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## Effect of P on nodule formation and N fixation in bean

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**Abstract** – The present study compares the demands for P of the initial nodule formation, and of the later growth and functioning of the nodulated root system in two inoculated lines of common bean (Coco blanc and BAT477). After germination and inoculation, seedlings were divided into two lots. One lot was grown under constant P supply, either 15 (low) or 250 (high)  $\mu\text{M}$  P, corresponding to provision of 120 and 2000  $\mu\text{mol}$  P, respectively. In the second, seedlings were cultivated on the same medium supplied with 15  $\mu\text{M}$  P for 24 days after germination, and then with 60 or 250  $\mu\text{M}$  (total provision of P: respectively, 390 and 1530  $\mu\text{mol}$ ). Nodule number and biomass were significantly diminished by the low P (120  $\mu\text{mol}$ ) treatment, as compared with the other treatments. However, the intrinsic characteristics of the nodules (individual biomass and size, P concentration and efficiency of N fixation) did not depend on P availability. Although the bean line BAT477 was distinguished from the Coco blanc line through higher nodule number, size, biomass, and nitrogen fixation, both lines displayed analogous responses to P availability.

*Phaseolus vulgaris* / P availability / nodulation / P allocation / symbiotic nitrogen fixation

### 1. INTRODUCTION

In legumes, phosphorus deficiency specially affects symbiotic fixation of  $\text{N}_2$  by limiting growth and survival of rhizobia (O'Hara et al., 1988), nodule formation (Drevon and Hartwig, 1997) and functioning (Tang et al., 2001a), and host plant growth (Tsvetkova and Georgiev, 2003). In addition, phosphorus deficiency has previously been reported to decrease nodule mass more than host growth in soybean (Drevon and Hartwig, 1997). Reports on P requirements for nodule formation and functioning are controversial: phosphorus deficiency may increase, decrease or not affect the nodule number per unit of shoot mass (Drevon and Hartwig, 1997; Gunawardena et al., 1992; Pereira and Bliss, 1989; Ribet and Drevon, 1995; Tang et al., 2001a; Vadez et al., 1996), as well as the specific nitrogenase activity and/or the amount of N fixed per nodule mass (Tang et al., 2001b; Vadez et al., 1996, 1997). Differences in the response of nodule formation and functioning to P deficiency appear thus to be related to legume species, line and experimental conditions. Additionally, the response of plants to P deficiency might depend on the duration and the severity of the stress. At early development stages, both P-efficient and P-inefficient common bean lines require relatively low P for optimal nodule initiation (Tang et al., 2001b). However, at later stages and in nodulated plants subjected to severe P deficiency, the further growth of already established nodules seemed to decrease (Valverde et al., 2002) or to be fully inhibited (Almeida et al., 2000).

The objective of the present study was to compare the demands for P of the initial nodule formation, and of the later growth and functioning of the nodulated root system. The plants were grown either in P-limiting or in P-sufficient conditions from sowing to flowering. In parallel treatments, P-limiting conditions were applied only during the initiation and early development of nodules, and then the plants were returned to P sufficiency conditions. Two inoculated lines of common bean (*Phaseolus vulgaris* L.) were used.

### 2. MATERIALS AND METHODS

#### 2.1. Plant material and growth conditions

Two lines of common bean were chosen: Coco blanc, commonly cultivated all around the Mediterranean sea, and BAT477, which is considered as more tolerant to a variety of abiotic constraints (Saadallah et al., 2001; Tang et al., 2001b). After germination and inoculation with *Rhizobium tropici* CIAT 899 (originating from the International Center of Tropical Agriculture, Colombia), seedlings were hydroaeroponically (Tang et al., 2001b; Vadez and Drevon, 2001) grown in a glasshouse under natural light. Each pot, containing 1 seedling, was filled with 1 liter of the following nutrient solution:  $\text{MgSO}_4$  (120.5  $\text{mg L}^{-1}$ ),  $\text{K}_2\text{SO}_4$  (122  $\text{mg L}^{-1}$ ),  $\text{CaCl}_2$  (180  $\text{mg L}^{-1}$ ),  $\text{H}_3\text{BO}_3$  (0.25  $\text{mg L}^{-1}$ ),  $\text{MnSO}_4$  (0.6  $\text{mg L}^{-1}$ ),  $\text{ZnSO}_4$  (0.16  $\text{mg L}^{-1}$ ),

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**Table I.** Different P levels used during the culture of two common bean lines (Coco blanc and BAT477). DAG: days after germination.

