Nitrogen and phosphorus availability as limiting factors of N_2 fixation in common bean.

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Summary

Nitrogen and phosphorus availability may significantly affect N_2 fixation in legumes. The interaction of N and P levels was studied in common bean considering their effects on nodulation and N_2 fixation, nitrate reductase activity, and the composition of N compounds in the xylem sap. The effect of N on plant P uptake was estimated by analysing rhizospheric pH and P concentration in the xylem sap and in plant shoots. Inoculated bean plants were grown in pots containing perlite/vermiculite in two experiments with different levels of P and N. In a third experiment, bean plants were grown on two soil types or river sand supplied with different N levels. At harvest, shoot growth, nodule number and mass, and nitrogenase activity were determined. Xylem sap was collected for the determination of ureides, amino acids, nitrate and phosphate concentration. Increasing P levels at low nitrate concentration in the medium promoted nodule formation and N_2 fixation, measured as ureide content in the xylem sap. However, with high nitrate concentration in the medium, nodulation and N_2 fixation did not improve with increased P supply. Glutamine and aspartate were the main organic N compounds transported in the xylem sap of plants grown in low nitrate (1 mM nitrate), while asparagine was the dominant N compound in xylem sap from plants grown in high nitrate (10 mM nitrate). Nitrate reductase activity in roots was higher than in shoots of plants grown with low P and high N. The increase on nitrate application in the soils and sand experiment reduced nodule mass and number, nitrogenase activity and xylem export of ureides but increased asparagine concentration in the xylem sap. Increasing P nutrition improved symbiotic N_2 fixation in bean only at low N levels of N. It did not alleviate the inhibitory effect of high nitrate concentration on N_2-fixation. A reduction in plant P uptake, as indicated by a lower P concentration in the xylem sap and shoots correlating with the level of

N supplied was observed. Simultaneously to the specific N inhibition of N_2 fixation, high nitrate levels might reduce P availability reducing its uptake, thus inhibiting even further the symbiotic association because of the high P requirement for nodulation and N_2 fixation.

Keywords: *Phaseolus vulgaris* L., N_2-fixation, ureides, amino acids, xylem sap composition, P deficiency, rhizospheric pH

Introduction

Nutrient limitation may be a major constraint of legume N_2 fixation and yield (O'Hara *et al.*, 1988). In common bean crops grown on Andalusian soils (Southern Spain), poor response to rhizobia inoculants was associated to high levels of residual nitrate in soils and low leaf concentration of P and B (Rodríguez-Navarro, 1996). Bean is usually considered a poor N_2 fixer being particularly sensitive to the amount of nitrate in comparison with other legumes (Graham, 1981; Temprano *et al.*, 1997). High N level in the soil affects the formation of nodules and the N_2 fixing process *per se* (Streeter, 1988). Increasing the supply of limiting nutrients may improve bean symbiotic N_2 fixation (Tsai *et al.*, 1993) in the presence of high N concentrations.

The level of P available to plant uptake is very low, about 10^{-5} to 10^{-4} M in fertile arable soils (Mengel & Kirkby, 1982). There is a pH-dependency of plant P uptake, with maximum rates between pH 5.0 and 6.0 (Marschner, 1995; Schachtman *et al.*, 1998). Root-induced changes of rhizosphere pH due to processes such as differential uptake of anions and cations, root respiration or organic acid exudation may strongly affect P uptake (Hinsinger, 1998). Legumes fixing N_2 acidify their rhizosphere because of a relative higher uptake of cations over anions (Marschner, 1995). Phosphorus requirement by legumes is affected by the mode of N nutrition (Cassman *et al.*, 1981). Nitrogen-fixing plants have a higher need for P than nitrate supplied plants (Israel, 1987) and the P requirement seems particularly high in bean (Graham & Rosas, 1979). Research on the effects of N and P interaction on N_2 fixation in legumes is limited (Gates & Wilson, 1974; Graham, 1981).

Ureide-producing legumes load different N solutes onto the xylem sap when feeding symbiotically by root nodules or when assimilating a combined source of N (Pate *et al.*, 1980;

Rodríguez *et al*., 1999). The analysis of N solutes in the xylem sap may provide the relative participation of N_2 fixation in plant N acquisition (Peoples & Herridge, 1990) but also indicate the root capacity for N assimilation (Thomas *et al*., 1979; Cookson *et al*., 1980). The concentration of some amino acids increases in P deprived plants (Rabe & Lovatt, 1986; Rufty *et al*., 1993). An understanding of N metabolism in the root could be obtained by studying xylem sap composition at varying P levels.

The main objectives of this work were to find out to which extent nitrate inhibition of N_2 fixation could be reverted by increasing P supply and to study how N and P interact affecting the overall plant N acquisition and metabolism.

Materials and methods

Experiment 1.

Surface-sterilized and pre-germinated bean seeds (*Phaseolus vulgaris* L. cv. Canellini) were transplanted to a perlite/vermiculite mixture (1/1, v/v), contained in black plastic bags, and inoculated with *Rhizobium etli* strain ISP-23 perlite-based inoculant (10^9 rhizobia g^{-1}). The Long Ashton nutrient solution (Hewitt, 1966) was modified to provide two different levels of nitrate (1 and 10 mM NO_3^-) combined with three levels of phosphate (0.01, 0.1 and 2 mM $H_2PO_4^-$). Ion balance was preserved with additions of K_2SO_4 and/or $CaCl_2$.

Plants were grown in a glasshouse keeping minimum temperature above 20ºC and irrigated with nutrient solutions every other day. At the end of the experiment (21 days), plants were harvested, separated in leaves, stem plus petioles and roots, weighed and their dry weights recorded after drying in an oven (70ºC, 48h). Nodules were separated from roots for counting and weighing.

Experiment 2.

A second experiment was designed to study the effect of N and P levels on nitrate reductase activity in leaves and roots and rhizospheric pH. It was run under similar conditions as Experiment 1, but modifying nutrient solutions to provide three levels of nitrate (0.2, 1 and 5 mM NO_3^-) and two levels of phosphate (0.01 and 0.1 mM $H_2PO_4^-$) in a 3 x 2 factorial experiment with 3 replicates. At harvest, plants were processed as indicated in Expt. 1.

