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Minimization of oxidative stress in cowpea nodules by the interrelationship between *Bradyrhizobium* sp. and plant growth-promoting bacteria

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ABSTRACT

Oxidative damage can result in various degenerative processes, including membrane lipid peroxidation, a process promoted by chain reactions initiated by reactive oxygen species that can rapidly affect many lipid molecules, resulting in damage to cellular structures. This study aimed to evaluate the symbiotic performance of cowpea plants inoculated and co-inoculated with *Bradyrhizobium* sp. and different combinations of plant growth-promoting bacteria (PGPB), *Paenibacillus graminis* or *P. durus*, based on biochemical variables related to protection/oxidative stress and senescence during and after the establishment of symbiosis. The experiment was conducted under greenhouse conditions. Cowpea seeds were disinfected and inoculated as follows: *Bradyrhizobium* sp.; co-inoculated with *Bradyrhizobium* sp. and *P. graminis* or *Bradyrhizobium* sp. and *P. durus*; or co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*). The plants were grown in Leonard jars containing washed and autoclaved sand. Uninoculated plants were used as an absolute control. The cowpea nodules were harvested at flowering point and the beginning of senescence. The results showed significant differences in the variables related to antioxidant metabolism in response to the treatments and harvest times. Although there was reduced ascorbate peroxidase, catalase and phenol peroxidase activities were observed in the plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* and *Bradyrhizobium* sp. and *P. durus* at flowering point, no accumulation of hydrogen peroxide or increase in lipid peroxidation was observed, indicating that the enzymatic activity was effective in controlling the possible oxidative damage in these plants. The cowpeas co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) showed a delay of the deleterious effects of senescence and, therefore, a better symbiotic performance.

1. Introduction

Rhizobia can interact positively with plant growth-promoting bacteria (PGPB) and have the ability to promote plant growth in legume species, contributing to increases in crop productivity (Lugtenberg and Kamilova, 2009). PGPB facilitate plant growth by direct stimulation of phosphate solubilization, iron sequestration, synthesis and/or modulation of phytohormones levels (Ferguson and Mathesius, 2003; Lugtenberg and Kamilova, 2009). Furthermore, PGPB can function in biological control, promote improvements in plant growth and development and provide increased nodulation and nitrogen fixation by affecting interactions between plant and rhizobia (Bashan et al., 2004; Marino et al., 2009; Compant et al., 2010; Liu et al., 2011). Compared to single inoculation, co-inoculation can result in positive effects by increasing the production phytohormones and nutrient absorption and mobilization, mainly phosphorus, nitrogen and carbon (Ott et al., 2005; Ladrera et al., 2007; Larrainzar et al., 2009; Figueiredo et al., 2010).

The use of PGPB as bio-protectors and growth stimulators is probably one of the most significant tactics of plant management in the world, mainly due to the growing necessity of sustainable agriculture focusing on environmentally-friendly practices, such as reducing on use of chemical fertilizers (Figueiredo et al., 2008a). Lebsky et al. (2001) demonstrated that occur a delay in the senescence events in the microalga *Chlorella vulgaris* in presence of the *Azospirillum brasilense*. *Azospirillum* is a free-living PGPB capable

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of affecting growth and yield of numerous plant species (Bashan et al., 2004). Strains of the *Pseudomonas* and *Bacillus* genera are the most well-known PGPB, which when co-inoculated with *Rhizobium* can improve growth of different legumes (Tilak et al., 2006; Wani et al., 2007; Guiñazú et al., 2010). Moreover, the association of *Paenibacillus* strains and *Rhizobium tropici* leads to higher growth in *Phaseolus vulgaris* compared to inoculation with *Rhizobium* alone (Figueiredo et al., 2008b).

During the symbiosis between legume plants and N-fixing bacteria, plants provide the bacteroids with sugars that are oxidized and used as an energy source to sustain the process of biological nitrogen fixation (BNF) (Larrainzar et al., 2009). Due to the highly reducing environment during BNF, reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), may be formed and can cause oxidative damage (Puppo et al., 2005; Møller et al., 2007). In addition, ROS can be produced by leghemoglobin autoxidation and some proteins with strong reducing potential (Chang et al., 2009). The ROS overproduction or oxidative stress is a secondary response that is normally stimulated under stress conditions or metabolic decline, such as nodule senescence (Becana et al., 2010; Ben Salah et al., 2011). ROS can play signaling functions in various cellular mechanisms (Mandon et al., 2009; Marino et al., 2009) and act as an important mediator in the interplay with phytohormones synthesized by PGPB (Torres, 2010). Furthermore, PGPB promote an enhancement of plant tolerance to biotic and abiotic stress, mitigating the levels of ROS (Jebara et al., 2005; Silveira et al., 2011).

