Bradyrhizobium improves nitrogen assimilation, osmotic adjustment and growth in contrasting cowpea cultivars under drought

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Abstract

This study investigated the effect of inoculation with *Bradyrhizobium* spp. on nitrogen metabolism and growth of two contrasting *Vigna unguiculata* cultivars exposed to both inoculation and water deficit. The study was carried out in Universidade Federal Rural da Amazônia (01°27′S and 48°26′W). The plants remained in greenhouse environment. The experimental design used was completely randomised with 2 cultivars (drought tolerant and sensitive) combined with 2 water regimes (water deficit and control), and 2 inoculation treatments (inoculated and non-inoculated), totalizing 8 treatments. The variables evaluated were leaf relative water content, nitrate reductase activity, nitrogen compounds, and growth parameters. Results of this study showed that both cultivars presented increases in nitrate and proline, compared with non-inoculated plants. Water deficiency induced more intense reductions in shoot dry matter of non-inoculated plants, compared with inoculated plants. This investigation confirmed the hypothesis that inoculation with *Bradyrhizobium* improves nitrogen assimilation, osmotic adjustment and growth parameters in *Vigna unguiculata* plants.

Keywords: Nitrogen metabolism; *Vigna unguiculata* [L.] Walp.; leguminous; biological fixation of nitrogen; inoculation; water deficit.
Abbreviations: N_2 _nitrogen; H^+_hydrogen; NH_4^+_ammonium; E.C. 1.6.6.1_nitrate reductase; GS_glutamine synthetase; ATP_ adenosine-5'_triphosphate; GOGAT_glutamate synthase; P5CR_pyrrolline-5-carboxylate reductase; NO_3^-_nitrate; NO_2^-_nitrite.

Introduction

Water deficit is an abiotic factor that affects agricultural production, influencing aspects related to plant development, such as decrease in photosynthesis rate, reduction in leaf area (Fontana et al., 1992), and stomata closing (Santos and Carlesso, 1998). As in other crops, performance in cowpea is affected by water deficiency, which can cause lower growth and development, with progressive reduction in leaf dry matter (Costa et al., 2011) and consequent reduction in yield and yield parameters such as the number of grains and pods per plant. Cowpea (*Vigna unguiculata* (L.) Walp.) plants are considered tolerant to water deficit, and important mechanisms have been developed by this species to tolerate inadequate water supply. For example, biochemical modifications in carbon metabolism, such as increase in sucrose (Lobato et al., 2009a); as well as interference in nitrogen metabolism, such as reduction of soluble proteins (Costa et al., 2011) and increase in total amino acids (Lobato et al., 2008) contribute to osmotic adjustment of *Vigna unguiculata* plants. The biological fixation of nitrogen is the capacity of a distinct group of microorganisms to divide the molecule of nitrogen (N_2) and to combine hydrogen atoms (H^+), forming ammonium (NH_4^+) (Albino and Campo, 2001), under symbiosis with root system. The *Bradyrhizobium* gender is described as soil bacteria that have ability to infect

root hair of leguminous plants, and it can induce nodule formation, with subsequent fixation of nitrogen (Mercante et al., 2002). Negative effects of water deficit are described in physiological (Ismail et al., 2004), biochemical (Hamidou et al., 2007), morphological (Lobato et al., 2008), and agronomical (Leport et al., 1998) aspects, and these modifications can influence yield components such as grains per pod and pod per plant. (Szilagyi, 2003; Showemimo and Olarewaju, 2007). In other hand, the responses in morphological parameters have not always been associated with nitrogen compounds such as increase in proline and reduction in proteins (Costa et al., 2011). Beneficial effects provided by the inoculation on growth parameters as leaf, stem and root are well known in leguminous plants (Ramos et al., 1999; Silveira et al., 2001; Moraes et al., 2010), but information on the specific aspects of this symbiotic process on essential compounds such as amino acids and proteins are limited. Figueiredo et al. (1999) reported that inoculation using *Bradyrhizobium* can alleviate the negative consequences in *Vigna unguiculata* plants induced by water deficiency, but Serraj and Sinclair (1996) revealed that water supply limits on symbiotic efficiency. This study aimed to investigate if inoculation with *Bradyrhizobium* spp. can improve the plant performance linked to nitrogen

assimilation, osmotic adjustment, and growth parameters of two contrasting *Vigna unguiculata* cultivars exposed to both inoculation and water deficit.

