

Metabolism of nitrogen and carbon: Optimization of biological nitrogen fixation and cowpea development



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ABSTRACT

Although the favorable role of plant growth-promoting bacteria (PGPB) in biological nitrogen fixation (BNF) is well established, their involvement in nodule metabolism is unknown. This study was performed to determine the relationship between C and N metabolism in cowpea nodules inoculated with only *Bradyrhizobium* sp. or with double and triple combinations of *Bradyrhizobium* sp., and two PGPB, *Paenibacillus graminis* and *Paenibacillus durus* during two critical phases of nodule development (flowering and beginning of senescence). The triple inoculation (*Bradyrhizobium* sp. and two PGPB) induced higher N content in nodules, total-N accumulation per plant and shoot dry weight compared with other combinations at the beginning of senescence. This increased N performance was positively correlated with the nodule sucrose content but not with the content of total soluble carbohydrates, reduced sugars and starch. The higher BNF under triple inoculation conditions was not significantly associated with sucrose synthase activity but was slightly associated with soluble acid invertase activity in nodules at the beginning of senescence. These enzymes exhibited opposite responses between flowering and the beginning of senescence in all treatments. Glutamate synthase, glutamine synthetase and glutamate dehydrogenase were stimulated by double (*Bradyrhizobium* sp. + *P. durus*) and triple inoculation compared with other treatments. Our data revealed that inoculation with *Bradyrhizobium* sp. and PGPB is favorable for BNF activity in cowpeas. This positive interaction requires a complex balance involving enzymes and metabolites related to C and N metabolism.

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1. Introduction

Nitrogen (N) is the most abundant element in Earth's atmosphere, and its deficiency results in changes in root formation, photosynthesis, the production and translocation of photo-assimilates, and plant growth rate (Shridhar, 2012). According to Andrews et al. (2009), N availability can occur in different ways depending on the plant species. Oxidized (NO_3^-) or reduced (NH_4^+) inorganic N can be absorbed from soil or obtained from atmospheric N_2 through biological nitrogen fixation (BNF). This process can be affected by physical, chemical and biological factors, and it is

more frequent when legumes are in symbiosis with fixing bacteria (Bernard and Habash, 2009; Rodrigues et al., 2013).

Due to the high price of N fertilizers, resulting from the consumption of fossil fuels during their production, and serious pollution problems caused by the intensive use of these fertilizers, BNF is a viable alternative for supplying the needs of plant species for N compounds. Thus, studies are necessary to examine maximization of BNF and thereby increase the availability of N to plants until the grain-filling period and the efficient use of carbohydrates from photosynthesis for energy production during BNF (Ladrera et al., 2007; Herridge et al., 2008; Franche et al., 2009; Larrainzar et al., 2009).

During rhizobia-legume symbiosis, plants provide carbohydrates mainly as sucrose (Shridhar, 2012). Sucrose is derived from leaves, transported by phloem and then released in the roots due to the action of sucrose synthase, a key enzyme in carbon flux regulation in root nodules that is associated with neutral invertases (Ben Salah et al., 2009, 2011). Sucrose is converted in hexoses,

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Table 1

Strain, growth conditions and source of the microorganism's species utilized for the inoculants preparation used in the experiment.

Species	Strain	Culture medium	Growth conditions	Source
<i>Bradyrhizobium</i> sp.	BR 3267	Yeast-Mannitol	200 rpm; 28 °C; 96 h	National Center of Agrobiolgy Research (CNPAB, RJ-Brazil)
<i>Paenibacillus durus</i>	C 04.50	Trypticase Soy Broth	200 rpm; 32 °C; 48 h	Federal University of Rio de Janeiro (UFRJ; Microbiology Institute)
<i>Paenibacillus graminis</i>	MC 04.21	Trypticase Soy Broth	200 rpm; 32 °C; 24 h	Federal University of Rio de Janeiro (UFRJ; Microbiology Institute)

which are oxidized in bacteroids as an energy source during BNF (Larrainzar et al., 2009; Lugtenberg and Kamilova, 2009). Furthermore, carbohydrates can produce organic acids that are utilized as carbon skeletons for the assimilation of ammonia produced during BNF (Herridge et al., 2008; Andrews et al., 2009; Larrainzar et al., 2009; Shridhar, 2012).

For efficient BNF, it is necessary to select efficient and competitive strains of rhizobia that have synergy with other microorganisms, thus resulting in increased BNF (Figueiredo et al., 2008; Rodrigues et al., 2013). However, it is necessary to understand the combination and compatibility of the bacterial strains involved in BNF (Figueiredo et al., 2010). The co-inoculation of rhizobia with plant growth-promoting bacteria can have positive effects by increasing phytohormone production and nutrient mobilization, mainly N and C (Ott et al., 2005; Ladrera et al., 2007; Larrainzar et al., 2009). However, the role of these organisms in nodule metabolism related to BFN has been scarcely studied.

The hypothesis that the interaction of rhizobia with plant growth-promoting bacteria (PGPB) could improve BNF, promote legume species growth and contribute to the delay of nodule senescence was tested and examined in this study. Cowpea plants inoculated with *Bradyrhizobium* sp. and PGPB (*Paenibacillus graminis* and *Paenibacillus durus*) were evaluate in terms of BNF associated with activities of the main enzymes and metabolites involved with C and N metabolism during two critical phases: plant flowering and the beginning of cowpea nodule senescence.

