Proton Release by Roots Differs among N₂-fixing Recombinant Inbred Lines of Common Bean (*Phaseolus vulgaris* L.) Under Phosphorus Deficiency

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Abstract: Changes in plant rhizosphere pH which result from an imbalance of ion uptake can be detected in the rhizosphere of several dicotyledonous plants. Acid production provided through legume roots, either directly or indirectly linked to N₂ fixation, makes contribution to subsoil acidification in the long term. This study was conducted to evaluate the influence of using high (250 µmol) and low (75 µmol) P supply on proton release, growth, nodulation and N concentration for six common bean recombinant inbred lines inoculated with *Rhizobium tropici* CIAT 899 in hydroaeroponic culture under glasshouse conditions. Data showed that all studied genotypes had similar shoot dry weight under P sufficiency but differed under P deficiency although root dry weight was independent of P supply. Total proton release generally increased with time regardless of P supplies. Thereafter, P deficiency plants released fewer protons than P sufficient ones. Nitrogen concentrations in shoots and nodules were affected by high P level while in roots there was a variation among genotypes. There were different correlations between P and N concentrations in nodules of the six genotypes (r²= 0.862, 0.78, 0.774, 0.736, 0.649 and 0.524). It is concluded that the nodulated common bean releases a substantial amount of protons into the rhizosphere that is linked to the symbiotic N₂ and differ among genotypes that were contrasting in their adaptation to P limitation.

Key words: acidification; common bean; hydroaeroponic culture; H⁺ exudation; N₂-fixation; nodulation; *Phaseolus vulgaris* L.; phosphorus nutrition.

INTRODUCTION

The availability of phosphorus is one of the most significant determinants of plant growth (Wang *et al*., 1998). Many plant species have developed various strategies of enhancing phosphorus acquisition from P-deficient plant culture. These strategies include increased root surface area, decreased rhizosphere pH and increased root exudation of carboxylates (Raghothama, 1999, Hinsinger *et al*., 2003). Plants supplied with nitrate ion (NO₃⁻) favor excess uptake of anions over cations and release hydroxyl ions to balance the electrical negative charges (Tang and Rengel, 2003). In contrast, legume plants reliant on N₂ fixation with adequate P supply take up more cations than anions and release protons at root-soil interface for compensation of electrical positive charges and regulation of cytosolic pH in the root cells. Of all nutrients, shortage of phosphorus has the biggest impact on legumes which generally rely on N₂ fixation for nitrogen nutrition.

The deficiency of a nutrient may limit N₂ fixation through its effects on growth and survival of rhizobia, nodule formation, nodule functioning and host plant growth. The effect of P supply on growth and N₂ fixation in legumes has been studied extensively, but the role of P in the symbiotic process remains unclear yet. Nevertheless, Israel (1987) demonstrated that symbiotic N₂ fixation in soybean requires more phosphorus for optimal functioning than either host plant growth or N assimilation in the plant. In addition, phosphorus deficiency has previously been reported to decrease nodule mass more than host growth in soybean and common bean (Drevon and Hartwig, 1997; Kouas *et al*., 2005).

A genotypic variability in P use efficiency for symbiotic nitrogen fixation was associated with a variation in nodulated-root proton efflux and respiration in cowpea rhizosphere (Alkama *et al*., 2009). In common bean, soybean, lupin and alfalfa, P deficiency has been shown to reduce the number and biomass of nodules as well as their nitrogenase activity (Kouas *et al*., 2005; Qiao *et al*., 2007). Acid production by N₂ fixing legumes is positively correlated with the excess cations or ash alkalinity in nutrient solutions. Acidification of soils under legumes has been shown to vary among species (Tang *et al*., 1997) and to be affected by the availability of nutrients under grown conditions and plant growth stages (Pierre and Banwart, 1973).

Fang *et al*., 1998 found a relation between total acid production and total content of excess cations in the whole plant, irrespective of P supply and plant species, and suggest that acid production by legumes under various P supplies is related to the imbalance of cation and anion uptake. Therefore the objective of this work

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was to address the genotypic variation on proton release by nodulated-roots, and its relation with P supply, the subsequent nitrogen concentration and growth of plant in six common bean Recombinant Inbred Lines RILs of the cross of BAT477 by DOR364.