Results

Leaf relative water content, nitrate reductase activity, and nitrate

The inoculated plants had higher values linked to leaf relative water content, compared with same treatments in non-inoculated plants (Fig 1 A), in both well-watered and water deficit treatments. Drought tolerant plants had a lower reduction in leaf water content under water deficit, compared with drought sensitive cultivars. Water deficit reduced nitrate reductase activity (Fig 1B) in all treatments, when compared to respective control. The inoculation produced an increase in nitrate of both cultivars (Fig 1C), compared to the same treatments without inoculation. Both non-inoculated cultivars had lower nitrate concentrations under water restriction compared with the well-watered controls.

Total soluble amino acids, total soluble proteins, and proline

The concentration of total soluble amino acids in plants subjected to inoculation was higher only in tolerant plants, compared with the same treatments on non-inoculated plants (Fig 2 A). Water deficit promoted a significant increase in this variable to all treatments, with exception in tolerant plants under inoculation. Water deficiency caused a significant decrease in total soluble proteins both cultivars in inoculated and non-inoculated plants, with a slightly greater decrease for sensitive plants (Fig 2 B). For proline the inoculated plants presented higher values, compared with same treatments in non-inoculated plants (Fig 2 C). The two cultivars demonstrated higher values under water limitation, compared with their respective controls.

Shoot dry matter, plant dry matter, and leaf number

The inoculation caused an increase in shoot dry mater, compared with the same treatments in non-inoculated plants (Fig 3 A). Water deficit resulted in a significant decrease in this parameter especially for the sensitive cultivar. The inoculation caused a slight increase in plant dry matter, with exception of the sensitive cultivar under irrigation (Fig 3 B), compared with same treatments in non-inoculated plants. Water deficiency induced a decrease in plant dry matter for all treatments, compared with control plants. Across all treatments, sensitive cultivars had lower plant dry matter compared with tolerant cultivars. Leaf number increased slightly after inoculation, with exception of sensitive plants under water deficiency (Fig 3 C). Water deficit reduced leaf number for all plants, compared with well-watered control plants. The tolerant cultivar had higher leaf number, compared with the sensitive cultivar.