2. Materials and methods

2.1. Preparation and application of inoculants

The strains used in the experiment were grown prior to inoculant preparation (Table 1). For inoculant preparation, two grams of exopolysaccharide (EPS) were dried, sieved, autoclaved (120 °C; 101 kPa; 15 min), then cooled in a fresh, dry location and stored at room temperature (24 °C). Autoclaved and distilled water (2.0 mL) was added to the EPS, and the mixture was homogenized and incubated for 30 h to allow the mixture to stabilize chemically and for the pH to reach optimum range (6.8–7.0).

After achieving the appropriate pH, 3.0 mL of the bacterial inoculum (in duplicate) was added to 1.0 g of the EPS, thereby forming the treatments. The treatments were (T1) only *Bradyrhizobium* sp. (1.5×10^8 CFU mL⁻¹ + 1.5 mL of sterile distilled water); (T2) *Bradyrhizobium* sp. and *P. graminis* (1.5×10^8 CFU mL⁻¹ of

Bradyrhizobium sp. + 1.5×10^7 CFU mL⁻¹ of *P. graminis*); (T3) *Bradyrhizobium* sp. and *P. durus* (1.5×10^8 CFU mL⁻¹ of *Bradyrhizobium* sp. + 1.5×10^7 CFU mL⁻¹ of *P. durus*); and (T4) *Bradyrhizobium* sp. plus *P. graminis* and *P. durus* (1.5×10^8 CFU mL⁻¹ of *Bradyrhizobium* sp. + 0.75×10^7 CFU mL⁻¹ of each *P. graminis* or *P. durus*).

After maturation at room temperature (24 °C) for 48 h, 1.0 g of the inoculant (EPS + bacteria, as described above) was dissolved in a saline solution (0.85% NaCl), kept in agitation (300 rpm; 28 °C; 30 min) until homogeneous and then used in the inoculation or co-inoculation of cowpea seeds, as described in section 2.2. Plate counts of the inoculants were performed using the drop plate method of dilutions (10^5 – 10^7) in yeast mannitol agar (YMA) culture medium with 0.25% Congo red.

2.2. Characterization of *Rhizobium tropici* exopolysaccharides (EPS)

The EPS synthesized by *Rhizobium tropici* (EI-6) was used for inoculant preparation. For characterization, the EPS were subjected to thermal analysis by differential scanning calorimetry, viscosity and viscoelasticity analysis in a rheometer (Haake®; model RS 150), acetyl and pyruvate lateral group analysis by colorimetric methods, and carbohydrate analysis by comparative thin-layer chromatography. As a result of these analyses, EPS was defined as a non-Newtonian fluid that was slightly viscous and pseudoplastic, with a melting point at 178 °C, and did not show true-gel behavior (Table 2). Furthermore, the chemical analyses indicated that EPS is a hetero-polysaccharide consisting of glucose and galactose with an absence of glucuronic and galacturonic acids and the presence of acetyl and pyruvate lateral groups. The EPS polymer was considered inert to bacterial strains and utilized as an alternate vehicle for inoculation because it promotes greater symbiotic efficiency, growth and productivity in cowpea plants (Rodrigues, 2012).

2.3. Experiment preparation, inoculation and planting

The experiment was conducted in a greenhouse at the Agro-nomical Institute of Pernambuco (IPA) at a temperature range of 27–36 °C with 50–70% relative humidity and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. Cowpea seeds (*Vigna unguiculata* subsp. *unguiculata* cv. IPA-206) were disinfected and sown in Leonard jars containing washed (pH 6.5) and autoclaved (120 °C; 101 kPa; 1 h) sand as the substrate. To each cowpea seed sown in Leonard jars, 2.0 mL of the inoculant (EPS + bacteria) was added. This inoculant was drained into the substrate as follows: only

Table 2Physical and chemical characterization of *Rhizobium tropici* (EI-6) exopolysaccharide (EPS).

	Physical characteristics			Chemical characteristics	
	Melting point	Viscosity	Viscoelasticity	Carbohydrates	Lateral groups
EPS	178 °C	Non-Newtonian fluid, slightly viscous, pseudoplastic	<6.0 Hz – viscous >6.0 Hz – elastic	Heteropolysaccharide of the glucose and galactose without uronic acids	Acetyl – 5.50% Pyruvate – 7.1%

Font: Rodrigues (2012).

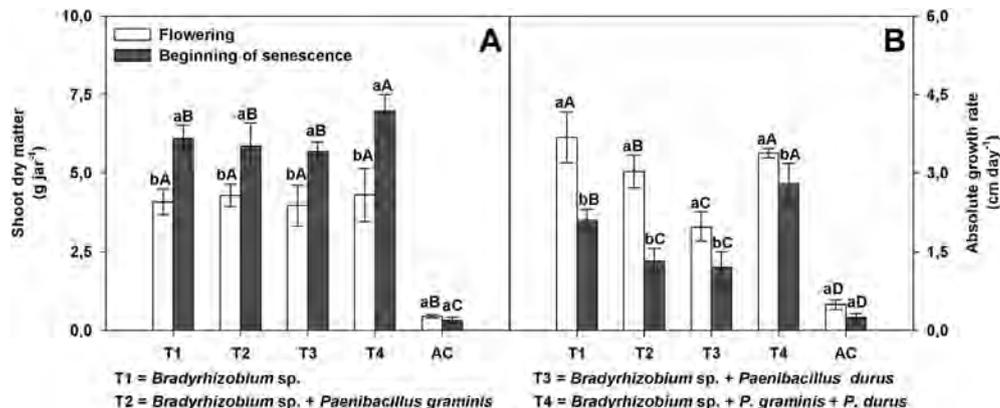


Fig. 1. Characterization of the cowpea plants. (A) Shoot dry matter ($CV^* = 11.06\%$) and (B) absolute growth rate ($CV = 14.10\%$) in cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Uninoculated plants were used as an absolute control (AC). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