In vivo nitrate reductase activity was assayed in leaves and roots (Nicholas *et al.* 1976). Leaf discs or roots (sections 2-3 cm from root tips) were vacuum infiltrated with a medium containing 100 mM K-phosphate buffer (pH 7.5), 5% propanol (v/v) and 50 mM KNO_3 and incubated at 30ºC during 30 and 60 min.

At harvest, rhizosphere pH was estimated by measuring pH in distilled water used for washing out roots immediately after extraction. A set of plants was transferred to a hydroponic system where the pH of a continuously aereated nutrient solutions was recorded during 7 days.

Experiment 3.

Bean plants cv Canellini were grown in pots containing 2.5 kg of soil or washed river sand in the greenhouse. The soils used for this study were from Castilblanco de los Arroyos (Palexerults) and Las Torres (Typic Chromoxerert). Data of soil chemical analysis are presented in Table 1. Phosphates were extracted and analyzed following Olsen & Dean (1965). The soils and sand were ammended with nitrate (KNO_3) to provide three different nitrate concentrations: 0, 40 and 80 ppm N. Seeds were surface sterilized, germinated and transplanted into pots. Inoculation was performed at the time of planting with 1 ml per seed of *Rhizobium leguminosarum* biovar. *phaseoli* strain ISP-14 culture (1.27 x 10^9 rhizobia ml^{-1}). Pots with soil were watered with deionized water (soil treatments), while pots containing sand were irrigated with diluted (1/4) nutrient solution (Rigaud & Puppo, 1975). Three replicates per treatment were run.

Plants were harvested after 3 weeks of growth under greenhouse conditions, weighing shoots and using roots for determination of nitrogenase activity (Hardy *et al.*, 1968). After analysis of nitrogenase activity, nodules were separated for counting and weighing.

Plant and xylem sap chemical analyses.

Total N and P in leaves, stems + petioles and roots was determined colorimetrically after Kjeldahl digestion of ground dried samples. Subsamples were taken for the analysis of nitrate (Cataldo *et al.*, 1975) after hot water extraction.

Xylem exudates were collected from stumps during 1 h in Eppendorf vials maintained on ice. The exudates were frozen (-20ºC) until analysis was performed. Concentration of ureides (allantoin and allantoic acid) was determined following Vogels & Van der Drift (1970) and amino acids according to Yemm & Cocking (1955). Amino acids were separated and quantitated by the Waters PICO.TAG method. Nitrate concentration was measured according to Cataldo *et al.* (1975). Phosphate present in sap was determined by the method of Murphy & Riley (1962).

Experimental design.

A randomized complete block design with five (Experiment 1) or three (Experiment 2 and 3) replicates per treatment was used. Statistical analysis was performed with a standard package.

Results

Experiment 1.

Significant increases in nodule mass, number and nodule size were observed by increasing P supply under low N concentration in the medium. High N concentration inhibited nodule formation in bean roots, and no positive effect on nodulation was observed by increasing P supply (Table 2). Increasing the level of P in the nutrient solution led to significant increases in leaf and stem biomass and shoot/root ratio at the highest N concentration (Table 2).

Leaf N concentration was not affected by increases in P supply at 1 mM NO_3^- (Table 3). However, at 10 mM NO_3^-, a significantly higher leaf N concentration was detected at the lowest P supply. Leaf P concentration increased following the increase of P supply in the nutrient solution in a rather similar way at both N levels. When combining high levels of N and P, a synergistic effect on leaf P was observed.

Increased concentrations of ureides and amino acids were observed in the xylem sap when increasing the level of P at low N concentration (1 mM NO_3^-), but the concentration of NO_3^- in the xylem sap decreased (Table 4). Changes in ureide and amino acids concentration paralleled the change in nodule mass and number produced by increasing P levels in the medium (Table 2). The lowest P level was clearly limiting in both N_2 fixation by nodules as indicated by the low level of ureides in xylem sap and in nitrate reduction and assimilation by roots as shown by the amount of NO_3^- present in the xylem sap. The ureide concentration was very low and no statistical differences between P levels was observed with 10 mM NO_3^- concentration in the medium,. The concentration of amino acids and NO_3^- was only affected when increasing P supply from 0.01 to 0.1 mM, suggesting limitations in the root capacity for nitrate assimilation at the lowest P level. A remarkable differential feature in the amino acid composition exported in xylem sap by low or high NO_3^- grown plants is shown in Figure 1. Glutamine was the main amino N compound present in xylem exudates of plants grown with low NO_3^-, followed by aspartate and asparagine in lower concentration. However, at 10 mM NO_3^-, the main amino N compound in the xylem sap was asparagine at all P levels, and its concentration increased with the level of P. The concentration of P in the xylem sap varied according to P supply (Table 4), and a reduction in P uptake occurred mainly at 0.1 mM P when increasing NO_3^- concentration. The level of sap P was closely associated with leaf P concentration ($r=0.978$, $P<0.001$, $n=9$).

Experiment 2.

Nodule formation and growth was affected by NO_3^- concentration (Figure 2) and increases in P did not revert the N inhibition of nodulation. For each NO_3^- concentration, nodulation was sligthly improved by increases in P supply (Figure 2).

Leaf nitrate reductase activity was more affected by P supply than root nitrate reductase (Figure 3). Nitrate reduction by roots closely followed NO_3^- availability in solution, and P availability only had minor effects on root nitrate reductase. At low N, nitrate reduction occurred predominantly in leaves. With high N level and low P supply, the amount of nitrate reduced in roots increased. With high P supply, the increase in N led to an increased plant capacity for nitrate assimilation. Nodulated plants grown with low NO_3^- significantly decreased the pH of the rooting medium at both P levels, while those plants grown with higher NO_3^- levels showed lower rhizosphere acidification (Figure 4). Significant differences in rhizosphere pH between treatments were recorded (data not shown), with actively N_2-fixing plants (low NO_3^-) presenting the lowest rhizosphere pH. The concentration of P in the xylem sap was significantly affected by N supply (Table 5). High NO_3^- concentration led to significant reduction of P concentration in sap at either P level in the nutrient solution independently from differences in sap flow rate (Table 5).

Experiment 3.