Discussion

The inoculation promoted attenuation of the negative effects induced by water limitation on leaf relative water content. The decrease of this variable can be explained by the reduction in water availability in substrate (Lawlor and Cornic, 2002), because the water deficiency induces turgor loss and consequent stomatal closing, which will affect other physiological processes such as photosynthesis and
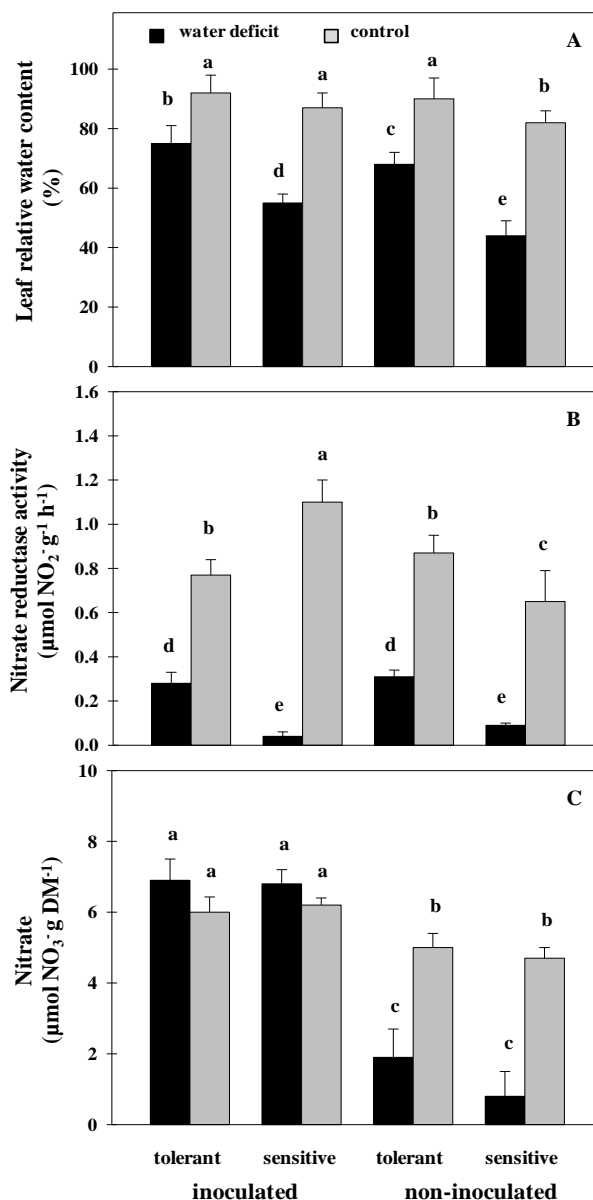
Fig 1. Leaf relative water content (A), nitrate reductase activity (B), and nitrate (C) in two contrasting *Vigna unguiculata* plants under water deficit and subjected to inoculation. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

transpiration (Nogueira et al., 1998). This reduction was also shown by Lobato et al. (2009b) studying two *Vigna unguiculata* cultivars exposed to water restriction, corroborating the data from this research. The lower nitrate reductase activity in inoculated plants could be due to nitrogen being absorbed in the N_2 form and transformed into NH_4 in the symbiotic process mediated by nitrogen fixing bacteria (Gerahty, 1992; Taiz and Zeiger, 2004), using the nitrogenase enzyme (Burris, 1999). In addition, Silveira et al. (2001) described an intense activity of the nitrate reductase in root and/or nodules can compensate the decrease of this enzyme in leaves. But further studies will be necessary to understand the regulatory mechanisms of this metabolic pathway. The reduction of this enzyme during water deficit is explained by Machado et al. (1992); by negatively affecting