Bradyrhizobium sp.; co-inoculated with *Bradyrhizobium* sp. and *P. graminis* or *Bradyrhizobium* sp. and *P. durus*; or co-inoculated with *Bradyrhizobium* sp. and both PGPB (*P. graminis* and *P. durus*). Uninoculated plants were used as absolute controls. After thinning the cowpea seedlings at seven days, two plants were retained in each Leonard jar.

During the experimental period, the plants were irrigated by capillary with N-free Hoagland and Arnon (1950) nutritive solution, as modified by Silveira et al. (1998). The plants were collected at two harvest times: (1) flowering, i.e., the period of highest N-fixation (36 days) and (2) the beginning of senescence, i.e., the period of N fixation decline (56 days). The roots were collected and the nodules were removed, weighed, frozen in liquid N₂ and stored in a freezer at -80°C until the analyses.

2.4. Biochemical determinations

2.4.1. Metabolite concentration

To characterize the levels of ammonia, free amino acids, ureides and soluble carbohydrates, fresh nodules were mixed with 1.5 mL of distilled water and brought to 95°C for 1 h to obtain the extract. Aliquots of the supernatant were analyzed for ammonia composition using the phenol-hypochlorite method (Weatherburn, 1967); free amino acids and ureides using the Yemm and Cocking (1955) and Young and Conway (1942) colorimetric methods, respectively; and soluble carbohydrates using the phenol-sulfuric acid method (Dubois et al., 1956). For starch determination, the resulting pellet was treated with perchloric acid and then aliquots of the supernatant were utilized (McCready et al., 1950).

The extraction and determination of leghemoglobin in the cowpea nodules was performed using Drabkin's reagent following the method described by Smaghe et al. (2009). Sucrose levels were determined according to van Handel (1968) using the extract obtained with an MCW (methanol, chloroform and water) solution. Reducing sugars were estimated by subtracting the concentration of sucrose from that obtained for total soluble carbohydrates. The extraction and determination of total N measurement was performed using a method proposed by Baethgen and Alley (1989). For soluble protein, fresh nodules were mixed with 1.5 mL of 100 mM phosphate buffer (pH 7.0), macerated until homogeneity and then centrifuged. The supernatants obtained were utilized for soluble protein determination as described by Bradford (1976).

2.4.2. Enzymatic activities

Fresh nodules were extracted with the appropriate buffers, and the supernatant was collected and used for the determination of the following enzymatic activities: glutamine synthetase (GS; EC 6.3.1.2), glutamate synthase (GOGAT; EC 1.4.1.14), aminant glutamate dehydrogenase (GDH; EC 1.4.1.2), phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), neutral invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13). All results were expressed as nodule fresh mass because the humidity was similar for all treatments.

The GS activity was determined as described by Elliott (1955) and was expressed as $\mu\text{mol } \gamma\text{-glutamyl hydroxamate g}^{-1} \text{FW h}^{-1}$. The GOGAT activity was measured following the oxidation of NADH and was expressed as $\mu\text{mol NADH g}^{-1} \text{FW min}^{-1}$ (Suzuki et al., 1994). The GDH activity was determined according to the methodology proposed by Coombs and Hall (1982) and was expressed as $\mu\text{mol NADH g}^{-1} \text{FW min}^{-1}$. PAL activity was determined based on the conversion of L-phenylalanine into *trans*-cinnamic acid and was expressed as $\text{nmol } \textit{trans}\text{-cinnamic acid g}^{-1} \text{FW h}^{-1}$ (Zucker, 1965; El-Shora, 2002). Soluble acid invertase activity was measured according to the method described by Zhu et al. (1997) and was expressed as $\mu\text{mol glucose g}^{-1} \text{FW h}^{-1}$. Sucrose synthase activity was determined in accordance with the method proposed by Hubbard et al. (1989) and was expressed as $\mu\text{mol glucose g}^{-1} \text{FW min}^{-1}$.

2.5. Statistical design and analysis

The experimental design consisted of randomized blocks with four replications using a 2×4 factorial arrangement: two harvest periods (flowering and beginning of senescence) and four treatment groups (one inoculation with only the *Bradyrhizobium* sp. and three co-inoculations of *Bradyrhizobium* sp. and PGPB). The data were subjected to analysis of variance (ANOVA) using the F test ($P < 0.05$), and the comparison of means was performed using Tukey's test ($P < 0.05$). All analyses were performed with ASSISTAT software.

