Inorganic Nitrogen Nutrition Enhances Osmotic Stress Tolerance in *Phaseolus vulgaris*: Lessons from a Drought-sensitive Cultivar

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Abstract. The nitrogen (N) requirement of legumes can be met by inorganic N assimilation and symbiotic N₂ fixation. Both N assimilation routes have diverse influences on drought tolerance because of the differences in the use of energy and the reduction in power characteristics of those pathways. We investigated N nutrition under osmotic stress and likely criteria conferring tolerance to osmotic stress in common bean. Osmotic stress tolerance in a drought-sensitive common bean cultivar (Coco blanc) was compared with beans relying on N nutrition, N₂ fixation, or fertilization with urea. Osmotic stress was applied by means of 25 (mild stress), 50 (moderate stress), or 75 (severe stress) mM mannitol for 15 days. At the end of the stress period, relative water content, total N contents, total soluble sugars, total chlorophyll, total protein, and potassium were assayed. Urea-fed plants grew better and had better tolerance to osmotic stress. This was attributed to maintaining higher N use efficiency, better leaf hydration, and adequate osmoregulation.

The N requirement of legumes can be met by inorganic N assimilation and symbiotic N₂ fixation, and in practice, legumes obtain their N through both processes. The extent of damage caused by water stress depends on N nutrition among other factors (Gonzalez-Dugo et al., 2010; Lodeiro et al., 2000). Both N assimilation routes have diverse influences on drought tolerance because of the differences in the use of energy and the reduce of power characteristics of those pathways (Schubert, 1995).

Several authors have addressed the inhibitory effect of osmotic stress on root nodule activity in legumes (King and Purcell, 2001, 2005; Marino et al., 2006; Serraj and Sinclair, 1997) including common bean (*Phaseolus vulgaris* L.) (Sassi et al., 2008, 2010a,b). However, these studies described interactions between osmotic stress and N₂ fixation without considering the effect of stress on plants dependence on inorganic N. The particular way in which osmotic stress is imposed might be of special importance in understanding the field response to drought and/or salinity and

also in evaluating the plant's capacity to acclimate to stress. For long term-aerohydroponic experiments, mannitol supplied to the nutrient solution could simulate water deficit stress (Sassi et al., 2008, 2010). Moreover, it was previously demonstrated that the potential for nodulation and N₂-dependent growth of legumes was highly expressed in hydroaerobic culture (Aydi et al., 2004).

The physiological consequences of N₂ fixation in comparison with the assimilation of NH₄⁺ or NO₃⁻ studies with legumes have shown that reliance on fixed N₂ alters both the pattern of photosynthate partitioning to plant parts (Layzell et al., 1985) and the efficiency in which photosynthate is used in the assimilation of N (Lawlor, 2002; Luo et al., 2013). In terms of energy demand, these studies have shown, in general, that N₂ fixation is slightly more expensive than NO₃⁻ assimilation and significantly more costly than NH₄⁺ assimilation. Common bean responds to osmotic stress by osmotic adjustment, which enhances stress tolerance (Sassi et al., 2010a, 2010b).

A previous study with common bean cultivars showed the relative sensitivity of cv. Coco blanc to mannitol-induced osmotic stress (Sassi-Aydi et al., 2008).

The main purpose of the present study is to determine whether osmotic stress limits plant growth and photosynthate partitioning by affecting the same or different parameters with different N sources. In the work reported here, we have compared the sensitivity of common bean to several osmotic stress levels through the stages of nodule development and the initiation of N₂ fixation under conditions requiring the use of different N assimilation

routes. We also looked for the adequate N nutrition under osmotic stress and the likely criteria conferring tolerance to osmotic stress to the drought-sensitive cultivar, Coco blanc.