Treatments	P provision per plant		
	From 5 to 24 DAG $\mu\text{mol week}^{-1}$	From 24 to 62 DAG $\mu\text{mol week}^{-1}$	Total (62 days) $\mu\text{mol}$
low-low	15	15	120
low-medium	15	60	390
low-high	15	250	1530
high-high	250	250	2000

$\text{CuSO}_4$  ( $0.16 \text{ mg L}^{-1}$ ),  $\text{CoCl}_2$  ( $0.016 \text{ mg L}^{-1}$ ) and  $\text{Na}_6\text{Mo}_7\text{O}_{24}$  ( $0.14 \text{ mg L}^{-1}$ ). The nutrient solution was renewed weekly, the pH was maintained near neutrality with  $\text{CaCO}_3$  ( $1 \text{ g L}^{-1}$ ), and the solution was constantly aerated at a flow of  $400 \text{ mL min}^{-1}$ .

## 2.2. Experimental design and P treatments

Three levels (low, medium and high) of P supply were used, respectively, corresponding to 15, 60 and  $250 \mu\text{M}$  P, as  $\text{KH}_2\text{PO}_4$ , in the medium. They provided each plant with, respectively, 15, 60 and  $250 \mu\text{mol}$  P. The nutrient solutions were supplemented with KCl to equalize K supply over these P treatments. After 5 days of germination on agar imbibed with water, seedlings were transferred for 2 weeks to a pretreatment solution with either low or high P level. 24 days after germination, the plants were transferred to treatment solution with low, medium or high P level, as described in Table I. Final harvest occurred 6 weeks later (61 days after germination). Urea ( $2 \text{ mM N}$ ) was added at 24 days after germination. Thereafter, the plants did not receive any external source of N.

## 2.3. Measured parameters

At the beginning of the flowering stage (52 days after germination), three plants from each treatment were used for the assessment of nodule nitrogenase activity, estimated by the

acetylene reduction assay (Hardy et al., 1968). Ten other plants were harvested 61 days after germination, and separated into roots, shoots and nodules. Samples were then dried at  $60^\circ\text{C}$  to constant dry weight. Dried nodules were counted, separated into two size classes using wire-mesh sieves (diameter  $<$  or  $>$   $0.5 \text{ mm}$ ) and ground. A  $25\text{-mg}$  dry weight nodule sample from each plant was digested in concentrated  $\text{HNO}_3$ . Phosphate was assayed using the vanado-molybdate method (Fleury and Leclerc, 1943).

For N determination, the sample was digested in hot concentrated  $\text{H}_2\text{SO}_4$ , as described by Kjeldahl. The symbiotic nitrogen fixation was estimated as the difference between N quantities ( $\text{mmol plant}^{-1}$ ) at 61 and 24 days after germination.

## 2.4. Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of data. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) procedure ( $P < 0.05$ ).