To maintain the normal function of the nodule, which harbor the bacteroids and to promote oxidative protection, it is necessary to control the ROS levels efficiently (Mandon et al., 2009; Becana et al., 2010; Silveira et al., 2011). Systems that control the ROS levels in the cell are needed to avoid possible oxidative damage and promote an adequate redox balance (Møller et al., 2007; Becana et al., 2010). Such redox balance is a result of the equilibrium between protective and reparative antioxidant systems that are formed by both non-enzymatic antioxidants, such as ascorbate and glutathione and enzymes, mainly catalase, superoxide dismutase and ascorbate peroxidase (Puppo et al., 2005; Mandon et al., 2009; Becana et al., 2010). It has been found that *Medicago* plants colonized by PGPB strains showed high antioxidant enzymes activity (Bianco and Defez, 2009). Indeed, an efficient protection against oxidative stress is required to maintain adequate BNF and delay senescence (Mandon et al., 2009; Marino et al., 2009).

The hypothesis that the co-inoculation of cowpeas with *Bradyrhizobium* sp. and PGPB is able to maintain adequate BNF during and after the establishment of symbiosis, resulting in the amelioration of the deleterious effects of oxidative stress, was tested in this study. Cowpea plants were inoculated with *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and PGPB and analyzed in terms of the compounds and enzymes that are indicative of oxidative protection during and after the establishment of symbiosis. It was demonstrated here that oxidative stress occurs in cowpea nodules and that this process is retarded by the presence of PGPB. The plants inoculated with *Bradyrhizobium* sp. and two PGPB (*Paenibacillus graminis* and *P. durus*) appeared to have their antioxidant defense systems stimulated during flowering point to maintain adequate levels of ROS and this may have triggered the favorable response in these plants at the beginning of senescence, thus avoiding possible degenerative processes.

2. Material and methods

2.1. Preparation and application of inoculants with exopolysaccharide (EPS) synthesized by rhizobia

The strains to be used in the experiment were multiplied prior to the preparation of the inoculant (Table 1). Exopolysaccharide (EPS, 2.0 g) synthesized by *Rhizobium tropici* (EI-6) was used for the preparation of the inoculants. The EPS utilized was characterized and described previously by the authors as a polymer of the glucose and galactose and considered an alternative vehicle for inoculation because it promotes greater symbiotic efficiency, growth and productivity in cowpea (data not published). The EPS was dried, sieved and autoclaved (120 °C; 101 kPa; 15 min) and then cooled in a fresh and dry location and stored at room temperature (24 °C). The 2.0 mL of autoclaved and distilled water was added to EPS and the mixture was homogenized and incubated for 30 h, time necessary for their chemical stabilization and for the pH reach the optimum range (6.8–7.0).

After achieving the appropriate pH, to 1.0 g of the EPS was added 3.0 mL of the bacterial inoculum in duplicate and therefore forming the treatments, as follows: 1.5×10^8 CFU mL^{-1} of *Bradyrhizobium* sp. in YM culture medium and 1.5 mL of sterile and distilled water for the inoculation with standard strain; 1.5×10^7 CFU mL^{-1} of *P. graminis* or *P. durus* in TSB culture medium and 1.5×10^8 CFU mL^{-1} of *Bradyrhizobium* sp. in YM culture medium for the co-inoculation; and, 0.75×10^7 CFU mL^{-1} each of *P. graminis* and *P. durus* in TSB culture medium and 1.5×10^8 CFU mL^{-1} of *Bradyrhizobium* sp. in YM culture medium for the co-inoculation with the two PGPB (*P. graminis* and *P. durus*) simultaneously.