3. Results and discussion

Cowpea plants inoculated and co-inoculated with plant growth-promoting bacteria (PGPB) exhibited alterations in growth (shoot dry weight) in response to the harvest times (Fig. 1). In comparing *Bradyrhizobium*-inoculated plants with those co-inoculated with

Bradyrhizobium sp. and both PGPB during the flowering, no significant differences in shoot dry matter or the absolute growth rate was observed (Tukey's test; $P < 0.05$). The plants co-inoculated with *Bradyrhizobium* sp. and both PGPB exhibited higher shoot dry matter accumulation at the beginning of senescence. This indicates that PGPB have a beneficial effect on the plant growth that is associated with an improvement in BNF and photosynthesis. Indeed, these two processes might act in synergy, as the nodules supplying N and photosynthetic processes ensure that sucrose is transported to the nodules for use as an energy source (Ladrera et al., 2007; Liu et al., 2011).

Plants co-inoculated with *Bradyrhizobium* sp. and either *P. graminis* or *P. durus* did not exhibit major alterations in shoot dry matter or absolute growth rate during the beginning of senescence compared to plants co-inoculated with *Bradyrhizobium* sp. and both PGPB, suggesting a single PGPB inoculum may be less effective than co-inoculation with multiple PGPB cultures. In plants co-inoculated with *Bradyrhizobium* sp. and both PGPB, the highest averages in variables related to growth were observed at the beginning of senescence. During senescence, physiological changes occur in the nodules, and this can lead to a loss of BNF efficiency (Franche et al., 2009). However, these changes were most likely minimized in plants co-inoculated with *Bradyrhizobium* sp. and both PGPB. This may be due to changes in the C and N flow between the plant and bacteroids in response to compounds released by PGPB (Lugtenberg and Kamilova, 2009; Shridhar, 2012).

The N-content in the cowpea shoot was altered in response to treatments (Fig. 2). Plants co-inoculated with *Bradyrhizobium* sp. and both PGPB exhibited higher N levels accumulated in shoots than plants inoculated solely with *Bradyrhizobium* sp. (Fig. 2A). This may represent a beneficial response triggered by substances released by the PGPB that are active in cowpea plants (Rodrigues et al., 2013). The effective association between rhizobia and PGPB can influence the BNF (Kuklinsky-Sobral et al., 2004), and a positive interaction between the plant and bacteria results in increased plant productivity and BNF (Herridge et al., 2008).

N-content at flowering did not differ significantly among the different treatments; however, there was a difference in the N-content at the beginning of senescence (Fig. 2B). Plants inoculated with *Bradyrhizobium* sp. and both PGPB had significantly higher N-contents at the beginning of senescence than following other treatments (Fig. 2B). These results reinforce the idea that co-inoculation with *Bradyrhizobium* sp. and both PGPB was more

effective than other combinations. According to Dawson et al. (2008), BNF is influenced by the symbiont genotypic characteristics and depends on the host range, specificity and symbiotic efficiency.

Nodule fresh matter was not significantly different in plants inoculated solely with *Bradyrhizobium* sp. than in those co-inoculated with *Bradyrhizobium* sp. and PGPB (Tukey's test, $P < 0.05$); however, significant differences were detected between the harvest times (Fig. 3A). For total N-levels in the nodules, plants inoculated with *Bradyrhizobium* sp. and both PGPB exhibited higher values ($70 \pm 12\%$) than those inoculated with only *Bradyrhizobium* sp. (Fig. 3B). Although the nodules had begun senescence, the results indicate that the energy flow directed to nodule production and growth in this treatment was maintained, suggesting a higher BNF efficiency.

BNF varies depending on biological and environmental factors, and this effectiveness can be measured by determining the concentrations of the compounds involved in this process (Liu et al., 2011). BNF was evaluated by measuring the total amount of N accumulated in both the shoot and the nodule (Fig. 4). There were no significant differences (Tukey's test, $P < 0.05$) in nodule total free amino acids at the time of flowering. Plants inoculated with only *Bradyrhizobium* sp. exhibited an increase in nodule total free amino acids of 39% when comparing the two harvest times, whereas this increase was approximately 30% in plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* (Fig. 4A). These results indicate the stimulation of protein catabolism and a reduction in amino acid exportation by the bacteroids to the host plant (Sarma and Emerich, 2005; Dawson et al., 2008).

Amino acids and ammonia exchange between the bacteria and host plant are important for a continuation of the BNF (Salavati et al., 2011). In this study, an increase in free ammonia was observed in response to different treatments and harvest periods (Fig. 4B). Plants co-inoculated with the *Bradyrhizobium* sp. and *P. graminis* symbiotic pair and those co-inoculated with *Bradyrhizobium* sp. and both PGPB had higher free ammonia in the nodules than other treatments at flowering (Fig. 4B). At the beginning of senescence, plants inoculated with only *Bradyrhizobium* sp. exhibited lower ammonia accumulation in their nodules than plants that received other treatments. Ammonia accumulation may be a reflection of frequent protein turnover, which provides amino acid reuse and maintains appropriate nodule metabolism (Cheng et al., 2010).