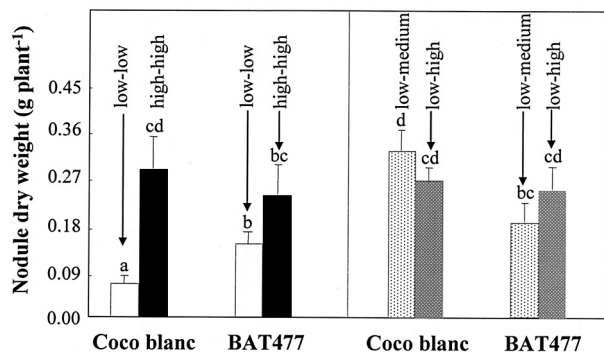
## 3. RESULTS AND DISCUSSION

### 3.1. Plant growth and nodule development

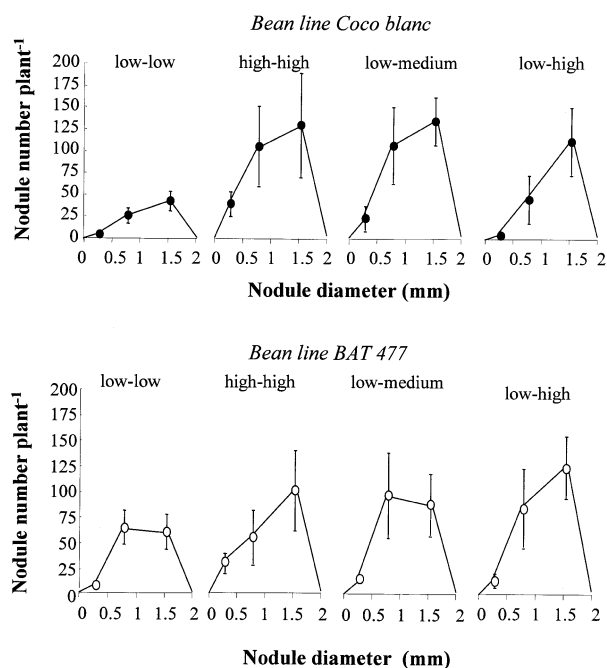
As compared with the other treatments, the low-low treatment restricted the whole plant growth of the bean line Coco blanc, but not that of the BAT477 line (Tab. II). Shoots rather than roots reflected this response to P shortage. An increase in the root/shoot ratio is known to frequently occur in P-starved plants (Tang et al., 2001b). The nodule biomass (Fig. 1) represented 4 to 8% of whole plant biomass when P was not limiting (not shown). These values are comparable with those published for common bean (Saadallah et al., 2001; Vadez and Drevon, 2001).

**Table II.** Whole plant, root, and individual nodule biomass and plant to nodule biomass ratio of two common bean lines (Coco blanc and BAT477) cultivated with low, medium or high P levels, as described in Table I. Data are the means  $\pm$  SE of 10 replicates. Mean values followed by the same letter are not significantly different at  $P < 0.05$ .

		Treatments			
		low-low	high-high	low-medium	low-high
Plant biomass (g dry weight)	Coco blanc	$1.8 \pm 0.6a$	$5.0 \pm 1.1cbd$	$4.3 \pm 0.4cb$	$4.17 \pm 0.4cb$
	BAT477	$4.3 \pm 0.4cb$	$3.9 \pm 0.9b$	$5.1 \pm 0.6cd$	$5.4 \pm 0.9d$
Root biomass (g dry weight)	Coco blanc	$1.1 \pm 0.1b$	$0.9 \pm 0.3ab$	$1.1 \pm 0.1b$	$1.1 \pm 0.1b$
	BAT477	$1.0 \pm 0.1ab$	$0.8 \pm 0.2a$	$1.0 \pm 0.1ab$	$0.9 \pm 0.1ab$
Mean biomass of individual nodules (mg dry weight)	Coco blanc	$1.23 \pm 0.58a$	$1.27 \pm 0.35ab$	$1.42 \pm 0.28ab$	$1.95 \pm 0.56b$
	BAT477	$1.14 \pm 0.17a$	$1.12 \pm 0.46a$	$0.91 \pm 0.20a$	$1.34 \pm 0.30ab$
Plant biomass / Nodule biomass	Coco blanc	$22.0 \pm 6.9ab$	$18.9 \pm 5.6ab$	$13.9 \pm 2.6a$	$21.5 \pm 9.4ab$
	BAT477	$32.8 \pm 7.4b$	$31.4 \pm 15.4b$	$32.9 \pm 16.0b$	$24.2 \pm 7.1ab$