Increases in plant growth by N application were only observed when raising N level from 0 to 40 ppm NO_3^- (Table 6). At the highest N concentration, plant growth did not increase (sand, soil B) or even decrease (soil A). Nodulation of bean plants and nitrogenase activity were significantly affected by the amount of N supplied (Table 6). With 80 ppm NO_3^-, there was a complete inhibition of nodule development in soil-grown plants. However in sand-grown plants, no differences were observed in nodule mass and nitrogenase activity between 40 and 80 ppm NO_3^-.

A significant increase in shoot N concentration was observed in soil-grown plants but not in sand-grown plants when increasing NO_3^- in the medium (Table 7). Similarly, the amount of N accumulated in shoot biomass (N content) significantly increased when increasing NO_3^- in the soil. The shoot P concentration in sand-grown plants was negatively affected by the increase in NO_3^- supply. Phosphorus shoot content increased when soil NO_3^- was raised from 0 to 40 ppm NO_3^-, but a further increase in NO_3^- from 40 to 80 ppm NO_3^- led to a decrease in P content (Table 7).

The variation in ureide concentration in the xylem sap was associated to the inhibition of N_2 fixation by NO_3^-, while NO_3^- concentration correlated to the amount of root available NO_3^- (Table 8). There was no significant effect of NO_3^- application level in the total concentration of amino acids found in xylem sap, but in soil-grown plants amino acid concentration in the xylem sap tended to increase from 0 ppm NO_3^- to 40 or 80 ppm NO_3^-.

Asparagine was the main organic N compound transported in plants receiving NO_3^- (Figure 5), and its concentration increased with N supply. Concentration of P in xylem sap was higher in sand-grown plants than in soils (Table 8), where the reduction of P concentration at increasing N supply was clear. The xylem sap concentration of P, as in the previous experiment, was closely related to leaf P concentration ($r=0.899$, $P<0.001$, $n=9$).

Discussion

Nodulation was improved by P supply at the low level of N in solution, whereas its effect at high N concentration was negligible. This effect suggests that P limited nodule formation and growth whereas shoot and root growth were little affected (Table 2). Similar to soybean, bean nodule formation was more sensitive to P deficiency than plant growth (Israel, 1987). The inhibitory effect of high N on nodulation could not be reversed by increased P availability (Table 2) as suggested by a previous report (Tsai *el al.*, 1993). So, it seems that P could partially counteract the detrimental effect of mineral N on N_2-fixation in bean. In the soil experiment, increasing N also limited nodulation (Table 6), but other factors might be involved, as important differences in nodule number and nodule mass were detected between soil and sand.

Xylem sap composition was related to the form of N nutrition (N_2 fixation or NO_3^- -N) as it was reported previously (Sarmiento *et al.*, 1998). Nitrogenous compounds in the xylem sap reflected changes in root N assimilation activity at different levels of available P. With low NO_3^- in solution, the products of N_2 fixation in plant sap (mainly ureides and glutamine) increased their concentration with the P levels. In common bean, the relationship between N_2 fixation and ureide and amino acids concentration in the xylem sap has been well established (Rodríguez-Navarro *et al*., 1999). A low concentration of ureides was found in sap of low P/low NO_3^- treated plants (Table 4) in close relation with nodule mass and number (Table 2). In soybean, P deficiency had negative effects on nodule initiation and growth and specific nitrogenase activity (Israel, 1987) and decreased ureide translocation in the xylem sap (Sa & Israel, 1995).

In experiments performed with soils and sand, a clear relation between nodulation and ureide concentration was found ($r=0.93$ $P<0.001$ and $r=0.88$ $P<0.01$, $n=9$, for ureide vs nodule mass or number respectively) (Tables 6 and 8). The highest N level completely inhibited nodule formation in soils but not in sand (Table 6). Low concentration of ureide was recorded in plants grown at high N level (Tables 4 and 8), which was obviously related to the inhibition of nodule formation and nitrogenase activity (Streeter, 1988).

The amino acid concentration in the xylem sap showed an increase with P supply (Table 4, Figure 1), which would suggest an increase in N_2 fixation and/or the rate of nitrate reduction in roots. In low N-grown bean plants, the increase in glutamine and aspartate with increasing P (Figure 1) may be associated to the improvement in N_2 fixation, as both N compounds like ureides are N export products derived from N_2 fixation (Pate, 1989). The high NO_3^- concentration in xylem sap under low P supply (Table 4) might be explained by the low nitrate reductase activity found in roots (Figure 3). High NO_3^- concentration in xylem sap may also be a consequence of restricted nitrate transport from roots to shoots as it was found in P-deficient soybean plants (Rufty *et al*., 1993) as changes in xylem sap flow suggest (data not shown). In high NO_3^--grown plants, the decrease in NO_3^- concentration and the increase in amino acids concentration (Table 4, Figure 1) found in sap when enhancing P supply was possibly due to the increase in root nitrate reductase activity (Figure 3). In fact, xylem sap from these plants transported mainly asparagine (Figure 1), the main N compound exported by NO_3^--fed bean (Pate, 1989; Sarmiento *et al.*, 1998).

In soil grown plants, increased NO_3^- supply led to increases in NO_3^- and a reduction of ureides in the xylem sap (Table 7, Figure 5). No statistically significant differences in amino acids concentration were observed (Table 7), although there was a tendency to increase following NO_3^- application. Asparagine was the dominant component in the amino compounds fraction in the xylem sap (Figure 5), with increasing concentration of asparagine at higher N supply. Rufty *et al.* (1993) found an increase in the concentration of asparagine in root and stem tissues in soybean plants deprived of P. Analysis of xylem sap composition is a particularly interesting approach for estimating N_2 fixation (Rodríguez-Navarro *et al.*, 1999) but also may reveal nutritional imbalances: low asparagine and high NO_3^- concentration in sap might be indicators of limited availability of P. Differences in the concentration of N compounds among plants grown with sand or soils (Table 8, Figure 5) are probably related to differences plant P uptake and use in N metabolism.