the nitrogen metabolism, as well as the nitrate reductase being extremely sensitive to water availability (Costa, 1999). Serraj and Sinclair (1996) reported that several tropical leguminous such *Vigna unguiculata* are partially affected by the water restriction. Ferreira et al. (2002) also obtained lower activity of this enzyme with water deficiency in two *Zea mays* genotypes. In this study, the nitrate concentration was increased by the inoculation, and this effect might be considered indirect. The increase in concentration of organic nitrate in inoculated plants under water deficit is a response induced by decrease in nitrate reductase (NR) activity, because the nitrate acts as substrate in reaction catalyzed by nitrate reductase enzyme, and enzyme is responsible for the reduction of nitrate (NO_3^-) from nitrite (NO_2^-) (Ferreira et al., 2002). In other words, the fall in nitrate reductase activity (Figure 1 B) promotes accumulation of organic nitrate. The reduction in nitrate in non-inoculated plants and under drought is associated with the lower assimilation of nitrogen in nitrate form. Similar results on the increase of nitrate in leaves were described by Delfini et al. (2010) investigating different tissues in *Arachis hypogaea* plants induced by inoculation of *Bradyrhizobium sp.* Inoculated drought tolerant plants showed increase in amino acids, and this can be attributed to biological fixation of nitrogen. The nitrogenase enzyme promotes the nitrogen absorption in form of nitrogen gas (N_2) and conversion to ammonium (NH_4^+). In addition, the increased formation of amino acids is probably linked to an increase in activity of the enzyme glutamine synthetase (GS), which depends ATP (adenosine-5'-triphosphate), and glutamate synthase (GOGAT). In addition, the increase in amino acids of plants exposed to inoculation is due to greater flux and better assimilation of nitrogen in form of ammonium, concomitantly with higher activity of GS and GOGAT enzymes. Ramos et al. (2005) evaluating the responses of *Glycine max* plants under water deficit and inoculation of *Bradyrhizobium japonicum* also observed an increase in concentration of total soluble amino acids. The concentration of total soluble amino acids in plants under water deficit increased in all treatments. This increment probably occurred due to increase in activity of protease enzymes, responsible for the breakdown of proteins to adjust the plant osmotically (Costa et al., 2006). For tolerant plants, this parameter was higher in inoculated than non-inoculated treatment, however an opposite tendency showed in sensitive plants exposed to non-inoculation and drought, being explained by the sensibility of this cultivar under condition of water restriction. Similar data on increase in amino acids were obtained by Costa et al. (1996) investigating *Vigna unguiculata* plants. Delfini et al. (2010) evaluating the responses of two *Arachis hypogaea* cultivars inoculated with *Bradyrhizobium sp.* showed significant increase in amino acids. The increase in total soluble proteins induced by inoculation suggests that bacteria action resulted in increase in nitrogen supply through a secondary route, that is regulated by the nitrogenase, because since there was no corresponding increase in activity of nitrate reductase after inoculation in this study. Hristozkova et al. (2006) evaluating the responses in *Pisum sativum* plants under inoculation and molybdenum application also obtained an increase in protein levels. The decrease in protein levels promoted by the water deficit is associated with a decrease of the protein synthesis combined with an increase of proteolytic enzymes, responsible by breakdown of soluble proteins in plants (Lechinoschi et al., 2007). Costa (1999) obtained similar results studying *Vigna unguiculata* subjected to water deficit, corroborating our results. The increase of proline levels induced by the inoculation was probably linked to better
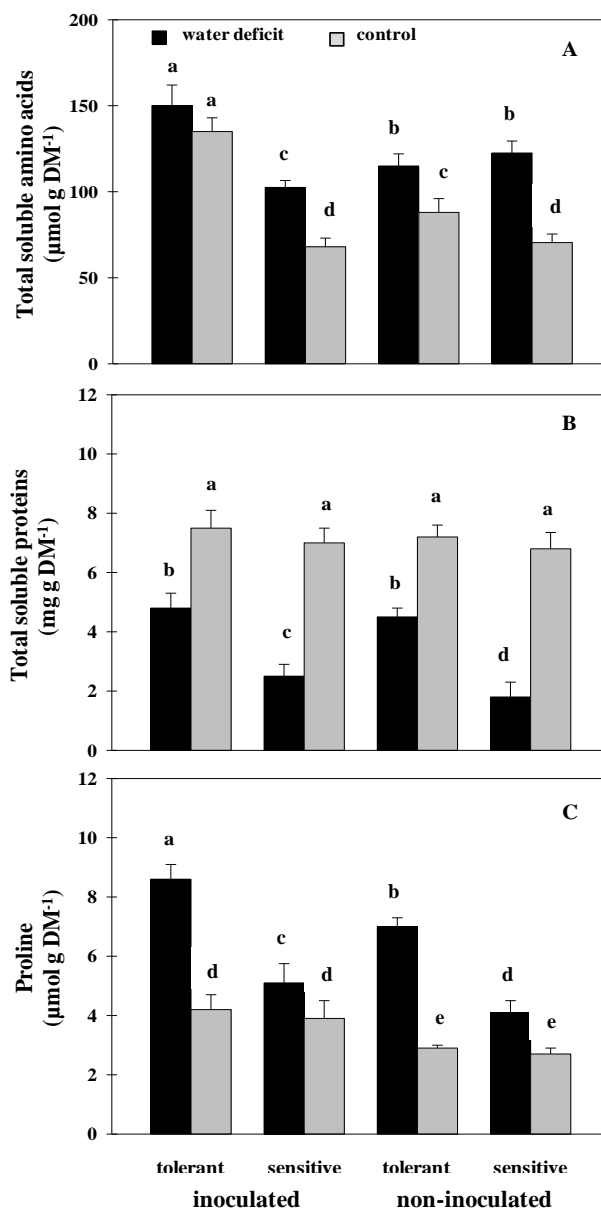
Fig 2. Total soluble amino acids (A), total soluble proteins (B), and proline (C) in two contrasting *Vigna unguiculata* plants under water deficit and subjected to inoculation. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