Materials and Methods

Plant growth and conditions of imposing osmotic stress. Common bean seeds of Coco blanc cv. were surface-sterilized and pre-germinated in 0.9% agar and then transferred to nutrient solution (Aydi et al., 2004) in 1-dm³ glass bottles wrapped with aluminium foil to maintain darkness in the rooting environment. The nutrient solution contained 0.25 mM KH₂PO₄, 0.7 mM K₂SO₄, 1 mM MgSO₄·7H₂O, 1.65 mM CaCl₂, and 22.5 mM iron for macronutrients and 6.6 mM manganese, 4 mM boron, 1.5 mM copper, 1.5 mM zinc, and 0.1 mM for micronutrients.

At the beginning of the experiment, 1 cm³ of *Rhizobium tropici* CIAT 899 [a reference strain largely used to study the N₂ fixation capacity of common bean (Sassi-Aydi et al., 2012)], containing ≈10⁸ cells/cm³, was added to the nutrient solution of only the symbiotic N-fixing plants. During the first 2 weeks, i.e., before nodule function, the nutrient solution was supplemented with 2 mM urea (Ribet and Drevon, 1996). For urea-fed plants, 2 mM urea was added to nutrient solution during the experiment. Neutral pH was maintained by adding 0.2 g·dm⁻³ CaCO₃ (Krouma et al., 2008). Bottles were aerated with a flow of 400 cm³·min⁻¹ of filtered air through a compressor and “spaghetti tube” distribution system. During the experiments, plants were grown in a controlled temperature glasshouse with night/day temperatures of circa 20/28 °C and a 16-h photoperiod. Supplemental irradiance of 400 μmol·m⁻²·s⁻¹ photosynthetic active radiation was supplied by mercury vapor lamps (OSRAM HQI-T400W/DH).

Osmotic stress treatments. Plants were distributed into eight treatments: two N nutrition forms (urea or symbiotic N fixation) and four osmotic treatments for each N form. Osmotic stress was applied by means of 25 (mild stress), 50 (moderate stress), or 75 (severe stress) mM mannitol, an osmotic component generally used to generate osmotic stress when added to nutrient solution. Mannitol was added to 25-d-old plants corresponding to the time of active N₂ fixation. Control plants were maintained in a mannitol-free solution. There were eight replicates for each treatment with only one plant per glass bottle. Nutrient solutions were renewed every 3 d and the bottles were distributed in a randomized block design depending on N nutrition.

At the beginning of flowering, 40 d after sowing, plants were harvested to determine growth parameters to compare stressed plants with non-stressed ones (controls).

Dry weight and leaf area. Two harvests were made: 1) at the beginning of treatment (initial harvest: six replicates); and 2) 15 d after the treatments (final harvest: eight replicates). Harvested plants were separated into leaves, petioles, stems, and roots and fresh

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weights (FWs) were determined. Leaf areas were determined with a portable Area Meter (Model LI-3000A; LI COR). Dry weights (DWs) of the different plant parts were determined after drying in an oven during 3 d at 70 °C.

Relative water content. The relative water content (RWC) was measured in the second or third youngest fully expanded leaf that was harvested in the morning. The RWC was determined using the following equation: $RWC(\%) = 100 \times [(FW - DW) / (TW - DW)]$

FW is the leaves fresh matter weight measured immediately at harvest and TW measured for the turgid fresh matter weight.

Turgid leaf weights were obtained by soaking the leaves in distilled water in test tubes for 4 h at room temperature (≈ 20 °C) under low light. Later, leaves were quickly and carefully blotted dry with tissue paper for determining turgid weight. Then, leaves were dried for 72 h in an oven at 70 °C for DW determination.

Nitrogen status. Nitrogen content was determined using the Kjeldahl Method (Bouat and Crouzet, 1965). Nitrogen uptake was calculated as the difference between N quantities (mmol per plant) measured at final harvest and the amount determined at the onset of osmotic treatment.