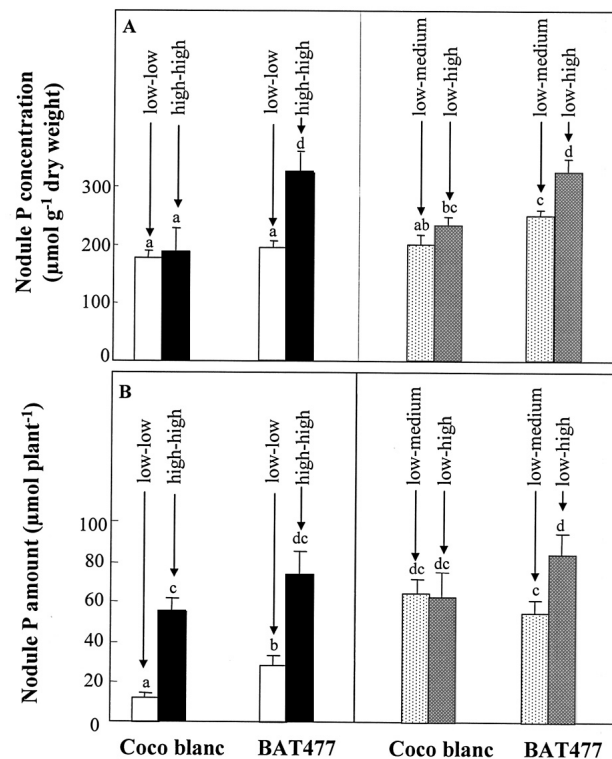


**Figure 1.** Effect of phosphorus availability on nodule biomass ( $\text{g plant}^{-1}$ ) of two common bean lines (Coco blanc and BAT477). Plants were cultivated with low, medium or high P levels, as described in Table I. Data are the means  $\pm$  SE of 10 replicates. Mean values followed by the same letter are not significantly different at  $P < 0.05$ .



**Figure 2.** Effect of phosphorus availability on nodule size (mm) distribution of two common bean lines (Coco blanc and BAT477). Plants were cultivated with low, medium or high P levels, as described in Table I. Data are the means  $\pm$  SE of 10 replicates.

The lowest numbers of nodules per plant were recorded when P shortage persisted during the whole plant cycle (low-low treatments) (Fig. 2). Treatments with P shortage at the early development stage of the plants followed by late application of 60 or 250  $\mu\text{M}$  P (low-medium and low-high) led to nodule production similar to that of the high-high treatment (Fig. 1). These results suggest that early restriction of nodule formation by



**Figure 3.** Effect of phosphorus availability on nodule P status of two common bean lines (Coco blanc and BAT477). Plants were cultivated with low, medium or high P levels, as described in Table I. (A) Nodule P concentration ( $\mu\text{mol g}^{-1}$  dry weight). (B) Nodule P amount ( $\mu\text{mol plant}^{-1}$ ). Data are the means  $\pm$  SE of 10 replicates. Mean values followed by the same letter are not significantly different at  $P < 0.05$ .

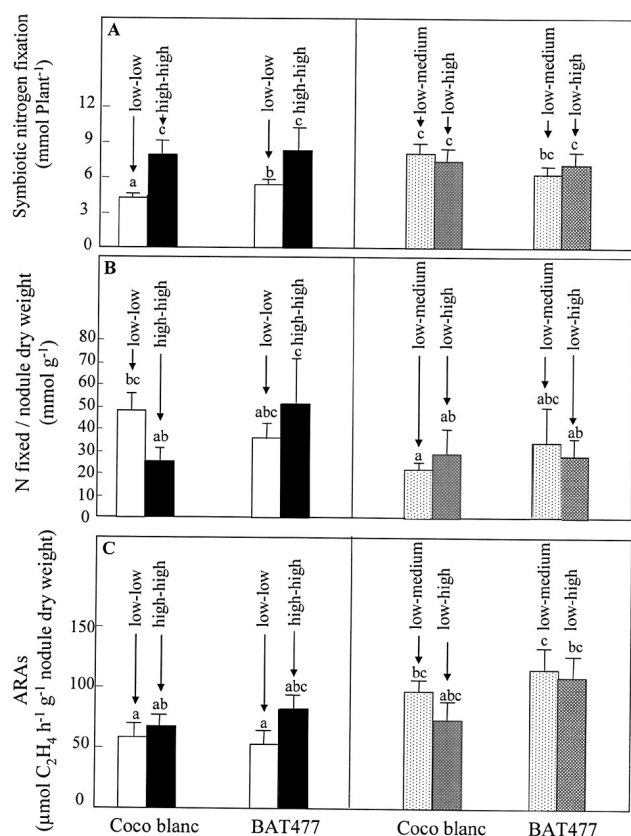
insufficient P provision was compensated by late nodule initiation, after returning the plants to P-sufficient conditions (low-medium and low-high treatments).