**MATERIAL AND METHODS**

**Hydroponic Culture:**

The experiment was conducted in a glass-house with six common bean (*Phaseolus vulgaris* L.) genotypes namely RILs 34, 75, 83, 104, 115, and 147 that were used in this study. Common bean seeds were sterilized and germinated. Four or five days after sowing (DAS), seedlings were mixed with an inoculant contains approximately 108 cells ml⁻¹ of *Rhizobium tropici* CIAT899, provided by International Center for Tropical Agriculture (CIAT), Colombia. After 30 min, common bean seedlings were carefully transplanted to 1L serum bottles by roots through the hole of rubber stopper on the bottle neck, and cotton wool was fitted at the hypocotyl level to maintain the root system suspended in nutrient solution (Vadez *et al*., 1996) corresponding to P limited and P non-limited treatments. Common bean seedlings were grown in a solution contains in ml L⁻¹; 1.0KH₂PO₄, 1.5K₂SO₄, 2.0MgSO₄·7H₂O, 0.75 CaCl₂, 0.5 (sequestrine Fe⁺³), 0.5 urea, 0.4 from each of the following oligo-elements i.e.; H₃BO₃ (4.0 mM); MnSO₄·H₂O (6.0 mM); ZnSO₄·7H₂O (1.0 mM); CuSO₄·7H₂O (1.0 mM); Na₂MoO₄·7H₂O (0.1 mM) and 75 and 250 µmol KH₂PO₄ plant⁻¹ week⁻¹ as described by Hernandez and Drevon (1991). Bottles were wrapped with aluminum foil to maintain darkness in the rooting environment and aerated with a flow of 400 ml min⁻¹ of filtered air *via* a compressor and spaghetti-tube distribution system. The nutrient solution was first replaced at 15 DAS and subsequently each week. The experiment was set up in a glasshouse under controlled conditions (28/20 C° day/night temperatures, 16 h photoperiod) with an additional illumination of 400 µmol photons m⁻² s⁻¹ and 70% relative humidity during the day as a complete factorial arrangement with two P levels and six bean genotypes in five replicate. The level of nutrient solution was readjusted to 1 liter with deionized water at least twice a week to maintain nodulated-roots in submerged conditions.

**Plant Sampling and Analytical Techniques:**

Common bean plants were harvested at flowering stage (45 days DAS) with the first pod measuring 3 cm long. Shoots were separated from the roots at the cotyledonary node, and then weighed after oven drying for 48 h at 70 °C. Nodules were separated from roots, then counted and weighted after oven drying for 48 h at 70 °C. The dried plant samples were milled agate mortar until they passed through a screen with a diameter of 0.5 mm sieve and kept in plastic containers until analyzed. Nitrogen (N) content was determined using CHO-O-Rapid elemental analyzer (Heraeus, Hanau, Germany). P was determined colorimetery using spectrophotometer in the digested solution of common bean plants using micro-node assisted acid digestion procedure. Sample masses around 50 mg were digested with 1.5 ml of 65% (mv⁻¹) HNO₃ and 10 ml distilled water in PFA vessels. The heating program was performed in three steps: 1 - ramp (150 °C / 5 min); 2- ramp (180 °C / 10 min); 3-cooling to 40 °C / 15 min) using MO-MET-ECH-PRE Micro-ondes 02 model 2005.

**Statistical Analysis:**

The results were statistically analyzed by analysis of variance and calculation of standard deviations of the means were compared by the least significant difference test (LSD) at *P*<0.05 according to the methods described by Gomez and Gomez, (1984). The regression model of nitrogen concentration in nodules as a function of phosphorus in nodules was performed with covariance analysis.

**Results:**

**Shoot and Root Growth:**

The P supply, RILs and the interaction between P supply and RILs affected significantly the shoot biomass. Figure 1A shows an increase in shoot biomass with the higher level of P in all genotypes by more than 17% for RIL 115 and about 15% for RILs 83 and 147 as compared with the limited level of P. On the contrary Fig. 1B shows no significant effect of P supply on the root biomass for RILs 83, 147 and 75. On the other hand under P deficiency, root biomass was significantly decreased for RILs 34, 104 and 115 by more than 15% as compared to the P sufficiency level. Thus, the ratio of shoot to root DW was significantly more decreased under P deficiency for RILs 83 and 75 than for RILs 34, 104 and 115 (Fig. 1C).

**Nodulation Status:**

The P supply, RILs and interaction between P supply and RILs affected significantly the nodule biomass and nodule numbers. Figure 2A shows an increase in nodule biomass with the higher level of P by more than 50% for RILs 34 and 83 and about 20% for RIL 104 as compared to the limited P supply. Figure 2B shows a significant increase with the P sufficiency in nodule numbers: nodule numbers were more than two folds higher.
than under P deficiency for RILs 34 and 75 and more than 20% for RIL 147 and about 15% for RILs 104 and 115.

![Graph](image)

**Fig. 1:** Dry weight (g plant\(^{-1}\)) of shoot (A), root (B) and shoot to root dry weight ratio (C) for six recombinant inbred lines common bean grown under P sufficiency and P deficiency. Data are means and error bars represent standard deviation (SD) of 5 replicates harvested at 45 days after sowing.

**Proton Release:**
Growing plants released protons and decreased the solution pH. Total proton release generally increased with time (Fig. 3) under P sufficiency. The proton efflux in the six genotypes was significantly greater under P sufficiency than under P deficiency (Fig. 3). RILs and the interaction between P supply and RILs affected significantly the H\(^+\) release cumulated during 12 days after transfer. For both P deficiency and sufficiency, the highest H\(^+\) release was observed for 115 against the lowest one for 83 with a difference of about 60% between
both RILs (Fig. 3). The H⁺ release of 115 was significantly higher than that of 75, 104, 34 and 147 by 18%, 27%, 39% and 44%, respectively.