Potassium determination. Dried samples of stems, leaves, roots, and petioles were finely ground. Potassium extraction was achieved in 0.5% (v/v) HNO₃ solution at room temperature for 72 h, then assayed by flame-emission photometry (Corning Flame Photometer, Model M410; Sherwood Scientific Ltd., Cambridge, U.K.).

Total soluble sugars determination. Total soluble sugars were quantified using the anthrone method. Samples of 20 mg gunpowder, from the different organs, were homogenated in deionized water and incubated in a water bath at 70 °C and then centrifuged at 3000 g for 10 min. The supernatant was collected and the pellet was re-suspended for additional extraction. This procedure was repeated a third time to allow full extraction of soluble sugars (glucose, sucrose, and fructose). A 100- μ L aliquot of the three mixed supernatants was added to 4 mL of anthrone solution and incubated in a boiling water bath. The absorbance of the samples was determined spectrophotometrically according to Aydi et al. (2010).

Total protein content assay. Soluble protein was determined using the Bradford method using Coomassie Brilliant blue (Kruger, 2002) with bovine serum albumin as the standard. Mannitol induced an osmotic stress on treated plant tissues that could change the total soluble protein (TSP) concentrations among treatments. To eliminate this “dilution” effect by water content, TSP content per unit of mass was evaluated on a DW basis rather a FW basis in subsequent analyses.

Statistical procedures. Statistical analysis was carried out using the Statistica software (Version 5; StatSoft, France). The analysis of variance and the least significant difference

of the means were used to determine statistical significance ($P < 0.05$) between treatments. Data are presented as mean values of eight replicates with their corresponding SES.

Results

Growth parameters. Without water stress, the N₂-fixing plants had lower biomass compared with plants fertilized with urea (Fig. 1A). Growth reduction was 18%. The application of osmotic stress showed marked differences between plants with different N sources. Urea-fed plants always had higher plant biomass compared with their respective N-fixing plants under mild, moderate, and high stress. Osmotic reduced plant biomass for urea-fed plants by 17%, 32%, and 46%, respectively, at 25, 50, and 75 mM mannitol, whereas plant biomass was reduced for N₂-fixing plants by 48% at 25 and 50 mM mannitol and at $\approx 70\%$ at the severe osmotic stress (75 mM mannitol) compared with controls. The reduced biomass was mirrored by leaf area and number (Fig. 1B–C). It should be noted that the leaf area seemed to be the most

affected parameter in the N₂-fixing plants under stressed conditions (Fig. 1B).

Relative water content. Both leaves of N₂-fixing and urea-fed plants showed progressive decrease of RWC as the mannitol concentration increased in the nutrient solution (Fig. 2). However, there were no significant differences between the inoculated and fertilized plants of cv. Coco blanc in terms of leaf RWC. Nevertheless, it should be noted that RWC dropped significantly in N₂-fixing plants at 75 mM mannitol (severe osmotic stress).

Total chlorophyll content. Total chlorophyll content (TCC) was fairly constant in urea-fed plants of cv. Coco blanc under mild and moderate osmotic stress, whereas a slight decrease was observed at severe osmotic stress, being $\approx 18\%$ compared with controls. Nevertheless, severe osmotic stress induced a significant decrease of TCC in N₂-fixing plants exceeding 65% compared with their respective controls. Controls of both plant groups did not exhibit similar TCC. Urea-fed plants had higher chlorophyll contents than symbiotic N₂-fixing ones (Fig. 3A).

Fig. 1. Plant biomass (A), leaf area (B), and leaf number (C) of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Results are the means of eight replicates. Vertical bars indicate SEMs. Values marked by different letters within a panel are significantly different at $P \leq 0.05$ according to least significant difference (LSD) analysis.