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

2.2. Experimental preparation, inoculation and planting

The experiment was conducted in a greenhouse at the Agronomical Institute of Pernambuco – IPA at a temperature range of 27–36 °C with 50–70% relative humidity and 800 μmol m^{-2} s^{-1} photosynthetically active radiation. The seeds of cowpea 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 was added 2.0 mL of the inoculant (EPS + bacteria), which drained into the substrate, as follows: only *Bradyrhizobium* sp.; co-inoculated with *Bradyrhizobium* sp. + *P. graminis* or *Bradyrhizobium* sp. + *P. durus*; or co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*). Uninoculated plants were used as an

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
Bradyrhizobium sp.	BR 3267	Yeast-Mannitol	200 rpm; 28 °C; 96 h	National Center of Agrobiology Research (CNPAB, RJ-Brazil)
Paenibacillus graminis	MC 04.21	Trypticase Soy Broth	200 rpm; 32 °C; 24 h	Federal University of Rio de Janeiro (UFRJ; Microbiology Institute)
Paenibacillus durus	C 04.50	Trypticase Soy Broth	200 rpm; 32 °C; 48 h	Federal University of Rio de Janeiro (UFRJ; Microbiology Institute)

absolute control. 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 nitrogen-free Hoagland and Arnon (1950) nutritive solution, as modified by Silveira et al. (1998). The plants were collected at two stages: (1) flowering point, the period of higher nitrogen fixation (at 36 days) and (2) the beginning of senescence, the period of nitrogen fixation decline (at 56 days). The roots were collected and the nodules removed, weighed, frozen in liquid N_2 and stored in a freezer at −80 °C until the analyses.

2.3. Biochemical determinations

2.3.1. Leghemoglobin concentration

The extraction and determination of the leghemoglobin concentration in the cowpea nodules was performed using Drabkin's reagent following the method described by Smagghe et al. (2009); the data were expressed in mg g^{-1} FW (fresh weight).

2.3.2. Determination of hydrogen peroxide and lipid peroxidation

To obtain the extract used in determining the hydrogen peroxide (H_2O_2) concentration and lipid peroxidation, the nodules were extracted with 5% TCA and the supernatant was collected and used for the determinations. The H_2O_2 measurement was performed after reaction of the titanium tetrachloride with H_2O_2 and formation of the hydroperoxide–titanium tetrachloride complex according to Brennan and Frenkel (1977), with minor modifications described by Bonifacio et al. (2011); the data were expressed in μmol H_2O_2 g^{-1} FW. Lipid peroxidation was determined by measuring of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex according to Heath and Packer (1968); the data were expressed as nmol MDA-TBA g^{-1} FW.

2.3.3. Enzymatic activities

Fresh nodules were extracted with 100 mM potassium phosphate buffer (pH 7.0) and the supernatant was collected and used for the determination of the following enzymatic activities: lipoxygenase (LOX; EC 1.13.11.12), protease (EC 3.4), uricase (EC 1.7.3.3), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.1), phenol peroxidase (POX; EC 1.11.1.7) and superoxide dismutase (SOD; EC 1.15.1.1).

The LOX activity was measured based on the hydroperoxide of linoleic acid (HLA) production and was expressed as nmol HLA g^{-1} FW min^{-1} (Axelrod et al., 1981). The protease activity was determined in accordance with the method described by Silveira et al. (2003) and was expressed as U g^{-1} FW min^{-1}. The uricase activity was measured following the method proposed by Schubert (1981) and was expressed as μmol uric acid g^{-1} FW min^{-1}. The CAT activity was measured following the oxidation of H_2O_2 and was expressed as μmol H_2O_2 g^{-1} FW min^{-1} (Havir and McHale, 1987). The APX activity was estimated in accordance with the method described by Nakano and Asada (1981) and was expressed as μmol ascorbate g^{-1} FW min^{-1}. The POX activity was in accordance with the method proposed by Amako et al. (1994) and was expressed as μmol pyrogallol g^{-1} FW min^{-1}. The SOD activity was determined by the inhibition of blue formazan production, as described by Giannopolitis and Ries (1977) and was expressed as U g^{-1} FW min^{-1}.

2.4. Experimental design and statistical analysis

The experimental design was randomized blocks, with four replications using a 2 × 4 factorial arrangement: two harvest periods (flowering point and beginning of senescence) and four treatments (one inoculation only with *Bradyrhizobium* sp. and three co-inoculations of *Bradyrhizobium* sp. and PGPB). Data were tested for normality using the Shapiro–Wilk test ($P < 0.05$). Only the catalase data were significantly different from a normal distribution and then subjected to square root transformation. The variables obtained were tested for homogeneity of variance using Levene's Test and, posteriorly, the means were subjected to analysis of variance (ANOVA) with the F test ($P < 0.05$). Comparison of treatment means was performed using Tukey's test ($P < 0.05$). All analyses described were performed with ASSISTAT software.

