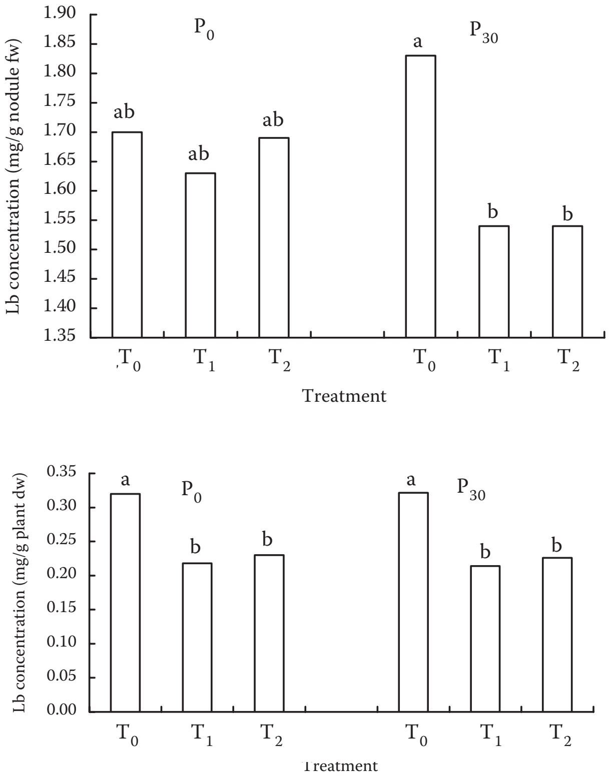

Figure 6. Leghemoglobin (Lb) concentration of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

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 P_{30}. 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: $T_0 > T_2 > T_1$, except for nitrogen concentration per unit weight at P_0 level (Figure 7). Regardless of P level, a significant difference of nitrogen concentration per plant was found between T_0 and T_1. For nitrogen concentration per unit weight, a significant difference was observed between T_0 and T_2 at both P levels (Figure 7). Seedling effect on P concentration per plant or per unit weight was low at P_0 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 T_0 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 T_0 seedlings was 1.316 mg/g, for T_1 it was 1.232 mg/g, and for T_2 it was 1.067 mg/g. Nodule number was significantly affected by P levels in T_0 and T_2, not in T_1 (Figure 2). This is because T_2 seedlings had a longer total root length, which provided more chances for bacteria infection than T_1. 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

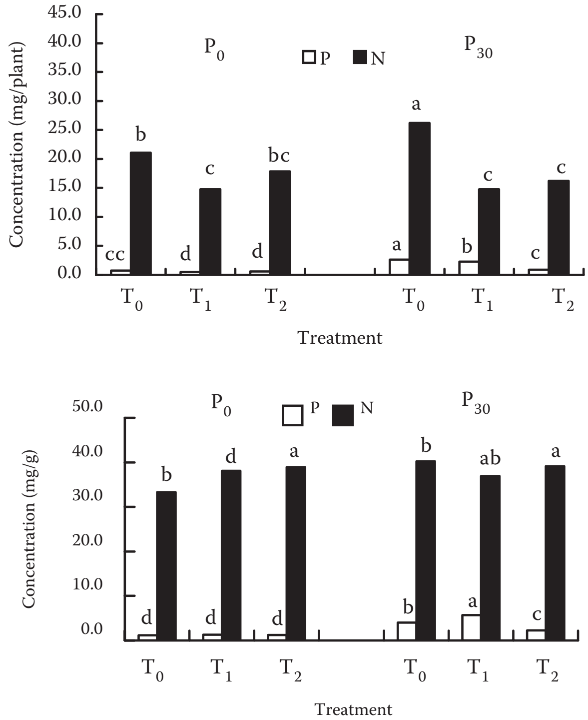

Figure 7. Nitrogen and P concentrations of soybean grown at T_0, T_1 and T_2 seedlings and P_0 and P_{30} levels in the 5th week; letters on the top of the bars indicate significant differences at 5% level

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 μmol/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).

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