Total soluble sugar content. Without mannitol induced stress, total soluble sugar (TSS) differed from plant N assimilation groups (Fig. 3B). Urea-fed plants had higher TSS content compared with N₂-fixing ones. The addition of increased mannitol concentration to the growing medium induced the accumulation of different patterns of TSS. In urea-fed plants, mild and moderate osmotic stress decreased sugar content by ≈20% to 25%, respectively, whereas severe osmotic stress (75 mM mannitol) increased the content of these solutes by ≈10% compared with their respective controls. For the N₂-fixing plants, TSS content was similar to controls in both mild and moderate stressed plants, whereas at severe osmotic stress conditions, the increase of TSS content was ≈60% (Fig. 3B).

Nitrogen content. At mild, moderate, and severe osmotic stress, the N content decreased in both fertilized and in N₂-fixing plants (Fig. 4). This decrease was higher in N₂-fixing plants, especially at severe osmotic stress (75 mM mannitol) exceeding 90%, whereas it did not reach 70% in urea-fed plants (Fig. 4).

Total protein content. Total protein content (TPC) declined significantly in leaves of both plant groups of cv. Coco blanc as a result of osmotic stress (Fig. 5). In urea-fed plants, mild osmotic stress had no significant effect on TPC, whereas in N₂-fixing plants, it seems that severe osmotic stress had harmful effects on the accumulation of soluble protein in leaves. The reduction reached ≈90% compared with their respective controls. Similar TPC was measured in the unstressed controls, either in urea-fed plants or in N₂-fixing ones.

Potassium accumulation. Without mannitol-induced stress, urea-fed plants and symbiotic N₂-fixing ones showed similar patterns of potassium accumulation (Fig. 6). In osmotic-stressed common bean plants, potassium content diminished with increasing stress level. Nevertheless, in moderate and severe-stressed urea-fed plants, potassium content remained unchanged.

The distribution of potassium in leaves, petioles, stems, and roots is shown in Figure 7. Results showed noticeable differences between urea-fed and N-fixing plants. In urea-fed plants, potassium seems to be more accumulated in roots under control conditions, whereas as mannitol concentration increased in the nutrient solution, more potassium is partitioned to the leaves (leaf and petiole). At the highest mannitol concentration, only a small amount of the potassium content was allocated to roots. In the N₂-fixing plants, potassium seems to be more equally distributed among the four plant parts in 0, 25, or 50 mM mannitol. However, as the stress level increased (75 mM mannitol), more potassium was allocated to leaves (leaf and petiole) compared with roots and stems.

Discussion

Relative sensitivity of the studied *Phaseolus vulgaris* genotype (Coco blanc) to osmotic stress was already reported by Jebara

Fig. 2. Relative water content (RWC) of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Values shown are means of eight replicates. Vertical bars indicate SEMs. Bars with different letters are significantly different at $P \leq 0.05$ according to least significant difference (LSD) analysis.

Fig. 3. Total chlorophyll content (TCC) (A) and total soluble sugar (TSS) content (B) of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Results are the means of eight replicates $\pm$ SD. Bars with different letters are significantly different at $P \leq 0.05$.

Fig. 4. Nitrogen content of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Numbers at the bars refer to nitrogen use efficiency (g dry biomass/mmol N). Values shown are means of eight replicates $\pm$ SD. Bars with different letters are significantly different at $P \leq 0.05$ according to least significant difference (LSD) analysis.

Fig. 5. Soluble protein content of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Results are the means of eight replicates $\pm$ SD. Values marked by different letters within a panel are significantly different at $P \leq 0.05$ according to least significant difference (LSD) analysis.

Fig. 6. Potassium content [$\mu\text{mol g}^{-1}$ dry weight (DW)] of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Results are the means of eight replicates $\pm$ SD. Bars with different letters are significantly different at $P \leq 0.05$ according to least significant difference (LSD) analysis.