3. Results

The cowpea plants inoculated and co-inoculated with plant growth-promoting bacteria (PGPB) showed different responses with regard to the indicators of senescence and/or oxidative protection during and after the establishment of symbiosis. As shown in Fig. 1A, during flowering point, the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* and with *Bradyrhizobium* sp. + *P. durus* exhibited lower H_2O_2 contents in their nodules than the plants inoculated solely with *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*). In contrast, the plants inoculated solely with *Bradyrhizobium* sp. had higher H_2O_2 levels than the other treatments at the beginning of senescence (Tukey's test, $P < 0.05$), as shown in Fig. 1A. In the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*), the H_2O_2 concentration was unchanged in the nodules when comparing the stages of flowering point and the beginning of senescence.

The lipid peroxidation in the cowpea nodules was altered in response to the different treatments (Fig. 1B). The plants inoculated solely with *Bradyrhizobium* sp. showed lipid peroxidation levels higher than the average of the others treatments at both flowering point and the beginning of senescence. As illustrated Fig. 1B, the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) exhibited increases in nodule lipid peroxidation of 15% when comparing the two harvest times, whereas this increase was approximately 30% in the plants inoculated only with *Bradyrhizobium* sp. and in the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* or with *Bradyrhizobium* sp. + *P. durus* (Tukey's test, $P < 0.05$).

According to Fig. 2, the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* or *Bradyrhizobium* sp. + *P. durus* and co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) showed an LOX activity that was significantly higher at flowering point in comparison to the plants inoculated only with *Bradyrhizobium* sp. (Tukey's test, $P < 0.05$). The plants co-inoculated with *Bradyrhizobium* sp. + *P. durus* and those co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) showed lower and higher LOX activities, respectively, at the beginning of senescence in the nodules (Fig. 2). The plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) exhibited an increase in 30% when comparing the stages of flowering point and the beginning of senescence.

At flowering point, a reduction in the leghemoglobin concentration was observed in the nodules of the plants co-inoculated with *Bradyrhizobium* sp. + PGPB when compared to the plants inoculated only with *Bradyrhizobium* sp. (Fig. 3). The plants inoculated only with *Bradyrhizobium* sp. showed a reduction in the leghemoglobin levels up to 50% at the beginning of senescence when compared to the flowering point (Fig. 3). Furthermore, when comparing the two harvest times, no alterations were observed in the leghemoglobin content in the nodules of the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* or *Bradyrhizobium* sp. + *P. durus*; in addition, those co-inoculated with

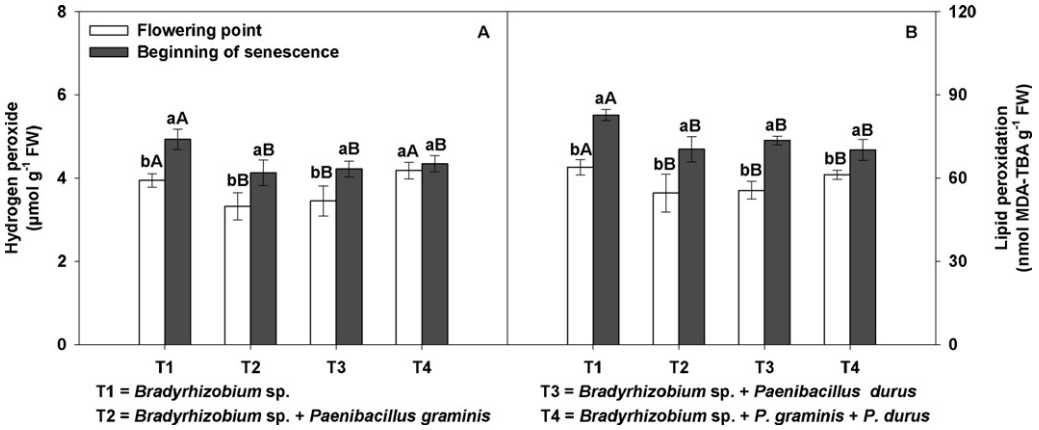

Fig. 1. Indicators of oxidative stress. (A) Hydrogen peroxide content (CV* = 6.08%) and (B) lipid peroxidation (CV = 5.65%) in nodules of the cowpea plants inoculated or co-inoculated, as follow: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *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 of 0.05. Data are mean of four replicates and were compared by Tukey's test. (*) Coefficient of variation.

Bradyrhizobium sp. and the two PGPB (*P. graminis* and *P. durus*) had a slight reduction (~17%) in the content of leghemoglobin (Fig. 3).