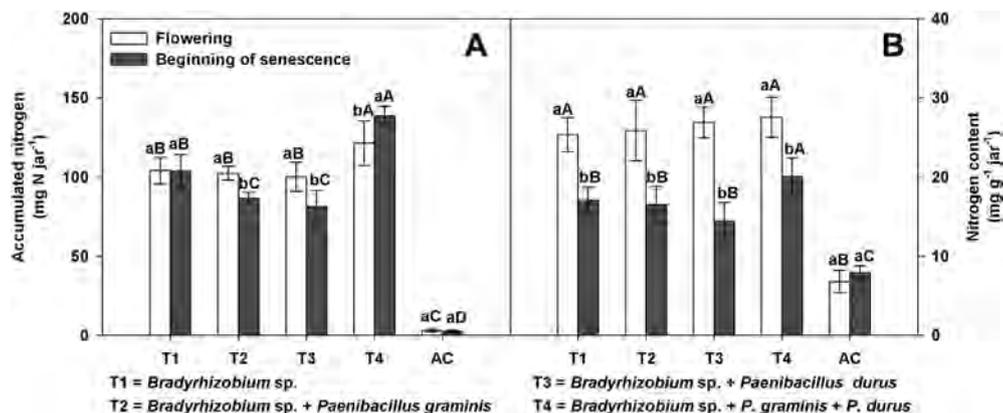


Fig. 2. Characterization of the cowpea plants. (A) Accumulated nitrogen ($CV^* = 9.20\%$) and (B) nitrogen content ($CV = 11.58\%$) in cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Uninoculated plants were used as an absolute control (AC). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

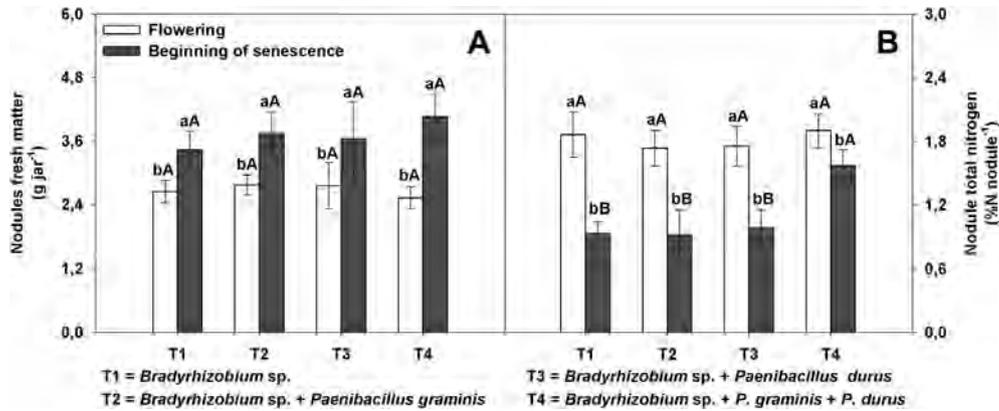


Fig. 3. Characterization of the nodules. (A) Fresh matter ($CV = 12.64\%$) and (B) total nitrogen ($CV = 11.81\%$) in nodules of the cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

Ammonia is produced during BNF, and after several reactions, it is incorporated into amines or ureides and exported via the xylem of the host plant (King and Purcell, 2005; Ladrera et al., 2007). According to King and Purcell (2005), ureides and asparagine are the main form of N transported from the nodules to the shoot. Ureides levels in the plant nodules harvested at flowering were not significantly different ($P < 0.05$); however, differences were observed in ureides levels in nodules harvested at the beginning of senescence (Fig. 4C). It has been proposed that high concentrations of some N compounds in nodules, such as amino acids and ureides, may function as an indicator of BNF decline (Larrainzar et al., 2009). Plants inoculated with *Bradyrhizobium* sp. and both PGPB exhibited

little difference in the levels of N compounds (amino acids, ammonia and ureides) when flowering and the beginning of senescence were compared (Fig. 4). These results indicate that the interaction among these bacteria in cowpea plants is more favorable and minimizes nodule senescence in comparison with inoculation with a single *Bradyrhizobium* sp.

For adequate nodule function, suitable protein content is required, and the synthesis of proteins may be increased due to the need for specific proteins that participate in the interaction between the plant and bacteria (Sarma and Emerich, 2005; Lugtenberg and Kamilova, 2009; Cheng et al., 2010). The concentration of soluble protein in cowpea nodules exhibited significant differences