High nitrate reductase activity in roots and a high concentration of reduced N compounds in the root probably was a critical factor affecting nodule formation. A feedback regulation of nodule growth and activity by phloem N suggested by Parsons *et al.* (1993) may explain the low nodulation in high N-grown plants. In fact, the high concentration of amino compounds in the xylem sap are probably a consequence of reduced transport (Rufty *et al.*, 1993) and lower transpiration rates (Radin, 1984) in P-deficient plants. These conditions would favour a xylem-to-phloem transfer of amino acids (Da Silva & Shelp, 1990) which could downregulate nodule development and N_2 fixation. A high downward translocation of amino acids was found in phloem of P-deficient plants (Jeschke *et al.*, 1997), which could additionally contribute to the feedback inhibition of nodule growth.

Acidification of the rhizosphere is a typical response of plants to P deficiency

(Marschner, 1995) even when plants are supplied with NO_3^- (Pilbeam *et al.*, 1993). Plants grown with 0.01 mM P showed stronger acidification than plants grown with 0.1 mM P (Figure 4), probably related to a P deficiency response. However, NO_3^- may have reduced the mechanism of acidification for enhancement of P acquisition in common bean. In nutrient solution with 1 and 5 mM NO_3^-, the acidification capacity of root systems seemed affected (Figure 4). The recorded differences in rhizosphere pH (Figure 4) and the sap P concentration (Table 5) between plants grown with different N and P combinations agree with a mechanism of P uptake limitation due to pH increases. At similar P concentration in the nutrient solution, raising NO_3^- concentration lowered the concentration of P in the xylem sap independently from changes in sap flow (Table 5). A relation was found for xylem sap P and transport from roots to leaves in barley (Mimura *et al.*, 1996). The relation between sap P and leaf P concentration observed here suggests that uptake and transport of P to shoots might be restricted in high N-grown plants. A possible explanation to the lowered P uptake might be rizospheric pH changes due to the amount of NO_3^- being reduced in the shoot and the root (Figure 3). With increasing NO_3^- in the medium, more NO_3^- is assimilated and the excess of OH^- generated and released as HCO_3^- by roots could have increased rhizosphere pH (Marschner, 1995). The increase in rhizosphere pH reduces P uptake, which has an optimum pH between 5-6 (Schachtman *et al.*, 1998), and at the same time can favour the formation of sparingly soluble P compounds (Hinsinger, 1998).

The concentration of P in leaves of soil-grown plants (Table 7) may be considered deficient (Reuter *et al.*, 1997), although P concentration in soils (Table 1) was apparently adequate (Olsen & Dean, 1965). The low leaf P concentration might explain the poor growth response to the increase in NO_3^- supply (Table 6) as P deficiency limits growth by affecting N uptake and assimilation (Israel, 1987; Rufty *et al.*, 1993).

In alkaline soils with high Ca levels, the immovilization of P as Ca phosphates (Hinsinger, 1998) might be favoured by a NO_3^--induced raise in rhizospheric pH. Nodule formation and N_2 fixation, with higher P requirement than NO_3^- assimilation, would be constrained by limited P availability and further by the accumulation of soluble N compounds at the root level. In Mediterranean soils, in which prevailing conditions of high pH and high Ca concentration reduce P availability, NO_3^--induced rhizosphere alkalinization may prove to be an aggravating factor limiting the establishment of nodulation.

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Table 1. Chemical properties of soils from Castilblanco de los Arroyos (Soil A) and Las Torres (Soil B) used in Experiment 3.

	pH	**Organic matter (%)**	**$CaCO_3$ (%)**	**N (‰)**	**NO_3^- (ppm)**	**NH_4^+ (ppm)**	**P (ppm)**
Soil A	6.4	2.19	1.5	1.26	1.1	8.5	17.8
Soil B	8.1	1.19	36.1	0.75	4.5	5.0	21.6

Table 2. Plant growth, shoot/root ratio, leaf area, nodule number and weight of inoculated bean plants cv Canellini grown with different levels of nitrate and phosphate.

Treatment (mM)			**Stem+**					**Nodule**
		Leaves	**petioles**	**Roots**	**Nodules**	**Shoot/**	**No**	**size**
N	**P**		**(g fresh weight plant^{-1})**			**root**	**Nodules**$^{(1)}$	**(mg)**
1	0.01	4.63a	2.55a	6.66a	0.24a	1.08a	78a	2.97a
1	0.1	4.75a	2.68a	6.77a	0.41b	1.10a	79a	5.14b
1	2.0	5.02a	2.80ab	6.42a	0.70c	1.22a	151b	4.67b
10	0.01	5.36a	3.52bc	6.93a	0.00d	1.30a	11c	0.00c
10	0.1	5.65a	3.42bc	7.04a	0.02d	1.32a	16c	1.18d
10	2.0	7.96b	4.07c	6.97a	0.00d	1.72b	4c	0.00c

Means in columns followed by the same letter are not significantly different at $p<0.05$ (LSD test).

(1)Nodules were detached and counted only when greater than 1 mm diameter.

Table 3. Nitrogen, phosphorus and nitrate concentration in leaves of inoculated bean plants cv Canellini grown with different levels of nitrate and phosphate.

Treatment (mM)		**Leaf concentration (%)**	
N	**P**	**N**	**P**
1	0.01	2.94a	0.18ab
1	0.1	2.88a	0.26b
1	2.0	2.89a	0.64c
10	0.01	4.81b	0.16a
10	0.1	4.16c	0.21ab
10	2.0	4.15c	1.44d

Means in columns followed by the same letter are not significantly different at $p<0.05$ (LSD test).

Table 4. Xylem sap composition of inoculated bean plants cv Canellini grown with different levels of nitrate and phosphate.

Treatments (mM)		**Ureides**	**Amino acids**	**Nitrate**	**Phosphate**
N	**P**	(μmoles ml^{-1} xylem sap)			
1	0.01	1.10ab	3.28a	19.68a	0.19
1	0.1	1.64a	5.54b	3.60b	0.74
1	2.0	2.26c	6.48b	2.61b	3.89
10	0.01	0.52bd	3.21a	34.4c	0.14
10	0.1	0.15d	4.83ab	27.6d	0.19
10	2.0	0.15d	5.47b	29.1d	6.56

Means in columns followed by the same letter are not significantly different at $p<0.05$ (LSD test).
Analysis of P concentration was performed on bulked sap samples (no replicates).