The enzymes evaluated in this study were differentially induced in response to all of the treatments. The protease enzyme activity was strongly induced in the plant nodules at the beginning of senescence, unlike during flowering point in which no significant difference was found for the different treatments (Fig. 4A). The plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* and co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) exhibited elevated proteolytic activity in their nodules in relation to the others treatments. As shown in Fig. 4B, the uricase activity in the plant nodules did not differ significantly (Tukey's test, $P < 0.05$) at flowering point, whereas uricase activity was not detected at the beginning of senescence.

The antioxidative enzymes evaluated in this study exhibited different patterns of response to the treatments. There were reductions in the CAT and APX activities in the nodules of the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* and with *Bradyrhizobium* sp. + *P. durus* at flowering point, whereas the APX and CAT activities remained equal in the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) to the values obtained for the cowpea inoculated only with *Bradyrhizobium* sp. (Fig. 5A and B). In general, the reduction was more dramatic for the CAT activity than the APX activity when comparing these enzymes at the two harvest times. The plants inoculated solely with *Bradyrhizobium* sp. showed a large decrease (~90%) in CAT activity at the beginning of senescence in relation to flowering point. In the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*), the activities of APX and CAT

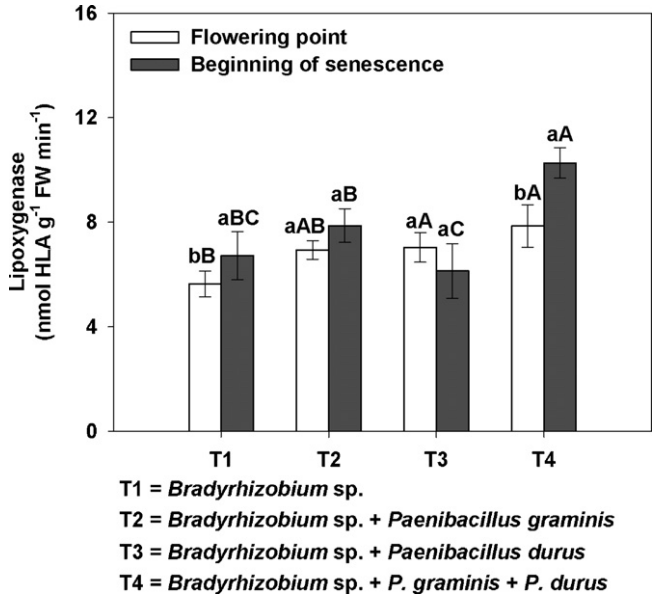

Fig. 2. Lipoxygenase activity (CV* = 9.72%) in nodules of the cowpea plants inoculated or co-inoculated, as follow: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (T4). Different lowercase letters represent significant differences among the harvest periods whereas different capital letters represents significant differences among the treatments, both at a confidence of 0.05. Data are mean of four replicates and were compared by Tukey's test. (*) Coefficient of variation.

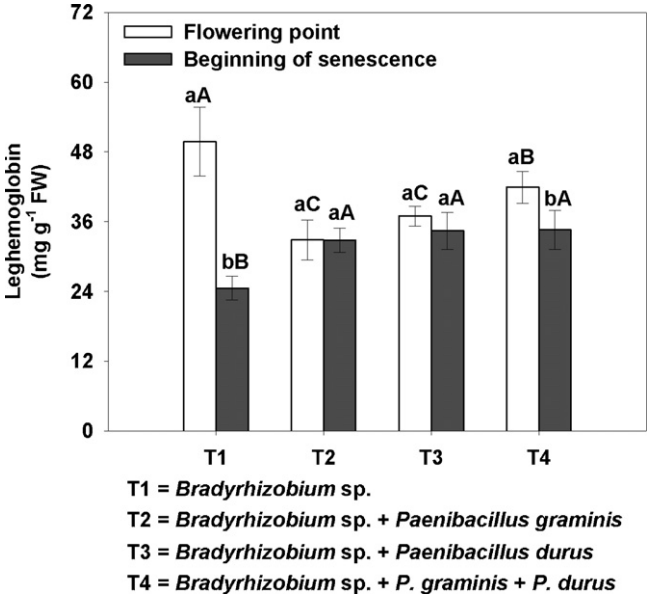

Fig. 3. Leghemoglobin concentration (CV* = 8.99%) in nodules of the cowpea plants inoculated or co-inoculated, as follow: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *P. graminis* + *P. durus* (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 of 0.05. Data are mean of four replicates and were compared by Tukey's test. (*) Coefficient of variation.

