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Effect of phosphorus deficiency on the growth, symbiotic N₂ fixation and proton release by two bean (*Phaseolus vulgaris*) genotypes

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Abstract – This study compared the growth, nodulation, N₂ fixation and proton release by two bean genotypes (*Phaseolus vulgaris* L., BAT477 and DOR364) in response to P deficiency in nutrient solution. For plants grown at 5 μM P for 42 days, shoot and root biomass were 55–62% and 82–88%, respectively, of that of plants grown at 25 μM P. Phosphorus deficiency decreased nodule biomass and size, with the decrease being smaller in BAT477 than in DOR364. Total nitrogenase activity of plants grown at 5 μM P was 50% of that of plants grown at 25 μM P, and reached the maximum earlier in BAT477 than in DOR364. However, nitrogenase activity per unit nodule mass was similar in both genotypes and was unaffected by P supply. Proton release was generally similar in both genotypes. There were high correlations between proton release and uptake of excess cations, with a slope of 1.44 for BAT477 and 0.85 for DOR364. It is suggested that the two genotypes differ in root exudation.

P deficiency / nitrogenase activity / proton release / cation-anion balance

Résumé – Effet de la carence phosphatée sur la croissance, la fixation de N₂ et la libération de protons chez deux génotypes de haricot (*Phaseolus vulgaris*). Cette étude avait pour objet de comparer en termes de croissance, de fixation de N₂ et de libération de protons la réponse à la carence en phosphore de deux génotypes de haricot (*Phaseolus vulgaris* L., BAT477 et DOR364) cultivés sur solution nutritive. Pour des plantes âgées de 42 jours, les croissances aériennes et racinaires des plantes recevant 5 μM P étaient respectivement 55–62 % et 82–88 % de celles des plantes recevant 25 μM P. La carence P diminuait la masse et la taille des nodosités. Cette décroissance était plus faible chez BAT477 que chez DOR364. L'activité nitrogénase totale des plantes recevant 5 μM P était 50 % de celle des plantes recevant 25 μM P. L'activité maximale était observée plus tôt avec BAT477 qu'avec DOR364. Néanmoins l'activité nitrogénase par unité de biomasse nodulaire était identique chez les deux génotypes et n'était pas affectée par la carence P. La libération de protons par les racines était corrélée significativement au bilan cations-anions prélevés. La pente de la droite de régression correspondante était de 1,44 avec BAT477 et de 0,85 avec DOR364. Il est conclu que les deux génotypes diffèrent par leurs excretions racinaires.

déficience en P / activité de la nitrogénase / équilibre cation-anion / libération de protons

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1. INTRODUCTION

Phosphorus deficiency is one of the most important factors limiting the production of bean (*Phaseolus vulgaris* L.) in many parts of the world. Phosphorus deficiency appears to affect symbiotic N₂ fixation in bean more than in other legume crops [23]. Plants deficient in P had a decreased nodule number [14, 21], and decreased nodule biomass when grown in soil, sand and alkaline solution [2, 14, 21].

Bean genotypes differ in N₂ fixation ability and P-use efficiency under P-deficiency [14, 23, 24]. When subjected to P-deficient conditions in the field, BAT477, a breeding line tolerant to drought, fixes more N₂ and yields better than DOR364, a disease-resistant cultivar in Central America (Beebe, personal communication). In addition, BAT477 produced a shallower root system than DOR364, which has been previously related to P-use efficiency [9].

Legume plants reliant on N₂ fixation take up more cations than anions and release H⁺ at the root-soil interface [16, 19]. The increased rhizosphere acidification is a root response to P deficiency and a strategy of an efficient plant to acquire P under such conditions [5]. Most of the previous work showing such responses has however been conducted in rather extreme conditions of P deficiency, comparing P-sufficient plants with plants that received no P at all [8, 12]. In addition, responses to low P supply have been little studied for legumes relying on N₂ fixation. It is unknown whether P deficiency in bean will induce more proton release due to an imbalance of cation-anion uptake by plants relying on N₂ and whether bean genotypes differ in proton release and thereby possibly in the capacity to acidify the rhizosphere and enhance P acquisition.