Table 5. Concentration of phosphate in xylem sap and sap flow of bean plants cv Canellini grown with different levels of nitrate and phosphate.

Treatments (mM)		**Sap $H_2PO_4^-$**	**Sap flow**
N	**P**	**(µmoles ml^{-1} xylem sap)**	**(µl h^{-1})**
0.2	0.01	0.23 a	40 a
0.2	0.10	0.88 b	95 b
1.0	0.01	0.07 c	47 a
1.0	0.10	0.42 d	88 b
5.0	0.01	0.09 c	95 b
5.0	0.10	0.09 c	60 a

Means in columns followed by the same letter are not significantly different at $p<0.05$ (LSD test).

Table 6. Shoot dry weight, nodule mass, number of nodules, and nitrogenase activity (ARA and SARA) in bean plants cv Canellini grown in pots with different nitrate levels using different substrates (sand and two soils).

Substrate	**Nitrate level** (ppm)	**Shoot dry weight** (g)	**Nodule mass** (g)	**No. Nodules**	**ARA** (μmol C_2H_4 h^{-1})	**SARA** (μmol C_2H_4 $g^{-1}h^{-1}$)
Sand	0	3.10a	490a	687a	4.70a	1.88a
	40	5.56b	158b	308b	1.30b	1.44ab
	80	5.34b	32dc	270b	0.27b	1.31b
Soil A	0	3.58a	114a	284a	0.56a	0.81a
	40	6.27b	58b	164b	0.84a	2.78b
	80	4.08a	0c	0c	0.00b	0.00c
Soil B	0	2.47a	70a	166a	0.35a	0.96a
	40	3.10a	21b	173a	0.15b	1.16a
	80	2.94a	0b	0b	0.00b	0.00b

Soil A, Castilblanco; Soil B, Las Torres.

For every substrate, means followed by the same letter are not significantly different at $p<0.05$ (LSD test).

Table 7. Concentration and content of N and P in shoots of bean plants cv Canellini grown in pots with different nitrate levels using different substrates (sand and two soils).

Substrate	**Nitrate level**	**N**		**P**	
	(ppm)	(%)	(mg/plant)	(%)	(mg/plant)
Sand	0	3.64a	112.8a	0.25a	0.78a
	40	2.52b	139.8b	0.17b	0.96b
	80	3.14c	167.6c	0.15b	0.78a
Soil A	0	1.83a	64.8a	0.13a	0.44a
	40	1.97a	123.8b	0.13a	0.80b
	80	3.59b	146.5c	0.09a	0.37a
Soil B	0	1.57a	39.3a	0.09a	0.22a
	40	2.68b	82.5b	0.13a	0.41b
	80	3.72c	108.1c	0.12a	0.35c

Soil A, Castilblanco; Soil B, Las Torres.

For every substrate, means followed by the same letter are not significantly different at $p<0.05$ (LSD test).

Table 8. Concentration of ureides, amino acids and nitrate in xylem sap of bean plants cv Canellini grown in pots with different nitrate levels using different substrates (sand and two soils).

Substrate	**Nitrate level** (ppm)	**Ureides**	**Amino acids**	**Nitrate**	**Phosphate**
			(μmoles ml^{-1} xylem sap)		
Sand	0	4.82a	5.40a	0.25a	2.65
	40	3.05b	5.54a	4.75b	1.07
	80	0.97c	4.63a	14.90c	1.84
Soil A	0	1.68a	3.08a	1.58a	0.77
	40	2.31b	8.08a	6.41a	0.60
	80	0.38c	7.82a	23.1b	0.31
Soil B	0	2.14a	3.89a	4.16a	0.38
	40	1.16a	6.07a	6.28a	0.32
	80	0.83a	7.65a	15.90b	0.50

Soil A, Castilblanco; Soil B, Las Torres.

For every substrate, means followed by the same letter are not significantly different at $p<0.05$ (LSD test).

Analysis of P concentration was performed on bulked sap samples (no replicates).

Captions to Figures.

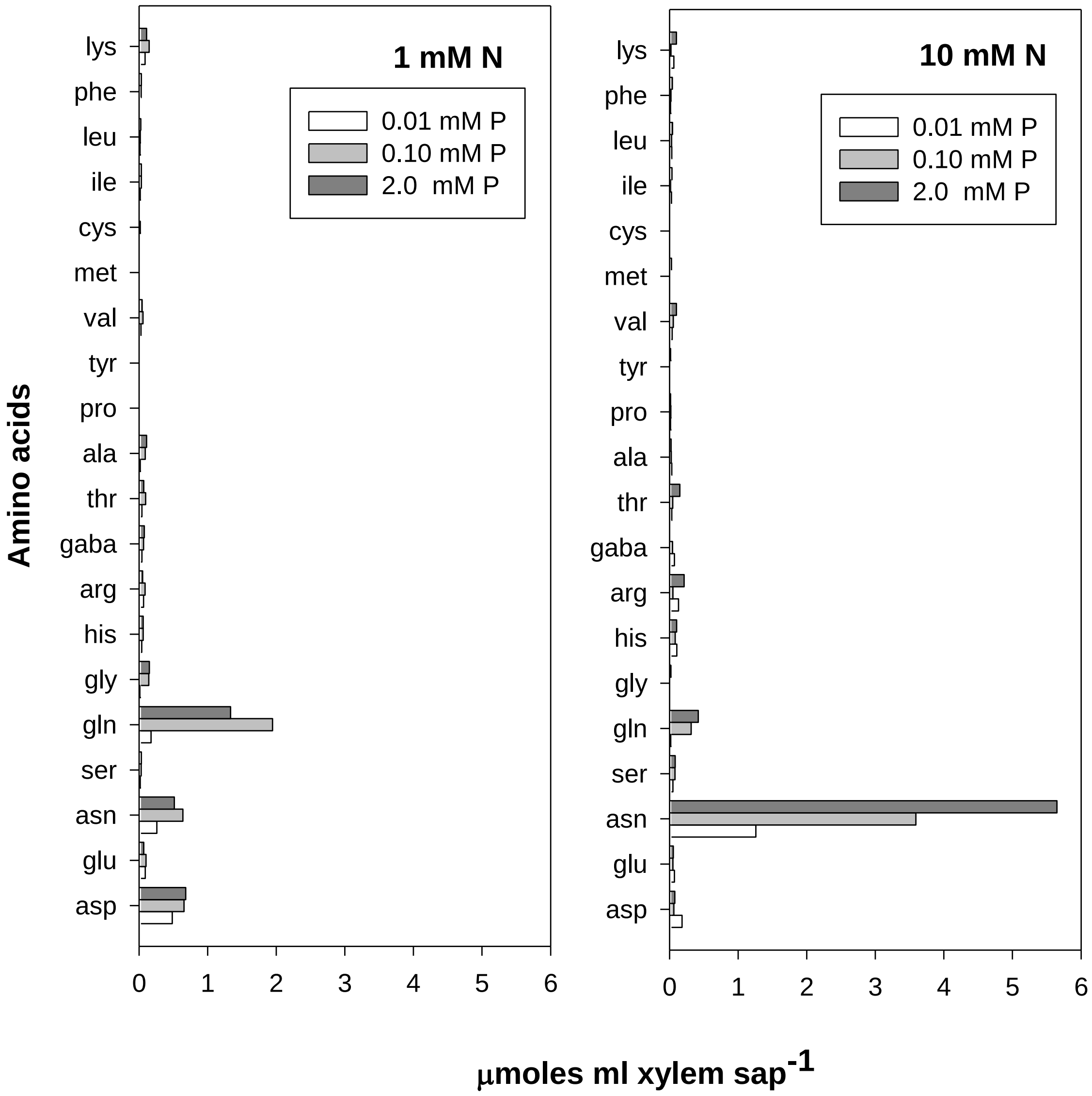
Figure 1. Effect of nitrate and phosphate concentration on amino acid composition of the xylem sap from bean plants.