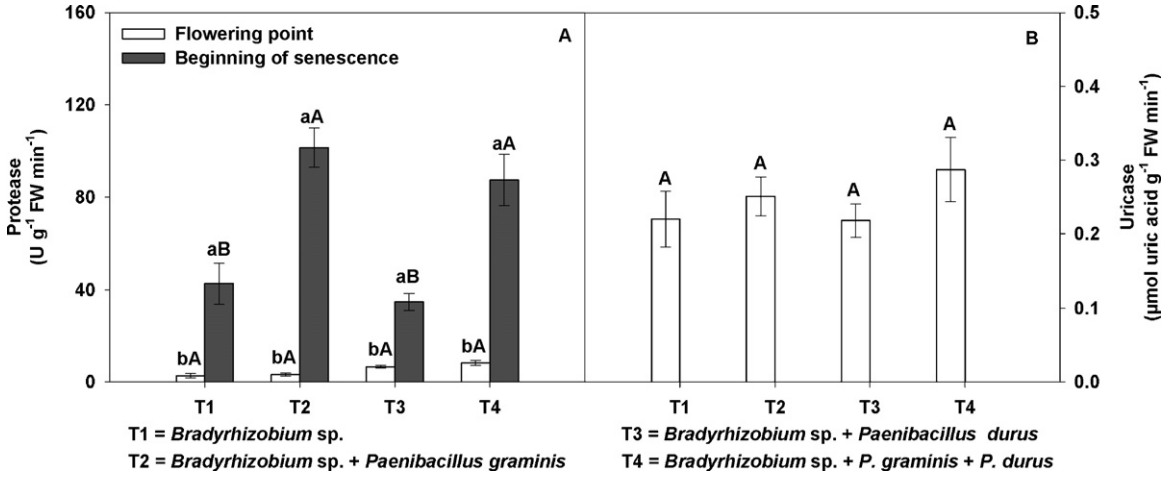

Fig. 4. Activity of (A) protease (CV* = 26.11%) and (B) uricase (CV = 13.47%) in nodules of the cowpea plants inoculated or co-inoculated, as follow: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* sp. + *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. + *P. durus* (T3); and *Bradyrhizobium* sp. + *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 of 0.05. Data are mean of four replicates and were compared by Tukey's test. (*) Coefficient of variation.

remained high compared to the other treatments at the beginning of senescence (Fig. 5A and B).

In the plants co-inoculated with PGPB, a decrease in the POX activity was observed in comparison to the plants inoculated only with *Bradyrhizobium* sp. at flowering point (Fig. 5C); however, at the beginning of senescence, the POX activity did not differ statistically among the treatments according to Tukey's test ($P < 0.05$). When comparing the two harvest times, an increase in the POX activity in the plants co-inoculated with PGPB was noted in relation to the plants inoculated only with *Bradyrhizobium* sp., particularly for the plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* or with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) (Fig. 5C). At flowering point, the SOD activity was greater in the plants co-inoculated with PGPB than in the plants inoculated only