The present study aimed to compare the growth and N₂ fixation of BAT477 and DOR364 in response to P deficiency and to study how P deficiency affected cation-anion uptake, and hence proton release in these two bean genotypes differing in tolerance to P deficiency.

2. MATERIALS AND METHODS

The study was conducted in a glasshouse with day/night temperatures of around 30/25 °C and 16 h photoperiod of natural light supplemented with mercury vapor lamps. The experiment was set up as a complete factorial with two P levels (5 and 25 µM) and two bean genotypes (*Phaseolus vulgaris* L. BAT477 and DOR364). Treatments were replicated five times and completely randomized.

Uniformly-sized seeds were germinated for 6 d in vermiculite moistened with deionized water and inoculated with a dense suspension of *Rhizobium tropici* (CIAT899), containing approximately 10⁸ cells·mL⁻¹. One seedling was carefully transplanted to each of 1000 mL glass bottles. The roots were gently passed through the hole of a rubber stopper on the bottle neck, and cotton wool was fitted at the hypocotyl level. The bottles were wrapped with aluminium foil to maintain darkness in the rooting environment. The bottles contained nutrient solution of the following composition (µM): K₂SO₄, 700; MgSO₄, 500; CaCl₂, 800; H₃BO₃, 4; Na₂MoO₄, 0.1; ZnSO₄, 1; MnCl₂, 2; CoCl₂, 0.2; CuCl₂, 1; FeNaEDTA (ferric monosodium salt of ethylenediamine tetraacetic acid), 10; and MES [2-(N-morpholino)ethane-sulfonic acid], 200. Phosphorus was added as KH₂PO₄. The rhizobial suspension was added to the solution at a rate of about 10⁸ cells·L⁻¹. This solution was kept unchanged for 3 d, then replaced by a similar solution without added rhizobia. The solutions were aerated [4, 22] and changed every second day. The solution pH was adjusted daily to 6.5 with KOH. Solution pH and amounts of KOH used were recorded.

Nitrogenase activity of nodules (acetylene reduction activity – ARA) was measured in situ, between 10:00 and 16:00 h as previously described by Drevon et al. [4], using a steady-flow gas mixture of 69 kPa N₂, 21 kPa O₂ and 10 kPa C₂H₂, with two replicates each day. To avoid nodule disturbance, the level of the solution was lowered to 70% of the bottle volume 1 d before the assay. The C₂H₄ concentration in the outflow was determined 2 to 50 min after C₂H₂ exposure with a Delsi 30 gas chromatograph (Delsi Instruments, Suresnes, France) equipped with a flame ionization detector. The ARA values were computed as C₂H₄ concentration by flow rate, and expressed as µmol C₂H₄·h⁻¹·plant⁻¹. The means of 5 replicates were plotted as a function of the median day of the measurement period.

The plants were harvested at 42 DAT (days after transplanting), and shoots, roots and nodules were separated. The plant material was dried and finely ground. A 200-mg subsample of ground material was digested in concentrated HNO₃ and then HNO₃-HClO₄ according to the AOAC procedure [1]. Phosphate was assayed using the vanado-molybdate method [1]. Sulphur was assayed by inductively coupled plasma emission spectrometry (Varian, Australia), while K, Na, Ca and Mg were assayed by flame atomic absorption spectrometry (Varian AA-600, Australia). For N determination, the 10–30 mg sample of root or shoot material was digested in hot concentrated H₂SO₄ [11], with an addition of salicylic acid (10%). Ammonium in the digest was determined colorimetrically using the phenol hypochlorite

method [10]. Chloride was measured by high performance ion chromatography (Dionex, USA) after extraction in water for 1 h.

Total amounts of protons released by plants were determined by summing up the amounts of KOH used for pH adjustment during plant growth and for titrating the used solution after plant growth to the pH of the no-plant control solution. Concentrations of excess cations in plant were calculated from individual elements as the sum of charge concentrations of Ca²⁺, Mg²⁺, K⁺ and Na⁺ minus the sum of H₂PO₄⁻, SO₄²⁻ and Cl⁻ [20].