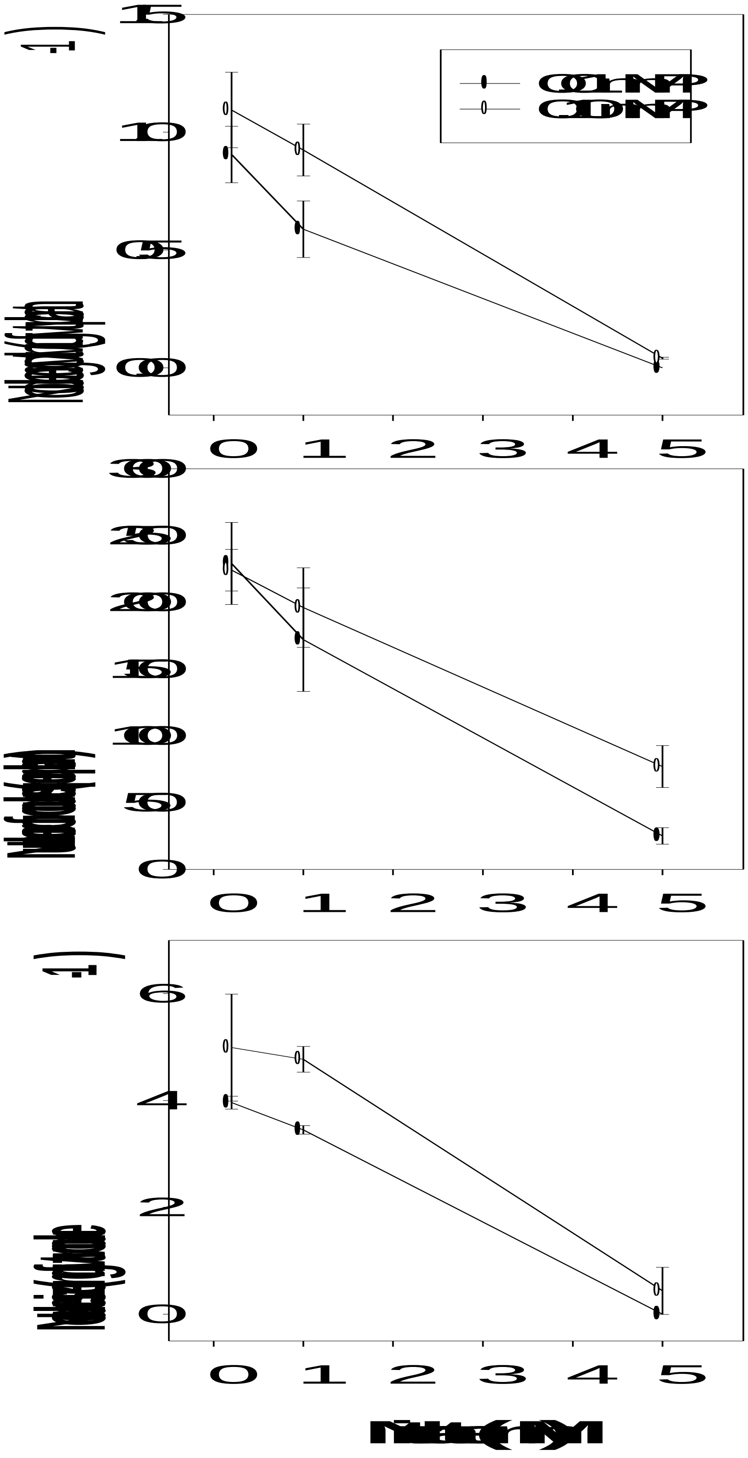
Figure 2. Effect of nitrate and phosphate concentration on nodulation parameters of bean plants cv Canellini.