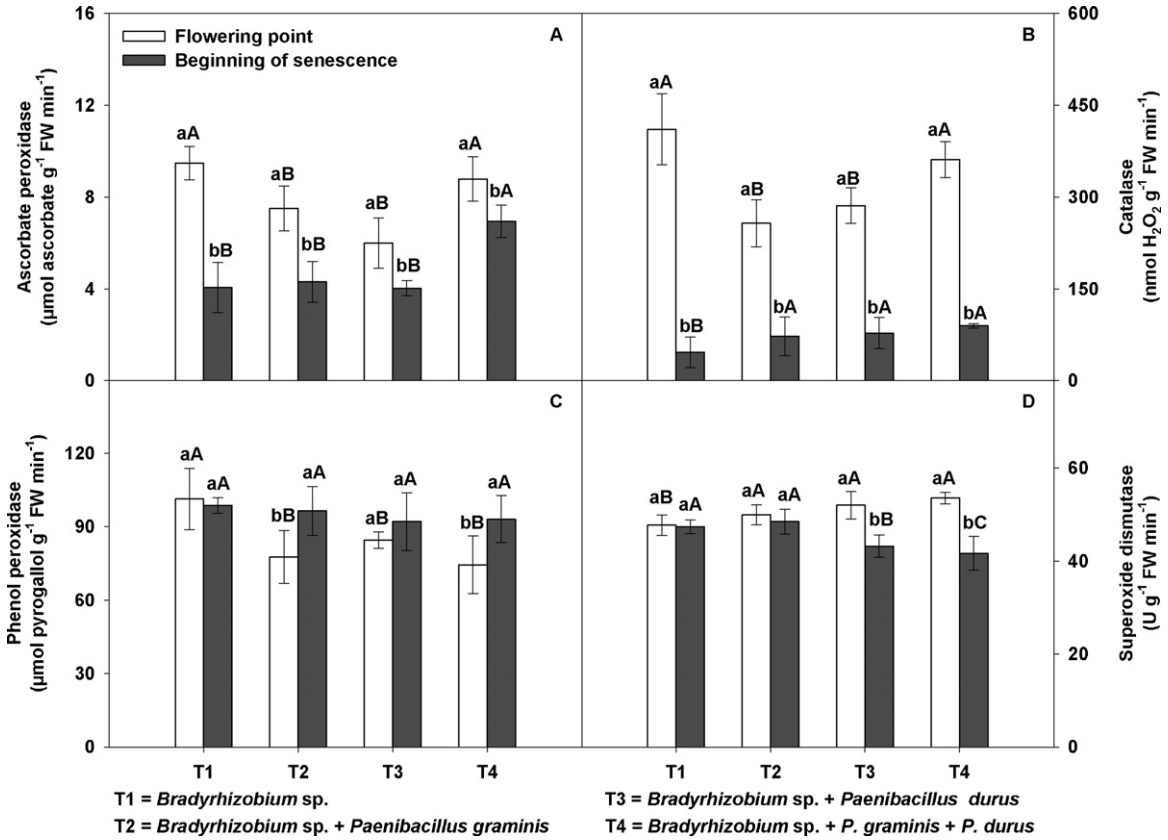

Fig. 5. Enzymes of the antioxidative metabolism: (A) ascorbate peroxidase (CV* = 14.71%), (B) catalase (CV = 9.71%), (C) phenol peroxidase (CV = 11.19%) and (D) superoxide dismutase (CV = 5.13%) in nodules of the cowpea plants inoculated or co-inoculated, as follow: only *Bradyrhizobium* sp. (T1); *Bradyrhizobium* 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 of 0.05. Data are mean of four replicates and were compared by Tukey's test. (*) Coefficient of variation.

with *Bradyrhizobium* sp. (Fig. 5D). At the beginning of senescence, the SOD activity in the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) was lower (~15%) than that for the other treatments (Fig. 5D).

4. Discussion

Reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet -}$), can react with innumerous molecules present in cells causing oxidative damage that can interfere with normal cell operation (Møller et al., 2007; Mandon et al., 2009; Marino et al., 2009; Silveira et al., 2011). In our study, the plants inoculated only with *Bradyrhizobium* sp. exhibited higher hydrogen peroxide levels than the other treatments at the beginning of senescence, suggesting that these plants lacked an adequate system to control the levels of H_2O_2, the production of which naturally increases when the nodules undergo senescence (Puppo et al., 2005; Mandon et al., 2009; Silveira et al., 2011). Besides being capable of causing damage to cellular structures, hydrogen peroxide has been highlighted as a powerful signaling molecule for several cellular events (Møller et al., 2007), with a central role in oxidative metabolism and detected during the infection, development and senescence of bacteroids (Chang et al., 2009; Becana et al., 2010).

Lipid peroxidation is a process that occurs by chain reactions initiated by ROS and can quickly affect many lipid molecules, resulting in damage to cellular structures (Puppo et al., 2005; Salavati et al., 2011). The means of lipid peroxidation in the nodules of the plants inoculated solely with *Bradyrhizobium* sp. increased when compared to the other treatments at both harvest times and this response may indicate a lower efficiency of the antioxidant system in the nodules of these plants. The plants co-inoculated with *Bradyrhizobium* sp. and two PGPB (*P. graminis* and *P. durus*) showed few alterations in their levels of lipid peroxidation when comparing the two harvest times. This response corroborates the findings of Ben Salah et al. (2011) for two *Medicago* species and may be related to a more efficient antioxidant system that minimized the harmful effects of oxidative stress and delayed nodule senescence in these plants.

Lipid peroxidation may be a response to the increased the activity of the lipoxygenase (LOX) enzyme, which is located in the lipid bodies, peroxisomes and plastids present in nodule cells (Feussner and Wasternack, 2002). LOX catalyzes the addition of O_2 to the pentadiene system of polyunsaturated fatty acids, forming the corresponding fatty acid hydroperoxides (Michalak, 2006). Overall, the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*) in our study exhibited the highest LOX activity in relation to the other treatments and this response appears to have mitigated the possible oxidative damage, mainly lipid peroxidation, which was not greatly altered in these plants. Indeed, the lipid hydroperoxides formed by LOX may be metabolized, resulting in molecular mediators, such as jasmonic acid, which act in plant defense in response to oxidative stress (Chang et al., 2009; Costanzo et al., 2012).