amino acid utilisation such as glutamic acid and arginine, glutamic acid being the precursor of the proline, while arginine can suffer from a reaction mediated by the enzyme, pyrroline-5-carboxylate reductase (P5CR) and consequently liberate proline (Ramos et al., 2005). Kohl et al. (1991) also observed higher amounts of proline in *Glycine max* plants inoculated with *Bradyrhizobium japonicum*, supporting this study. The increase of proline in plants under water deficit is a response to loss of cell turgidity (Oliveira et al., 2006). Nogueira et al. (1998) stated that proline accumulation was related to drought tolerance in higher plants, actuating as osmoregulator agent to keep water in plant tissues (Muchow and Carberry, 1993). Similar behaviour was described by González et al. (1998) working with *Pisum sativum* plants under water restriction. Shoot dry matter was maximised after the inoculation, and this fact can be explained by increase in
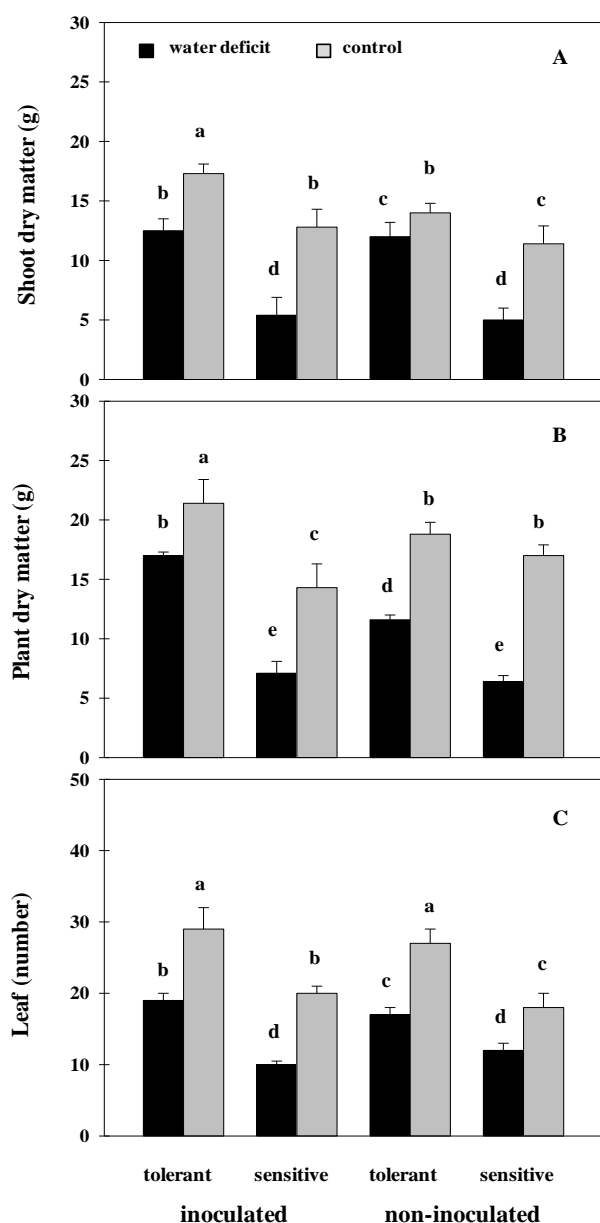
Fig 3. Shoot dry matter (A), plant dry matter (B), number of leaves (C) in two contrasting *Vigna unguiculata* plants under water deficit and subjected to inoculation. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