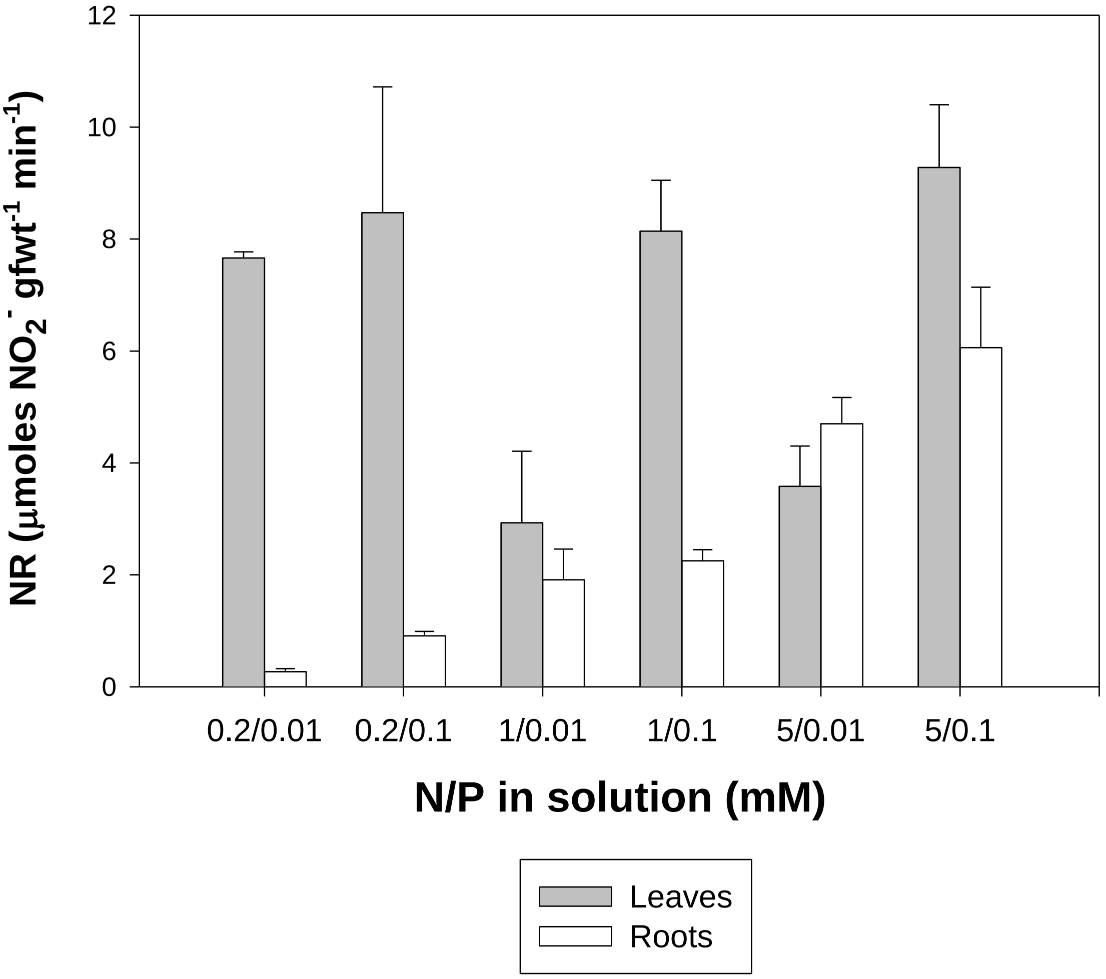
Figure 3. Nitrate reductase acitivity in leaves and roots of of bean plants cv Canellini grown with different levels of nitrate and phosphate.

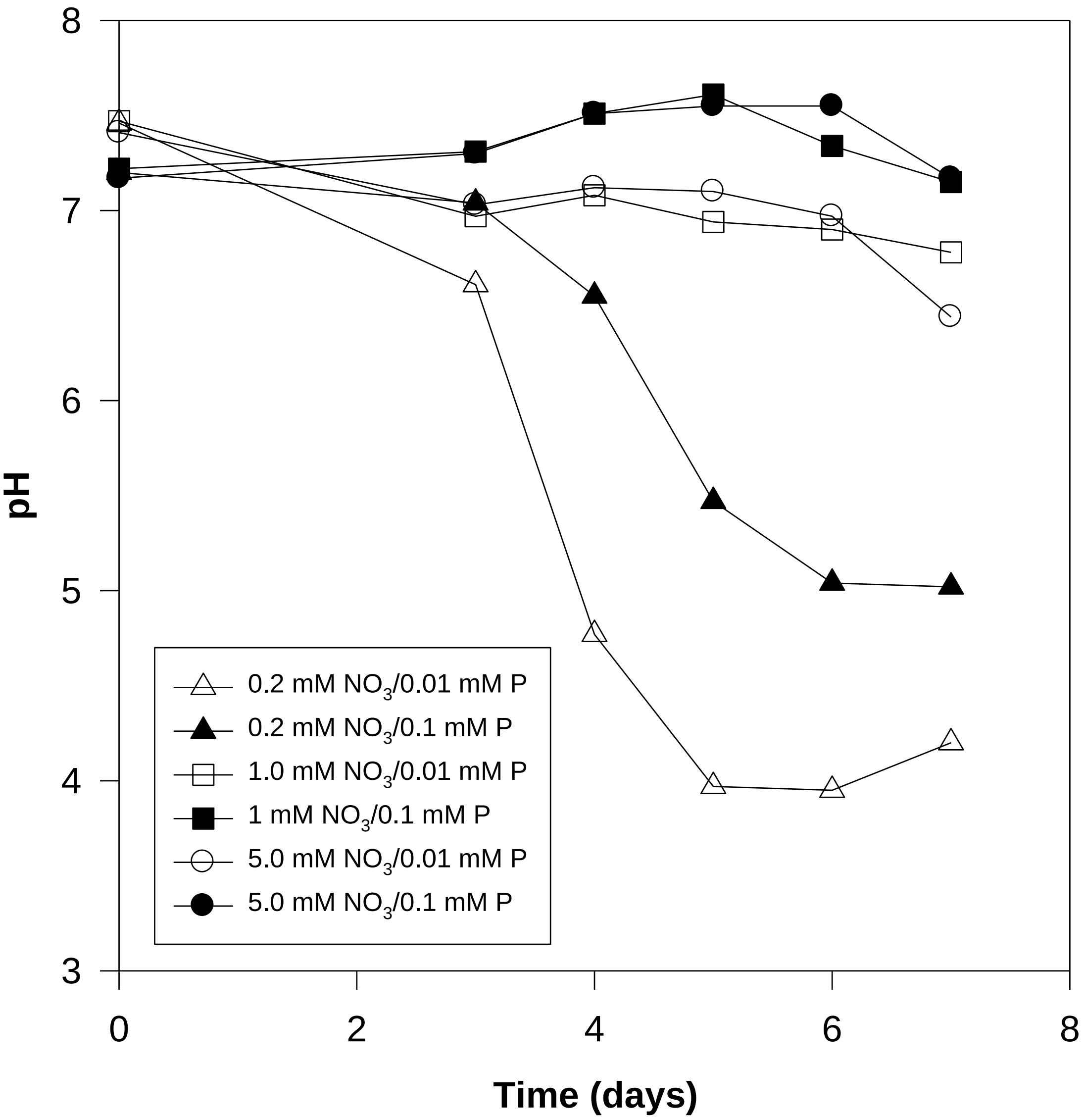
Figure 4. Changes in pH of aereated nutrient solutions after transferring bean plants cv Canellini grown with different levels of nitrate and phosphate.