Fig. 7. Distribution of potassium in different plant parts (leaf, petiole, stem, root) of common bean plants cultivated using different nitrogen sources: urea or dependent on symbiotic nitrogen fixation (SNF). Plants were cultivated under non-stressed conditions (control), low (25 mM mannitol), medium (50 mM mannitol), or high osmotic stress (75 mM mannitol). Results are the means of eight replicates.

et al. (2010) for salt stress and by Sassi et al. (2008) for mannitol-induced osmotic stress. In crop legumes, atmospheric N may be fixed through the nitrogenase pathway in the root nodules, and/or inorganic N in solution may be principally assimilated through the nitrate reductase reaction sequence in leaves.

In the present study, non-ionic osmotic stress was imposed by three mannitol concentrations for 15 d on inoculated bean plants at the stage of nodule functioning and active N_2 fixation and on non-inoculated bean plants fertilized with 2 mM urea. To avoid different initial vigor of the treatment plants, the osmotic stress was applied at the active N_2 fixation period and evading the stage of the onset of nodule functioning. Sassi-Aydi et al. (2006) demonstrated that osmotic stress damage depends on the developmental stage at which the stress is applied with the effect being more harmful if the stress occurs throughout the nodule growth stage.

The work reported here displayed a clear difference between the damage caused by osmotic stress on urea-fed and N_2 -fixing plants in terms of growth, leaf area, and leaf number (Fig. 1). Several works showed different tolerance to osmotic stress among legume genotypes when they were inoculated with efficient rhizobia or N-fertilized; Sulieman and Schulze (2010) in barrel medic; and Lodeiro et al. (2000) and Tejera et al. (2004) in common bean. These authors showed differences in osmotic stress tolerance between N assimilation pathways. Osmotic stress tolerance under each pathway seems to depend on the developmental stage when stress was applied at the level of the stress and the crop legume genotype. Our results showed better tolerance of urea-fed plants compared with ones grown solely on symbiotic N_2 fixation as a N source. Preferential N assimilation by either one of the mentioned routes has diverse influences on osmotic tolerance. This could be the result of the differences in the use of energy and the reduce of power characteristic of those pathways, as described in some lupins species (*Lupinus luteus*, *Lupinus angustifolius*, and *Lupinus albus*) (Schubert, 1995) and soybean (*Glycine max*) (Kirova et al., 2008). The total chlorophyll content decreased more in the N_2 -fixing plants (Fig. 3A) showing more harmful effect of osmotic stress on photosynthesis. In terms of energy demand, previous studies have shown that symbiotic N_2 fixation, by common bean, soybean, and lupins, is slightly more expensive than NO_3^- assimilation and significantly more costly than NH_4^+ assimilation (Le Roux et al., 2009; Nielsen et al., 2001). Those studies have shown that reliance on fixed N_2 alters both the pattern of photosynthate partitioning to plant parts and the efficiency in which photosynthates used in the assimilation of N. At the highest osmotic stress (75 mM mannitol), the urea-fed plant group had higher nitrogen use efficiency (NUE) than the N_2 -fixing plants, (Fig. 4). It seems that in high-stressed conditions, urea-fed plants were more efficient in N assimilation. Previous studies, conducted on soybean,

report that sole dependency on N_2 fixation is more costly in terms of NUE (Matsunami et al., 2004, and references therein). Our results demonstrated that N_2 -fixing bean plants were relatively deficient in N as indicated by the lower NUE (Fig. 4). This deficiency seems to be inherent in the N assimilation system of N_2 -fixing bean and soybean plants (Bellaloui et al., 2013; Olivera et al., 2004), which in turn could limit the potential development of photosynthate and limit water and nutrient-absorbing organs. This was mirrored by lower RWC mainly at severe osmotic stress in N_2 -fixing bean plants (Fig. 2). Those findings imply that uptake and translocation of water could be more efficient in urea-fed plants and suggest that urea-fed leaves could be more resistant to dehydration than N -fixing leaves. However, differences in RWC values could also be a consequence of different initial cell sap osmolality, cell wall elasticity, or osmoregulation (Sassi et al., 2010a).