total soluble proteins, with consequent better supply of nitrogen, in which is essential to adequate plant development (Epstein and Bloom, 2006; Souza et al., 2008). Similar data linked to shoot dry matter were reported by Figueiredo et al. (1999) with *Vigna unguiculata* plants exposed to *Bradyrhizobium* inoculation. Drought reduced the production of shoot dry matter due to inhibition in biochemical processes such as biological fixation of nitrogen (Costa et al.,1996), indirectly modifying the partitioning of photo-assimilates in root and shoot, and consequently resulting in a decrease in accumulation of shoot biomass (Correia and Nogueira, 2004a). Similar results were found by Mendes et al. (2007) working with two *Vigna unguiculata* cultivars subjected to water deficiency during two stages. The inoculation increased total dry matter, and this may be linked to better development

and efficiency of root system, providing higher nitrogen absorption through the nodulation process. In addition, the higher nitrogen fixation will produce an increase in amino acids and proteins (Delfini et al., 2010), and also photoassimilate availability. Similar responses were described by Sassi et al. (2010) investigating two *Phaseolus vulgaris* cultivars subjected to inoculation with bacteria of *Rhizobium* genus. Plants under water deficit frequently have the production of dry matter reduced, due to the decrease in several metabolic processes such as water and nutrient absorption, which are fundamental to maintain adequate growth and development rates. Nascimento (2009) also reported that water deprivation affects the osmotic mechanism, and by consequence reduces the CO_2 supply, essential for the photosynthetic process. Similar results were found by Leite and Virgens Filho (2004) studying *Vigna unguiculada* plants exposed to water limitation. The increase in leaf number promoted by inoculation may be probably due to the higher number of nodules in root, and consequently due to the better biological fixation of nitrogen (Ferreira et al., 2011). Araújo et al. (2009) studying *Vigna unguiculata* and *Leucaena leucocephala* plants also reported an increase of this variable, confirming our data. The lower leaf number after water deficiency is caused by the process of leaf abscission, due to insufficient water and nutrient to the plant (Santos and Carlesso, 1998). Correia and Nogueira (2004b) obtained similar results with *Arachis hypogaea* plants under water deficit.

Materials and methods

Growth conditions

The study was carried out in Instituto de Ciências Agrárias (ICA) of the Universidade Federal Rural da Amazônia (UFRA), Belém city, Pará state, Brazil (01°27′S and 48°26′W). The plants remained in greenhouse environment under natural sunlight conditions, with air temperature minimum and maximum of 24.1 and 38.2°C, respectively. Air relative humidity oscillated between 72 and 89%. The photoperiod was 12 h of light.

Plant materials, substrate and pot

The *Vigna unguiculata* (L.) Walp. seeds used in this study were Pitiuba and Pérola cultivars, in which are tolerant and sensitive to water deficit, respectively (Lobato et al., 2009b). The substrate used for the plant growth was composed by a mixture of sand and silic in the proportion of 2:1, respectively, and it was autoclaved at $120°C\ atm^{-1}$ for 40 min. The container used for the plant growing was Leonard pot with 2 L capacity, adapted in Laboratório de Fisiologia Vegetal Avançada (LFVA).

Experimental design

The experimental design used was a completely randomised with 2 cultivars (drought tolerant and sensitive), 2 water regimes (water deficit and control), and 2 inoculation treatments (inoculated and non-inoculated), making up 8 treatments, with 6 replicates and 48 experimental units, in which each experimental unit consisted of 1 plant pot^{-1}.

Inoculation and conduction of plant

Three seeds per pot were sowed and thinned out to 1 seedling after germination. The seedlings were inoculated with 1 ml of

Bradyrhizobium spp. (BR 3256) suspension with concentration of 1.0×10^9 CFU by 3 times, and at regular intervals on the 5th, 10th and 15th day after the experiment started. The control and water deficit treatments received macro and micronutrients from nutrient solution of Hoagland and Arnon (1950), for 30 days, and the nutrient solution was changed with 2 days of interval, always at 09:00 h. The pH of the nutrient solution was adjusted to 6.0 ± 0.1 with addition of HCl or NaOH. On the 30th day after the experiment started, the plants of the treatment under water deficit were submitted to period of 5 days without nutrient solution, in which the deficit was simulated from the 30th until 35th day after of the experiment started.