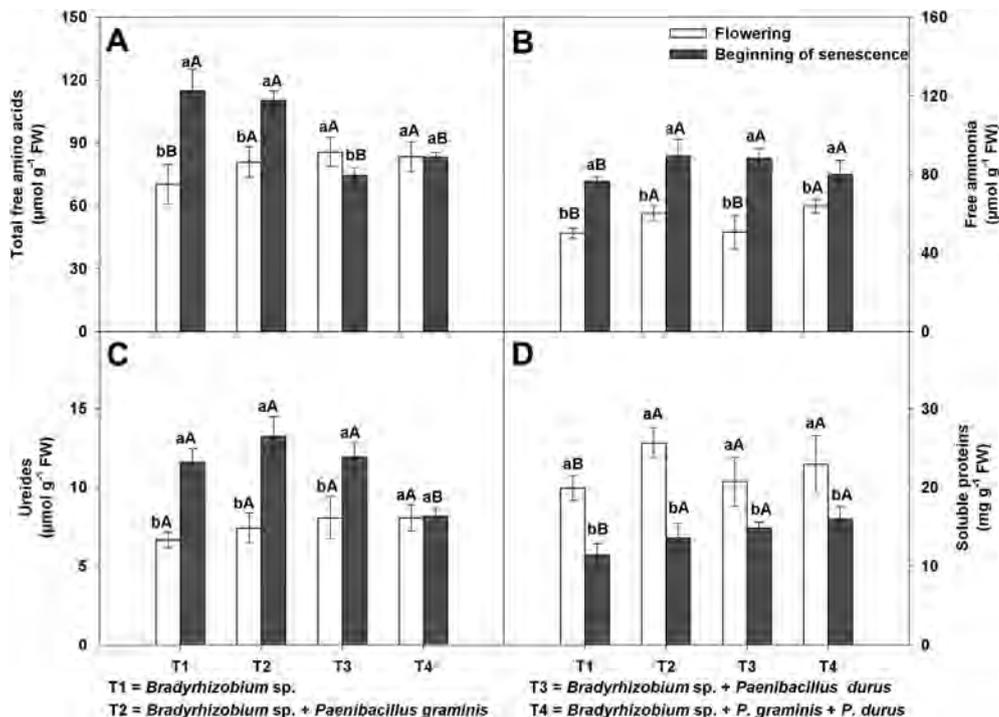


Fig. 4. N metabolism compounds. (A) Total free amino acids ($CV = 7.96\%$), (B) free ammonia ($CV = 7.52\%$), (C) ureides ($CV = 10.57\%$) and (D) soluble proteins ($CV = 12.04\%$) in nodules of the cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

($P < 0.05$) among the different treatments and harvest periods (Fig. 4D). At flowering, plants co-inoculated with *Bradyrhizobium* sp. and PGPB exhibited higher soluble protein content than those inoculated with only *Bradyrhizobium* sp. A reduction in soluble protein in nodules was observed at the beginning of senescence in comparison to flowering, whereas plants co-inoculated with *Bradyrhizobium* sp. and both PGPB had higher soluble protein levels than the plants receiving other treatments.

The establishment of an effective symbiosis between plants and bacteria is a complex process that involves increasing the synthesis of proteins related to different signaling and recognition pathways (Herridge et al., 2008; Compant et al., 2010). This process may have been stimulated by PGPB, particularly in the plants co-inoculated with *Bradyrhizobium* sp. and both PGPB because the soluble protein levels increased in the nodules of co-inoculated plants compared to those inoculated with only *Bradyrhizobium* sp. (Fig. 4D). In this study, the mixture of strains again favored this response, most likely successfully colonizing the root system.

Changes were recorded in the activity of enzymes involved in ammonia metabolism, and consequently BNF, on bacteroids in response to different treatments and harvest periods except for phenylalanine ammonia-lyase, an enzyme that releases ammonia by its reaction, which showed no changes (Fig. 5). At flowering, only plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* exhibited a reduction in glutamine synthetase (GS; Fig. 5A), glutamate synthase (GOGAT; Fig. 5C) and aminant glutamate dehydrogenase (GDHa; Fig. 5D) activities compared to plants receiving other treatments. These enzymes may be differentially modulated in response to numerous endogenous and environmental stimuli (Franche et al., 2009; Larrainzar et al., 2009).

The GS/GOGAT system activity increases as the nodules develop with GS being considered the key enzyme of BNF (Larrainzar et al., 2009). Together with GOGAT, GS converts ammonia into amino acids while avoiding its excessive accumulation within the bacteroids, which can result in toxicity and BNF inhibition (Prell and Poole, 2006; Bernard and Habash, 2009). In addition to these enzymes, GDHa can also metabolize ammonia, especially under conditions where there is excess ammonia; however, some authors have noted that this enzyme is less important than others (Sarma and Emerich, 2005; Bernard and Habash, 2009; Shridhar, 2012). The GS, GOGAT and GDHa activities were measured at the beginning of senescence; however, only GS was detected in the nodules of plants inoculated with only *Bradyrhizobium* sp. and in those co-inoculated with *Bradyrhizobium* sp. and PGPB (Fig. 5). Partial GS inhibition, combined with the absence of GDHa and GOGAT, seemed to be a direct response to the start of senescence, whereas during this phase of nodule development, there is a reduction in soluble protein levels and an increase in ammonia concentration in bacteroids.

Nodule growth and BNF in bacteroids consume considerable amounts of C. This is due to the need for energy and specific metabolites to supply the BNF, and these metabolites are provided through the respiratory catabolism of carbohydrates produced by plants (Franche et al., 2009). In this study, the concentrations of total soluble carbohydrates, sucrose and reducing sugars at flowering were significantly reduced in the nodules of cowpea plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* and not in those co-inoculated with *Bradyrhizobium* sp. and both PGPB compared to plants inoculated with only *Bradyrhizobium* sp. (Fig. 6A–C). The nodules of plants co-inoculated with *Bradyrhizobium* sp. and both PGPB had less starch at flowering than plants receiving other