Soluble sugars have been shown to increase under osmotic stress and are potentially important contributors to osmotic adjustment (OA) in legume (*Medicago truncatula*) and non-legume (*Triticum aestivum*) species (Aydi et al., 2010; Bajji et al., 2001; Khoshro et al., 2013; Sayed et al., 2013). Data herein showed decreased sugar contents at mild and moderate osmotic stress in leaves of both-treated plant groups, whereas osmotic stress-accumulated sugar contents was observed at high stress (Fig. 3). Various hypotheses have been proposed to account for these observations. Decreased sugar content could be the consequence of osmotic stress-induced reduction in photosynthesis and the subsequent shortage of photoassimilates in the aerial parts (Hussin et al., 2013; Tejera et al., 2006), either in urea-fed or in plants growing solely on symbiotic N_2 fixation. It could also be the result of equilibrium between produced and used soluble sugar. In urea-fed plants, quaternary ammonium compounds could be accumulated for osmotic adjustment instead of soluble sugars at mild and moderate osmotic stress because those plants seems not to be N-deficient (Fig. 4). Hence, higher total protein (reduced N) was measured in urea-fed plants at all stress levels (Fig. 5), indicating that more N had been metabolized by urea-fed than by N_2 -fixing plants during the 6-week growth period. The accumulation of sugars in severe osmotic-stressed plants seems to depend on the reduction in the use of assimilates induced by osmotic stress in relation to an inhibition of sucrose synthase or invertase activities mainly in nodules of symbiotic N_2 -fixing plants such as pea (*Pisum sativum*) (Gálvez et al., 2005) common bean (Sassi et al., 2008), and the wild legume *Lotus japonicus* (Tsikou et al., 2013) or by slowing their transport to nodules in chickpea (*Cicer arietinum*) and soybean (Gil-Quintana et al., 2013; Soussi et al., 1999) because RWC of those plants dropped at severe stress (Fig. 2). Such a reduction leads to the subsequent inhibition of growth (Fig. 1) and may contribute to the observed increase of sugar content (Fig. 3B). Concerning urea-fed plants, the

accumulation of soluble sugars observed with severe stress may be the result of stress-induced inhibition of phloem loading (Bajji et al., 2001; Lemoine et al., 2013; Martorell et al., 2014) and/or to a stimulation of sucrose phosphate synthase activities (EC 2.4.1.14) (Fu et al., 2010; Huber and Huber, 1996; Vandoorne et al., 2012). An increase in soluble sugar content for OA could prevent RWC dropping under severe osmotic stress (Fig. 2), which would preclude leaf dehydration (Fresneau et al., 2007).

Unlike organic solutes, inorganic solutes (notably potassium content) did not seem to play as an important role in OA because their amounts were either unaffected or even reduced in stressed leaves of both plant groups (Fig. 6). Generally, increased concentrations of inorganic ions as a consequence of water deficits have not been commonly found in the tissue of higher plants. According to Hu and Schmidhalter (2005), decreased potassium availability in plants with decreasing soil water content is the result of decreasing K^+ mobility. This was not fully verified in our study because Figure 7 showed preferential allocation of potassium to photosynthetic organs (leaves and petioles) as the stress level increased, notably in the urea-fed plants showing better potassium mobility as compared with N_2 -fixing plants. Other inorganic solutes (SO_4^{2-} , Cl^- , and PO_4^{3-}) were present at much lower concentrations than K^+ (data not shown) and their amounts slightly decreased or remained unchanged in response to osmotic stress and their contributions to OA, if any, would therefore be limited.

Conclusion

Urea-fed and N_2 -fixing common bean plants had markedly different terms of growth, chlorophyll content, N content, and solute accumulation in response to osmotic adjustment. Urea-fed plants grew better, especially at high osmotic stress. This was attributable essentially to 1) higher NUE; 2) better leaf hydration; and 3) adequate osmoregulation. We suggested that reliance on fixed N_2 alters both the pattern of N partitioning to plant parts and the efficiency in which N is used to sustain growth.

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