Number of leaves

At the end of the experimental period, the plants were harvested and number of leaves counted, including the first pair of leaves. Subsequently, leaf fresh matter was used to determine leaf relative water content and nitrate reductase activity.

Leaf relative water content

Leaf relative water content was evaluated on leaf disks of 10 mm diameter from each plant; 40 disks were removed and the calculation was done using the formula proposed by Slavick (1979): $LRWC = [(FM - DM)/(TM - DM)] \times 100$
Where: FM is fresh matter, TM is turgid matter evaluated after 24 h saturation in deionised water at 4°C in dark, and DM is the dry matter determined after 48 h in oven with forced air circulation at 80°C.

In vivo nitrate reductase activity

reductase enzyme (E.C. 1.6.6.1) was extracted from 200 mg of leaf and root samples. and incubated in 5 mL of extraction buffer (KH_2PO_4 at 0.1 M, KNO_3 at 50 mM, isopropanol at 1% (v/v) and pH 7.5) for 30 minutes at 30ºC, and all the procedures were carried out in the dark. The quantification of the enzyme activity was in accordance to the method of Hageman and Hucklesby (1971) with absorbance at 540 nm using spectrophotometer (Quimis, model Q798DP).

Shoot dry matter, plant dry matter, and sample preparation

Leaf, stem and root were divided and dried in an oven with forced air circulation at $70 \pm 2°$ C by 96 h. After drying, shoot dry matter (leaf and stem) and plant dry matter (leaf, stem and root) were determined. Subsequently, plant tissues were triturated, with the resulting powder kept in glass containers. These containers remained in the dark at 15ºC until they were ready for biochemical analysis.

Nitrate, total soluble amino acids, and proline

In determination of nitrate, amino acids and proline were performed using 50 mg of leaf dry matter powder, and incubated with 5 mL of sterile distilled water at 100ºC for 30 minutes. After incubation, the homogenised was centrifuged at 2.000 *g* for 5 minutes at 20ºC and supernatant was removed. The quantification of the nitrate was carried out at 410 nm in accordance with Cataldo et al. (1975), with KNO_3 (Sigma Chemical) as standard. Quantification of the total soluble amino acids was carried out at 570 nm according to the method of Peoples et al. (1989), and L-asparagine + L-

glutamine (Sigma Chemicals) was used as standard. The quantification of proline was performed after measuring the absorbance at 520 nm according to Bates et al. (1973) based on L-proline (Sigma Chemicals) as standard.

Total soluble proteins

Determination of the total soluble proteins was carried out with 100 mg of powder, incubated with 5 mL of extraction buffer (Tris-HCl at 25 mM and pH 7.6). This was homogenised and kept in agitation for 2 h, and centrifuged to 2.000 *g* by 10 minutes at 20ºC. Quantification of the total soluble proteins was carried out at 595 nm in accordance with Bradford (1976), with albumin bovine (Sigma Chemicals) as standard.

Data analysis

Data were subjected to variance analysis and when significant differences were detected, Scott-Knott test at 5% level of error probability was applied (Steel et al., 2006). Standard errors were calculated for all means. All statistical procedures were carried out with the SAS software (SAS, 1996).

Conclusions

Inoculation with *Bradyrhizobium spp.*induced increases in shoot dry matter, and also higher levels of nitrogen compounds such as nitrate and proline, compared with the same treatments in non-inoculated plants. This investigation confirmed the hypothesis that inoculation promotes improved nitrogen assimilation, osmotic adjustment and growth parameters in *Vigna unguiculata* plants.

Acknowledgments

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