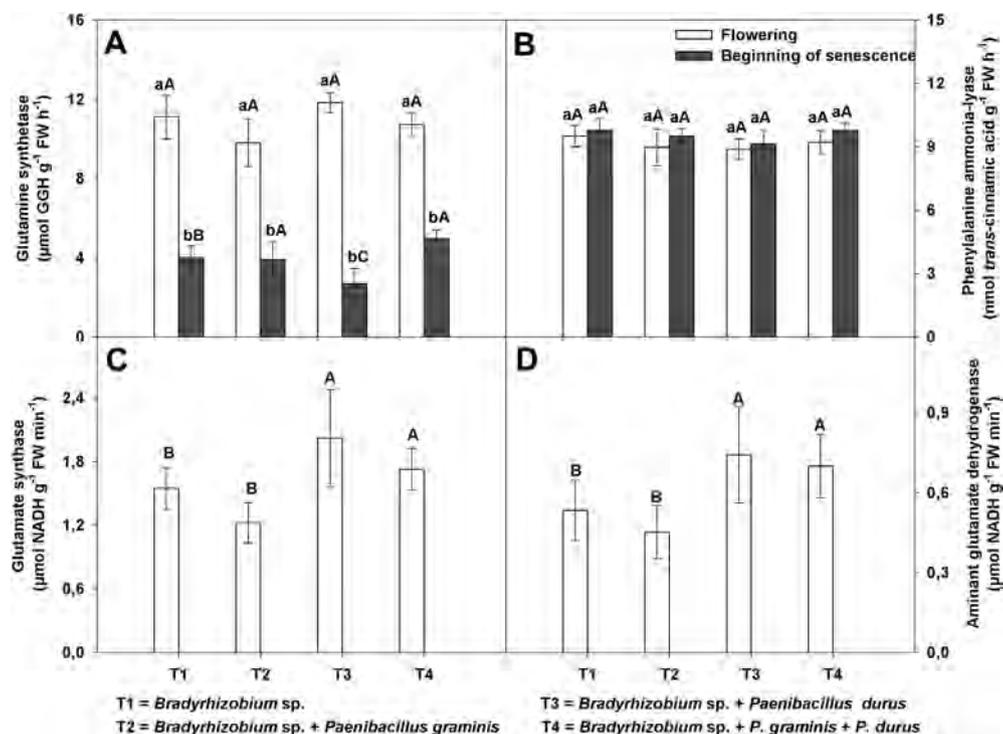


Fig. 5. Enzymes involved in N metabolism. (A) Glutamine synthetase (GS; CV² = 10.95%), (B) phenylalanine ammonia-lyase (PAL; CV = 6.00%), (C) glutamate synthase (GOGAT; CV = 17.47%) and (D) aminant glutamate dehydrogenase (GDHa; CV = 21.76%) in nodules of the cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. ²Coefficient of variation.

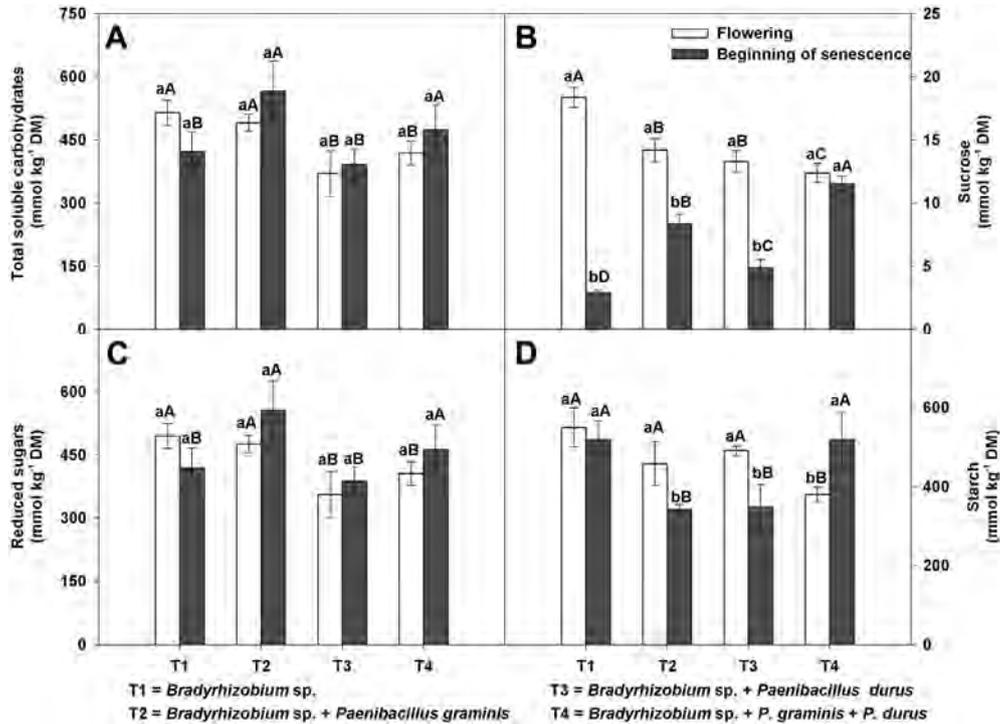


Fig. 6. Carbon metabolism compounds. (A) Total soluble carbohydrates ($CV^* = 10.73\%$), (B) sucrose ($CV = 6.91\%$), (C) reduced sugars ($CV = 10.97\%$) and (D) starch ($CV = 10.20\%$) in nodules of the cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

treatments (Fig. 6D). This response may represent a higher energy demand for cell division during PGPB colonization in the host (Prel and Poole, 2006; Larrainzar et al., 2009).