3. RESULTS

3.1. Plant growth and nodulation

Shoot dry weights of plants grown at 5 µM for 42 days was 55–62% of that of plants grown at 25 µM P (Tab. I). Dry weights of roots were also lower at 5 µM P as compared with 25 µM P, but the decrease in root weight by P deficiency was less than the effect on shoot weight. Consequently, the root/shoot dry weight ratio increased as P supply decreased (Tab. I).

Nodules first appeared at 4 DAT. At 42 DAT the nodule number was not influenced by P supply or genotype (Tab. I). However, dry weights of nodules decreased

with decreasing P supply. Whereas nodule sizes were similar in both genotypes grown at 25 µM P, at 5 µM P the nodule size of BAT477 was twice as much as that of DOR364 (Tab. I).

3.2. Nodule nitrogenase activity

Total acetylene reduction activity (ARA) per plant decreased with decreasing P supply (Fig. 1). The plant ARA of BAT477 increased with time and reached a maximum at 31 DAT. Whereas ARA values at 24 and 42 DAT were similar in both genotypes, ARA of BAT477 at 31 DAT was 22 and 65% higher than that for the P-sufficient and P-deficient plants, respectively, of DOR364. By contrast, nodule ARA, i.e. ARA per gram nodule dry weight (µmol C₂H₄·h⁻¹·g⁻¹ nodule) measured at 42 DAT was not significantly affected by P supply or genotypes (Tab. I).

3.3. Proton release

The two genotypes displayed a similar pattern of proton release during the experimental period (Tab. I and Fig. 2). Total proton release was not affected by P supply before 20 DAT for BAT477 and 22 DAT for DOR364.

Table I. Plant growth, nodulation, nitrogenase activity and proton release by two genotypes (BAT477 and DOR364) of *Phaseolus vulgaris* grown at 5 or 25 µM P for 42 days.

Genotypes	BAT477		DOR364		LSD (<i>p</i> =0.05) for any two means	Significance		
	5 µM	25 µM	5 µM	25 µM		Geno	P	Interaction
<i>Plant growth</i>								
Shoot dry weight (g·plant ⁻¹)	1.93	3.50	1.76	2.82	0.81	n.s.	***	n.s.
Root dry weight (g·plant ⁻¹)	0.80	0.98	0.79	0.90	0.20	n.s.	*	n.s.
Root:shoot DW ratio	0.41	0.29	0.45	0.32	0.06	n.s.	***	n.s.
<i>Nodulation</i>								
Nodule number (no plant ⁻¹)	49	60	69	66	n.s.			
Nodule dry weight (mg·plant ⁻¹)	202	341	154	312	68	n.s.	***	n.s.
Nodule size (mg·nodule ⁻¹)	4.3	5.8	2.2	5.2	1.5	*	***	n.s.
<i>Nitrogenase activity</i>								
Specific (µmol C ₂ H ₄ ·h ⁻¹ ·g ⁻¹ nodule)	99	86	133	104	n.s.			
<i>Proton release</i>								
Total (mmol·plant ⁻¹)	2.52	3.17	2.39	3.01	0.45	n.s.	***	n.s.
Specific (mol·kg ⁻¹ shoot+root)	1.20	0.87	1.23	0.96	0.19	n.s.	***	n.s.
Specific (mol·kg ⁻¹ root)	4.56	4.43	4.34	4.35	n.s.			
Specific (mol·kg ⁻¹ nodule)	12.9	9.5	15.9	9.9	3.4	n.s.	***	n.s.

n.s., not significant; *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001.