For both lines, the mean biomass (Tab. II) and size distribution (Fig. 2) of the individual nodules did not significantly differ between the treatments. For the nodule size distribution, the modal class was that of the larger size value (diameter larger than 1 mm) in all treatments, including low-low. Thus, nodule growth was not restricted by P shortage, even when it lasted during the whole plant cycle. Only the nodule number was dependent on P supply.

The ratio of plant biomass to nodule biomass estimates the intrinsic capacity of nodules to support plant growth, i.e. the symbiosis efficiency. This parameter was consistently higher in the bean line BAT477 than in the Coco blanc line, but for both lines, there was no significant difference between treatments (Tab. II).

### 3.2. Nodule P status

P concentration in nodules was much less sensitive to P shortage (low-low treatment) than the amount of P accumulated in nodules (Fig. 3). For instance (Coco blanc), P concentration



**Figure 4.** Effect of the P supply mode on symbiotic nitrogen fixation of two common bean lines (Coco blanc and BAT477). Plants were cultivated with low, medium or high P levels, as described in Table I. (A) Nitrogen amount (mmol plant<sup>-1</sup>). (B) N fixed / nodule dry weight (mmol g<sup>-1</sup>). (C) Specific nitrogenase activity, ARAs (μmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> g<sup>-1</sup> nodule DW). Data are the means ± SE of 10 replicates. Mean values followed by the same letter are not significantly different at  $P < 0.05$ .

in nodules was the same for the low-low and high-high treatments (Fig. 3A). On the contrary, the amount of P associated with the nodule fraction of low-low plants was ca. 20% that of high-high plants (Fig. 3B). In the bean line BAT477, the P concentration in low-low nodules was 60% that of high-high nodules, while P amount was only 39%. Thus, restricting P availability limited nodule biosynthesis more than nodule enrichment in P. This effect was more marked in the bean line Coco blanc than in the BAT477 line.

### 3.3. Symbiotic nitrogen fixation

For both lines Coco blanc and BAT477, the symbiotic nitrogen fixation capacity (mmol N plant<sup>-1</sup>) was lower in the plants submitted to continuous P deficiency (low-low) than in plants with early (high-high) or late (low-medium, low-high) sufficient P provision (Fig. 4A). This trend was no longer apparent when the amounts of fixed N were expressed on a nodule mass basis (Fig. 4B), suggesting that early P deficiency did not

restrict nodule functioning. Indeed, acetylene reduction activity estimation did not discriminate between treatments with early vs. late P provision (Fig. 4C). The acetylene reduction activity assay in a closed system may underestimate the nitrogenase activity, owing to acetylene-induced inhibition of this enzyme. However, this assay is useful for comparative purposes (Minchin et al., 1983). Since our above conclusion relies on relative acetylene reduction activity values, we hypothesize that it is not affected by a possible nitrogenase inhibition.

## 4. CONCLUSION

Our results indicate that, when P is limiting for whole plant growth, the number of nodules is severely restricted, resulting in lowered symbiotic nitrogen fixation capacity for the whole plant. However, return to normal P nutrition permits a compensatory nodule initiation and growth, so that there may be no after-effect of the P shortage period. Nodule growth, as distinguished from nodule initiation, is not restricted by P shortage. Even when the plant growth is limited by P availability during the whole cycle, the few nodules formed present normal growth. Priority seems to be given to preserving the P nutrition of the nodules. The functional characteristics of the nodules are also independent of P supply: neither the symbiosis efficiency nor the estimated specific nitrogenase activity are significantly diminished by P restriction. In conclusion, under our experimental conditions characterized by relative low growth activity of the plant, nodule initiation but not nodule growth and functioning depends on sufficient P supply in common bean. Perhaps there is some regulation of nodule initiation which adjusts the number of nodules to P availability, so that optimal nodule performance is maintained.

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