At the beginning of senescence, plants inoculated with only *Bradyrhizobium* sp. exhibited a reduction in sucrose ($85 \pm 15\%$) while the levels of total soluble carbohydrates, starch and reduced sugars did not significantly change in comparison to those during flowering (Fig. 6). In plants co-inoculated with *Bradyrhizobium* sp. and both PGPB, there was an increase in starch ($45 \pm 9\%$), but the levels of total soluble carbohydrate, sucrose and reduced sugar did

not differ significantly (Tukey's test; $P < 0.05$). The photosynthates are imported into nodules and are used as carbon skeletons in ammonia assimilation (Larrainzar et al., 2009). When they are not metabolized, due to partial or complete BNF blockage, the accumulation of starch can occur (Ben Salah et al., 2009).

The appropriate amount of BNF in bacteroids can be achieved by maintaining the levels of photoassimilates, mainly sucrose (Ben Salah et al., 2011). Sucrose is formed in the leaves by photosynthesis and is translocated to the roots in a process regulated by the sucrose synthase. A decrease in sucrose synthase may not

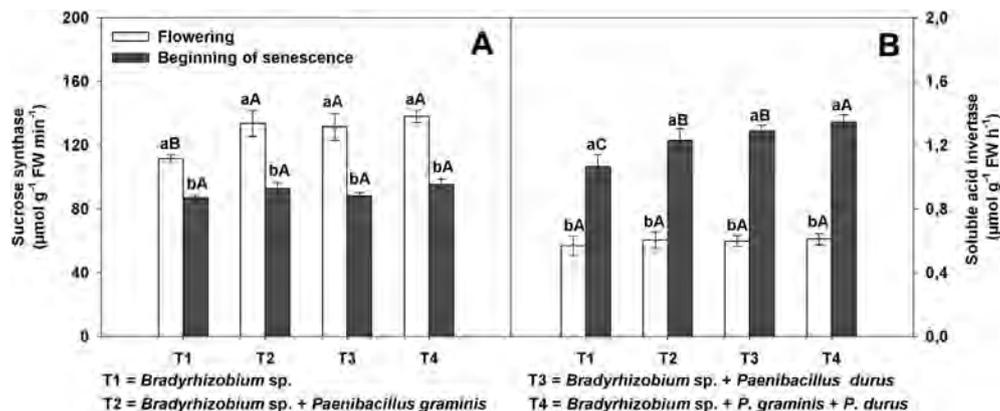


Fig. 7. Enzymes involved in carbon metabolism. (A) Sucrose synthase ($CV^* = 5.68\%$) and (B) soluble acid invertase ($CV = 5.91\%$) in nodules of the cowpea plants inoculated or co-inoculated as follows: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods, whereas different capital letters represents significant differences among the treatments, both at a confidence level of 0.05. The values shown are the means of four replicates and were compared using Tukey's test. *Coefficient of variation.

necessarily be offset by neutral and soluble acid invertase (Ben Salah et al., 2009). Sucrose synthase is the key enzyme regulating C metabolism and is crucial for adequate BNF in nodules (Marino et al., 2009).

In this study, the C-metabolism enzymes sucrose synthase and soluble acid invertase were evaluated in the nodules of cowpea plants inoculated with *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and PGPB (Fig. 7). The results showed that sucrose synthase activity at flowering remained higher in nodules of plants co-inoculated with PGPB than in plants inoculated with *Bradyrhizobium* sp. (Fig. 7A). For soluble acid invertase at flowering, there was no significant difference (Tukey's test; $P < 0.05$) among the different treatments (Fig. 7B). Although invertase can cleave sucrose, sucrose synthase is the major enzyme responsible for this process (Duncan et al., 2006). Thus, it is possible that the high sucrose synthase activity in plants inoculated with PGPB inhibited soluble acid invertase at flowering.

Sucrose synthase activity was reduced while soluble acid invertase activity increased significantly at the beginning of senescence (Fig. 7). It is likely that an increase in soluble acid invertase activity recorded at the beginning of senescence compensated for the reduction in sucrose synthase activity and maintained the sucrose supply at optimal levels to prevent starch synthesis inhibition, which is necessary when the photosynthate consumption in nodules is reduced due to BNF decline in bacteroids (Duncan et al., 2006). In plants co-inoculated with *Bradyrhizobium* sp. and both PGPB, the increase in soluble acid invertase activity ($121 \pm 18\%$) was more pronounced than in plants receiving the other treatments, and this response must have been crucial in maintaining an unchanged C skeleton, thereby avoiding changes in BNF in cowpea nodules.

4. Conclusions

Plants co-inoculated with *Bradyrhizobium* sp. and PGPB, particularly those co-inoculated with *Bradyrhizobium* sp. and both PGPB, exhibited improved results with respect to N content and C metabolites. Although there was a decline in enzymes involved in N and C metabolism at the beginning of senescence, plants co-inoculated with *Bradyrhizobium* sp. and both PGPB exhibited a delay of the deleterious effects of nodule senescence, thus ensuring N availability for a longer period. The data suggest that co-inoculation with PGPB results in increased symbiotic performance and BNF optimization in cowpea plants.

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