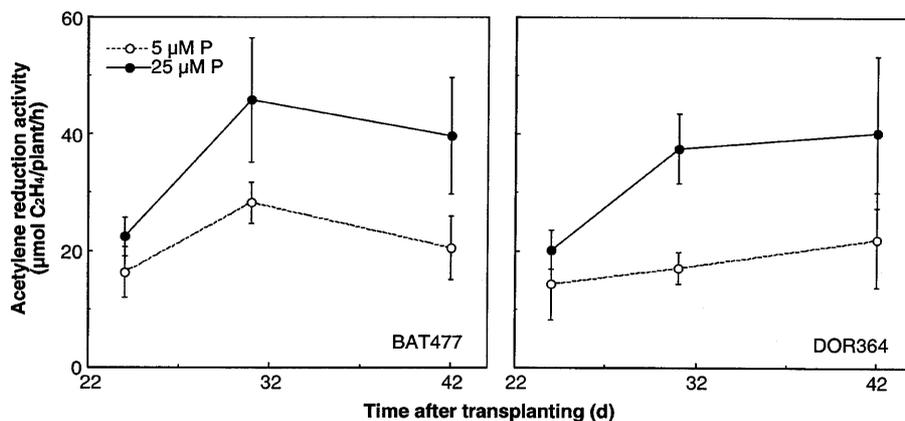


Figure 1. Acetylene reduction activity (ARA) of nodules of BAT477 and DOR364 grown at 5 and 25 μM P for 24, 31 and 42 days. ARA values represent the average measured between 4 and 50 min after C_2H_2 exposure. Vertical bars represent \pm standard errors of means of 5 replicates.

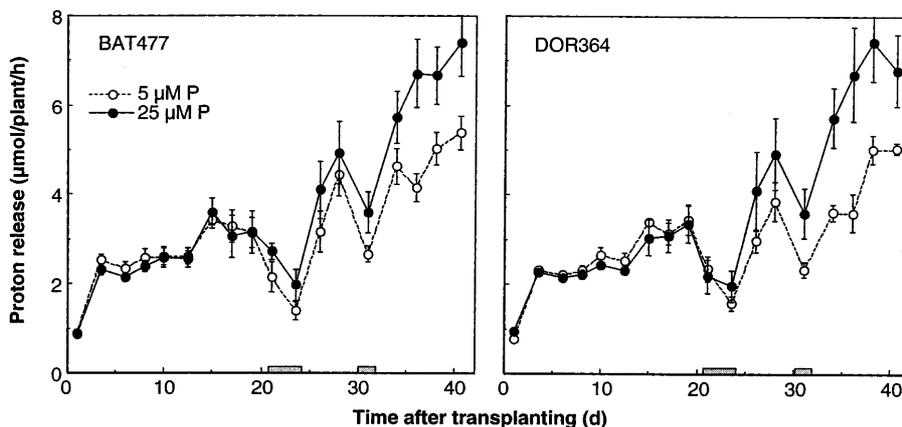


Figure 2. Time course of total proton release of BAT477 and DOR364 grown at 5 and 25 μM P for 42 days. Dotted bars on the X axis indicate the time of the acetylene reduction measurements. Vertical bars represent \pm standard errors of means of 5 replicates.

Thereafter, P-deficient plants released less protons than P-sufficient plants (Fig. 2). Total proton release generally increased with time (Fig. 2). The exceptions were that the proton release markedly decreased after each of the ARA measurements, indicating that the ARA measurement was detrimental to root activities.

Total amounts of protons released during the 42-d growth period was 21% lower in P-deficient plants than in P-sufficient ones (Tab. I). Proton release per unit plant biomass (roots plus shoot) was 28–38% higher at 5 μM P than at 25 μM P. By contrast, proton release per unit root biomass was not affected by P supply (Tab. I). Both total

and per unit biomass proton release were not significantly different between the two genotypes (Tab. I).

3.4. Chemical composition

Nitrogen concentrations in shoots and roots were not affected by P treatments. The two genotypes had a similar nitrogen concentration in shoots, but BAT477 had higher N concentration in roots than DOR364 (Tab. II). Total amounts of N fixed during the experimental period, as determined by the N budget deduced from plant analysis, were 61–76% higher at 25 μM P than at

Table II. Chemical compositions of two genotypes (BAT477 and DOR364) of *Phaseolus vulgaris* grown at 5 or 25 μM P for 42 days.