During the senescence process in the nodules, a natural reduction in the leghemoglobin concentration occurs (Ott et al., 2005; Ben Salah et al., 2011; Liu et al., 2011). Leghemoglobin is an oxygen-carrying hemoprotein that is responsible for creating a barrier to oxygen diffusion, making it the ideal environment for the development of bacteroids (Ma et al., 2011). Leghemoglobin can be attacked by ROS, producing oxyleghemoglobin that, when deprotonated, generates superoxide and then hydrogen peroxide (Marino et al., 2009). In this study, the plants inoculated with PGPB showed reduced leghemoglobin contents in relation to the plants inoculated only with *Bradyrhizobium* sp. at flowering point and few alterations in the leghemoglobin concentration at the beginning of senescence, unlike what was observed for the plants inoculated only with *Bradyrhizobium* sp. when comparing the two harvest times. This response may represent a way for the plants inoculated with PGPB to contain the levels of hydrogen peroxide in the nodules or be a result of increased proteolysis via the proteases present in the bacteroids, as suggested by Ott et al. (2005).

Proteases are enzymes that hydrolyze the peptide bonds of proteins, releasing small peptides and/or amino acids (Cheng et al., 2010); in the present study, these enzymes were tightly induced in the nodules of the cowpea plants at the beginning of senescence. This induction may have been responsible by not detection of the uricase activity in the plants inoculated solely with *Bradyrhizobium* sp. and in combination with PGPB at the beginning of senescence. Uricase is an enzyme present in nodules that acts in ureide synthesis from uric acid and this reaction results in the production of hydrogen peroxide (Werner and Witte, 2011). The H_2O_2 produced may be removed by the ascorbate peroxidase (APX) and catalase (CAT) enzymes via different mechanisms, which both result generate water (Møller et al., 2007; Ben Salah et al., 2011). In addition to the APX and CAT activities, phenol peroxidase (POX) can degrade H_2O_2 using phenolic compounds or ascorbate as the electron donors (Michalak, 2006).

Although there was reduced activity of APX, CAT and POX in the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* and *Bradyrhizobium* sp. + *P. durus* at flowering point, H_2O_2 accumulation or an increase in lipid peroxidation was not observed, indicating that these enzymes were effective in controlling the possible oxidative damage in these plants. Indeed, APX activity protects leghemoglobin and other proteins from H_2O_2 and may be involved in maintaining the oxygen diffusion barrier present in the bacteroids (Marino et al., 2009; Becana et al., 2010). Additionally, corroborating the data presented here, according to the results of Jebara et al. (2005), a reduction in the CAT activity can reduce the symbiotic efficiency in bacteroids.

In addition to the APX, CAT and POX activities, superoxide dismutase (SOD) operates in the enzymatic antioxidant system for the detoxification of excess ROS (Puppo et al., 2005; Michalak, 2006) and acts in the control of several metabolic pathways because the enzyme can control the gene expression related to oxidative stress (Puppo et al., 2005; Salavati et al., 2011). We observed few alterations in the SOD activity; however, in the plants co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*), there were reductions in the activity of this enzyme that may explain the lack of alteration in the H_2O_2 content in these plants when comparing the two harvest times. According to Chang et al. (2009), the presence of SOD in bacteroids has a critical protective role in nodulation and biological nitrogen fixation efficiency and also acts in the delay of senescence.

5. Conclusions

Although there was reduced ascorbate peroxidase, catalase and phenol peroxidase activities in the plants co-inoculated with *Bradyrhizobium* sp. + *P. graminis* and with *Bradyrhizobium* sp. + *P. durus* at flowering point, there was no H_2O_2 accumulation or increase in lipid peroxidation, indicating that the activities of these enzymes were effective in controlling the possible oxidative damage in these plants. The plants co-inoculated with *Bradyrhizobium* sp. + PGPB, particularly the co-inoculated with *Bradyrhizobium* sp. and the two PGPB (*P. graminis* and *P. durus*), showed better results for the biochemical indicators related to antioxidant metabolism. Thus, it can be suggested that the co-inoculation with PGPB in cowpea resulted in the delay of the deleterious effects of senescence.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2012.12.018.

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