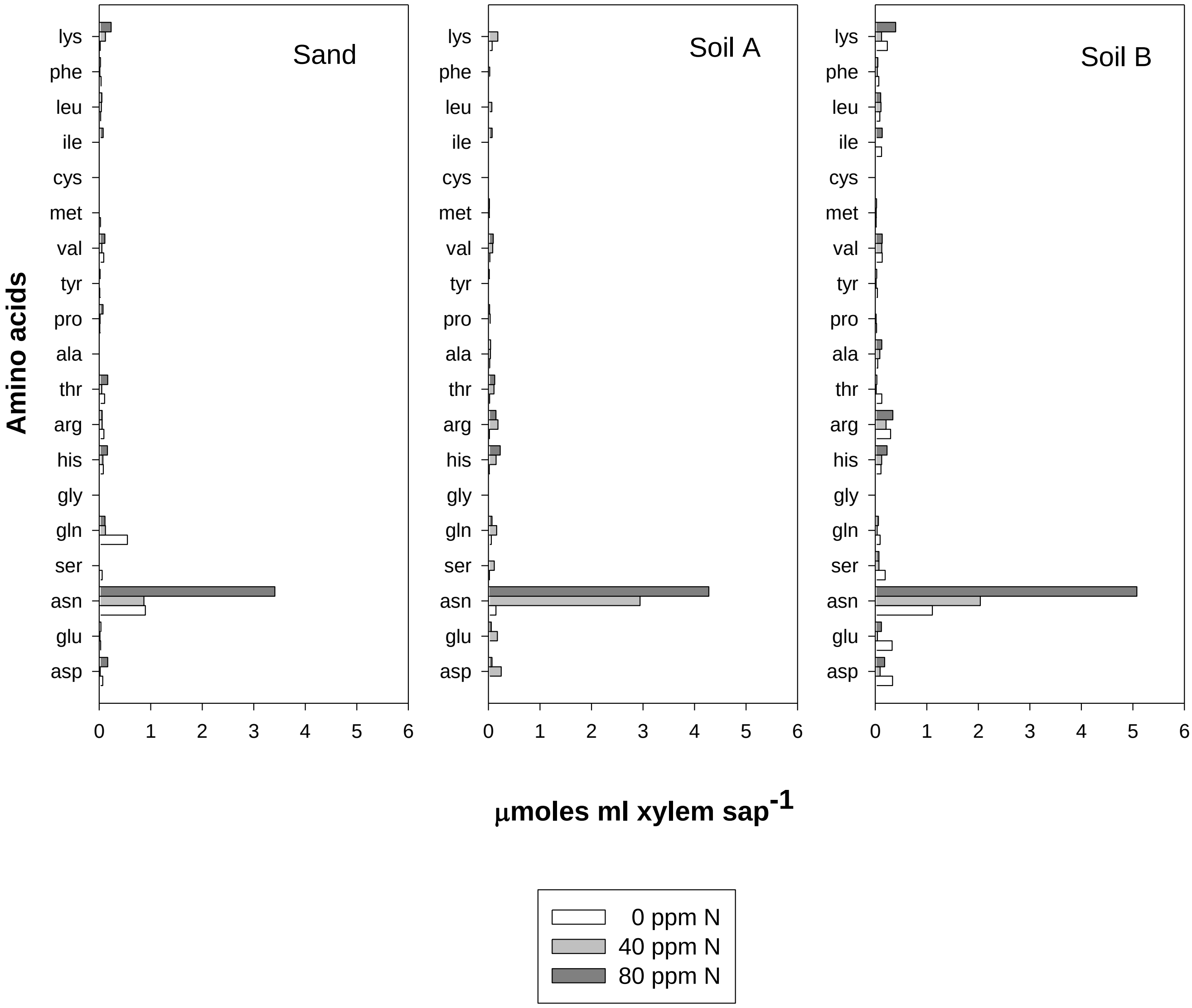
Figure 5. Effect of increasing nitrate concentration on amino acid composition of the xylem sap from bean plants grown in sand and two different soils.


1 mM N
10 mM N
0.01 mM P
0.10 mM P
2.0 mM P
Amino acids
lys
phe
leu
ile
cys
met
val
tyr
pro
ala
thr
gaba
arg
his
gly
gln
ser
asn
glu
asp
0
1
2
3
4
5
6
μmoles ml xylem sap-1


15
10
05
00
30
25
20
15
10
5
0
6
4
2
0
0
1
2
3
4
5
Nitrate (mM)


NR (μmoles NO_2^- gfwt^{-1} min^{-1})
0
2
4
6
8
10
12
0.2/0.01
0.2/0.1
1/0.01
1/0.1
5/0.01
5/0.1
N/P in solution (mM)
Leaves
Roots


8
7
6
5
4
3
pH
0
2
4
6
8
Time (days)
0.2 mM NO_3/0.01 mM P
0.2 mM NO_3/0.1 mM P
1.0 mM NO_3/0.01 mM P
1 mM NO_3/0.1 mM P
5.0 mM NO_3/0.01 mM P
5.0 mM NO_3/0.1 mM P


Sand
Soil A
Soil B
Amino acids
lys
phe
leu
ile
cys
met
val
tyr
pro
ala
thr
arg
his
gly
gln
ser
asn
glu
asp
0
1
2
3
4
5
6
μmoles ml xylem sap^{-1}
0 ppm N
40 ppm N
80 ppm N