Genotypes	BAT477		DOR364		LSD ($p=0.05$) for any two means	Significance		
	5 μM	25 μM	5 μM	25 μM		Geno	P	Interaction
<i>N</i> concentration ($\text{mg}\cdot\text{g}^{-1}$)								
Shoot	17.0	15.5	18.3	17.6	n.s.			
Root	17.8	18.2	15.7	16.7	2.4	*	n.s.	n.s.
<i>P</i> concentration ($\text{mg}\cdot\text{g}^{-1}$)								
Shoot	1.36	2.45	1.48	3.08	0.48	*	***	n.s.
Root	1.43	5.20	1.38	5.21	1.07	n.s.	***	n.s.
Nodule	3.16	5.38	3.37	6.33	0.79	*	***	n.s.
Total <i>N</i> fixed during days 14–42 ($\text{mg}\cdot\text{plant}^{-1}$)								
	30.2	53.2	30.3	48.9	6.6	n.s.	***	n.s.
Molar ratio of H^+ exuded to <i>N</i> fixed								
	1.19	0.87	1.12	0.91	0.23	n.s.	**	n.s.
Excess cations ($\text{cmol}\cdot\text{kg}^{-1}$)								
Shoot	90	68	109	91	16	**	**	n.s.
Root	40	7	45	32	13	**	***	*

n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

5 μM P, but were not significantly different between the two genotypes (Tab. II). The amounts of protons released per unit N fixed (molar ratio) were, on average, 23–37% higher at 5 μM P than at 25 μM P and were not significantly affected by genotype (Tab. II).

Concentrations of P in plant parts markedly decreased with decreasing P supply (Tab. II). Nodules had higher P concentration than roots at 5 μM P, and higher than shoots at both P levels. DOR364 had higher P concentrations in shoots and nodules than BAT477. The amounts of N fixed per unit P taken up in both genotypes were 2.4 times higher at 5 μM P than at 25 μM P (data not shown).

Decreasing P supply generally increased concentrations of K in shoots and Mg in shoots and roots, and decreased concentrations of S and Cl in roots. Compared to BAT477, DOR364 had higher concentrations of Ca, K and Mg in shoots and K in roots, but had lower concentrations of Ca, S and Cl in roots (data not shown). Decreasing P supply increased the concentration of excess cations in plants (Tab. II). DOR364 had higher concentrations of excess cations than BAT477 in both shoots and roots (Tab. II), with Ca and S contributing most to such differences in shoots and roots, respectively. There were good relationships between the amounts of protons released (Y) and excess cations taken up by

the plant (X), with a slope of 1.44 for BAT477 and 0.85 for DOR364 (Fig. 3).

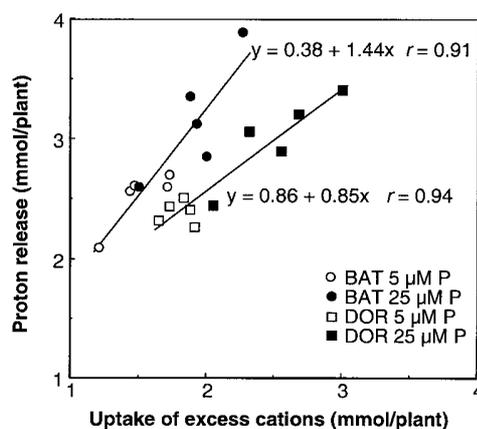


Figure 3. Relationships between total proton release and uptake of excess cations, i.e. excess of positive cationic charges, by BAT477 and DOR364 grown at 5 and 25 μM P during the experimental period. The slopes of the two lines are different at $p < 0.05$.

4. DISCUSSION

4.1. Proton release

The interesting finding in this study is a genotypic difference in the relationship between proton release and the uptake of excess cations. It was observed that BAT477 released more protons per unit excess cations taken up (a slope of 1.44, Fig. 3) than DOR364 (a slope of 0.85). This indicates that a part of the protons released by BAT477 did not result from the uptake of excess cations. One possible explanation is that BAT477 roots might have released organic anions that were not accounted for in the cation-anion balance. When exuded at large fluxes, organic anions can contribute some significant proportion of cation-anion balance as shown for citrate in white lupins [5, 18].