Fig. 2: Dry weight (g plant⁻¹) (A), and number (B) of nodule for six recombinant inbred lines common bean grown under □ P sufficiency and ■ P deficiency. Data are means and error bars represent standard deviation (SD) of 5 replicates harvested at 45 days after sowing.

**Nitrogen Content in Shoots, Roots & Nodules:**

The six genotypes showed a pattern of nitrogen concentration in shoots under P limited supply lower than that under the P sufficient level Fig (4 A, B and C). On the other hand, there was a variation among the six genotypes of common bean. Nitrogen content in shoots was similar (around 15 mg g⁻¹) under P deficiency, except for RIL147 that had N content up to 20 mg g⁻¹. Under P sufficiency, N accumulation in shoots was significantly high for RILs 83, 75 and 104 with about 45, 30 and 25% respectively, compared to P-deficiency treatment (Fig. 4A).

Nitrogen content in roots of three genotypes (RILs 75, 83 and 147) showed a similar pattern in response to high and low phosphorus level. While, RILs 34, 104, 115 showed a significantly high nitrogen content in roots under high P level as compared with low P level.

In case of N content in nodules, all genotypes showed significant high values under the high P level. Figure 4C shows that nitrogen in nodules was significantly higher (around 60 mg Kg⁻¹) than in shoots and roots regardless of the RIL or the P supply. Under P sufficiency, the significantly highest values of nodule N content was 59.8, 58.0 and 57.5 mg g⁻¹ (LSD = 0.39, p < 0.05) for RILs 104, 34 and 83, respectively. Whereas under P deficiency, the significantly highest values were 44.5, 37.2 and 36.2 mg g⁻¹ (LSD = 0.45, p < 0.05) for RILs 83, 75, and 147, respectively.

**Relation Between Phosphorus and Nitrogen in Nodules:**

Data in Fig (5) showed a high correlation (r² = 0.862, 0.78, 0.774, 0.736) and a fair correlation (r² = 0.649 and 0.524) between P and N concentration in nodules of the six genotypes for RILs 115, 83, 104, 147, 34 and 75 respectively, under P sufficiency. On the other hand under P deficiency, the six genotypes in Fig (5) showed very low.
**Fig. 3:** Temporal evolution of cumulated H⁺ efflux of 6 common bean genotypes under □ P sufficiency versus ■ P deficiency. Data are the means of 5 replicates harvested at 45 days after sowing.

**Discussion:**

This study reports the response of six different common bean genotypes to two different P supply on proton release by roots, nitrogen concentration, plant growth and nodule development. It was observed that lower values of growth were with P deficiency (75 µmol) than with P sufficiency (250 µmol). Genotypes having large root systems or high root/shoot ratio and with greater exposure to P sufficient solution could be expected to benefit more. Genotypes with high P uptake rates per unit root length would also be favored (Christiansen and Graham, 2002). The results showed that the nodule biomass per unit plant biomass was stimulated by an increase in P supply, whereas increasing the P supply had no effect on the root dry weight, indicating that the effect of external P on the nodulation was specific to and independent of the host plant growth as proposed by Gentili and Huss-Danell (2003).
Fig. 4: N content (mg kg⁻¹) in shoot (A), root (B) and nodules (C) of six common bean genotypes grown under □ P sufficiency and ■ P deficiency. Data are the means of error bars replicates standard deviation (SD) of 5 replicates harvested at 45 days after sowing.

The higher H⁺ release in all studied genotypes correlated with their better adaptability to P limitation, suggesting an involvement of the root acidification capacity in the adaptation of common bean to this a biotic constraint (Kouas et al., 2008). There are several previous reports of increased H⁺ release under P deficiency (Neumann and Romheld, 1999, Bertrand et al., 1999 and Hinsinger, 2001). Several of these authors stressed that decreased NO₃⁻ uptake was the major cause of the observed increase of H⁺ under P deficiency.

Cultivar variation in N₂ fixation is well documented in beans (Chaverra and Graham, 1992) and is amenable to plant improvement (Elisondo-Barron et al., 1999).

The present study illustrates that the P supply in the solution had an important role in nitrogen fixation. This was supported with two lines of evidence. Firstly, increasing the P supply increased the N concentration in shoots and roots and secondly, the N in shoots correlated well with P. However, the N fixed per nodule biomass significantly decreased at a higher P supply, suggesting that P did not have a specific role in the nodule function.
Therefore, the increased N fixation relative to P supply resulted from the enhanced number of nodules. These results were consistent with the previous findings by Hogh-Jensen et al., (2002) and Miao et al., (2006).

correlation ($r^2 = 0.0.328, 0.315, 0.292, 0.044, 0.003$ and $0.001$) for all genotypes.

**Fig. 5:** Effect of P nutrition on the regression parameter of nodule N content (mg kg$^{-1}$) as a function of nodule P (mg kg$^{-1}$) of common bean recombinant inbred lines 83, 75 and 147 (A and B) and 34, 104 and 115 (C and D) under P sufficient (A and C) or P deficient (B and D). Data are individual values of 5 replicates harvested at 45 days after sowing.

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