In the present study, BAT477 and DOR364 displayed the same pattern of proton release in response to P supply. Both genotypes released virtually the same amounts of protons under both adequate and inadequate P supply when grown in acid solution (pH < 6.5). Further, the proton release per unit root biomass was not increased under P deficiency. The results suggest that the rhizosphere acidification may not be an important cause of the genotypic difference in P acquisition observed in the field. The present results would, however, suggest that BAT477 released more organic anions than DOR364 (cf. Fig. 3). Organic acids in root exudates can mobilize soil inorganic P through either ligand exchange desorption or complexation of metal cations such as Fe or Al and dissolution of P-bearing minerals such as sesquioxides [5]. This process may thus be important in P mobilization, especially in acidic soils where beans are commonly cultivated. The better efficiency of BAT477 than DOR364 to cope with low P soils might therefore arise from such difference in their exudation pattern.

Both genotypes showed an increase in the root/shoot ratio under P deficiency. This is consistent with observations in many plant species efficient in P utilization [8, 15]. This increased root growth relative to shoot growth would thus enhance the total surface area for exploration and acquisition of P from soil.

4.2. Nodulation and N₂ fixation

Nodule number in both genotypes was not decreased by decreasing P supply in the present study. Supply of 5 µM P appeared to be sufficient for optimal nodulation when a large number of nodule bacteria were present. In another solution culture study, nodule number of the same genotypes measured 10 days after rhizobial inoculation was not affected by the absence of P (unpublished

data). Indeed, the growth and survival of rhizobia requires less than 0.5 µM P [13]. The results suggest that both P-efficient and P-inefficient bean genotypes require relatively low P for optimal nodule initiation.

The effect of P on nodule biomass per plant showed the same pattern as the effect on the shoot growth, indicating that P deficiency indirectly affects subsequent nodule development by restricting metabolite supply from the host plant. However, DOR364 formed smaller nodules than BAT477 under P deficiency. To compensate for small nodules, P-deficient plants of DOR364 appeared to be able to form more nodules.

Decreasing P supply decreased total N₂ fixation, with BAT477 apparently reaching the maximum N₂ fixation earlier than DOR364. Furthermore, P did not appear to have a specific role in nodule functioning in the present study because (i) nodule nitrogenase activity measured at 42 DAT did not decrease with a decrease in external P concentration from 25 to 5 µM, and (ii) the amounts of N fixed per unit nodule mass during 42 days were similar in both P treatments. Similarly, Pereira and Bliss [14] observed that the amount of N fixed per nodule mass was unaffected by P deficiency in bean plants grown in a sand-alumina system for 33 days. In other studies where different genotypes were grown in alkaline solutions for various periods, P supply decreased, increased or did not affect the specific nitrogenase activity and/or the amounts of N fixed per nodule mass [21, 22]. It appears that the role of P in bean nodule functioning depends on genotypes used, growth conditions and time of measurements.

Nodules appeared to be a strong sink for P since higher P concentrations were observed in nodules than roots and shoots when P supply was limited. This is in agreement with previous reports in a wide range of legumes [3, 6, 7, 17, 21, 23]. Whatever the P supply, nodules represented 6–8% of the total biomass and contained 12–14% of the total P. By contrast, P deficient shoots decreased their contribution to total biomass but increased their P content, the inverse being observed for roots. Thus, the plants favored P allocation to the shoots at the expense of the roots.

In conclusion, BAT477 and DOR364 showed similar responses to P deficiency of N₂ fixation and proton release when grown in acid solutions. Therefore, the difference between the two genotypes at the field level may be related to better adaptation of BAT477 to drought, or a difference in their efficiency to absorb and/or use the soil nitrogen. However, BAT477 released more protons per unit excess cations taken up than DOR364. It appears that BAT477 may have released more organic anions than DOR364 to maintain the charge balance. The interactions between P and exudation of organic anions in

these two bean genotypes thus deserve further investigation. Direct evidence, and qualitative and quantitative assessments of the organic anions exuded by BAT477 and DOR364 would be needed in the first place.

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