

Full Length Research Paper

Rhizobium tropici* exopolysaccharides as carriers improve the symbiosis of cowpea-*Bradyrhizobium*-*Paenibacillus

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Received 24 May, 2015; Accepted 31 August, 2015

Exopolysaccharides (EPS) may represent a viable inoculant carrier to replace peat and reduce production costs. In addition, the use of plant growth-promoting bacteria (PGPB), as *Paenibacillus*, in association with rhizobia represents a new technology that can improve the biological nitrogen fixation (BNF) and crop productivity. Thus, the present research aimed to characterize the EPS that are produced by *R. tropici* and evaluate their effectiveness as carriers for cowpea inoculation. Initially, EPS were defined as a heteropolysaccharide polyanionic inert to bacterial strains. In addition, EPS were successful as vehicles for cowpea inoculation with *Bradyrhizobium* sp. compared to peat, enhancing the growth parameters and nitrogen fixation of these plants. Thus, EPS were used for cowpea inoculation with *Bradyrhizobium* sp. and *Paenibacillus* species to test the effectiveness of these carriers on unsterile soil. Cowpea development and productivity were observed after cowpea inoculation with *Bradyrhizobium* sp., co-inoculation with *Bradyrhizobium* sp. and *P. graminis* or with *Bradyrhizobium* sp. and *P. durus*, and simultaneous co-inoculation with *Bradyrhizobium* sp., *P. graminis* and *P. durus* using EPS as carriers. Cowpea plants were collected in two stages, flowering and grain filling, and the plants that were co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp. produced better results. Simultaneous co-inoculation with *Bradyrhizobium* sp., *P. graminis* and *P. durus* stood out from all treatments. Overall, the use of EPS that were produced by *R. tropici* and *Paenibacillus* species represents an emerging technology for the improvement of cowpea-rhizobia symbiosis due to the positive effects on BNF and crop productivity.

Key words: Inoculant, plant growth-promoting bacteria, biological nitrogen fixation.

INTRODUCTION

Carrier materials can influence the success of bacterial inoculum, making it more favorable for microbial colonization (Albareda et al., 2008; Kumar et al., 2012; Bashan et al., 2014). Peat-based inoculants are habitually

utilized as carriers for rhizobia inoculation worldwide and have been produced in Brazil since the 1950s. Peat is a fossil material that is extracted from natural systems and may have biological or chemical impurities with negative

effects on microbial physiology (Albareda et al., 2008; Fernandes Júnior et al., 2012). Considering the possibility of the exhaustion of peat sources and mainly the minimization of environmental damage, alternative carriers have been studied, and emphasis has been given to microbial exopolysaccharides (EPS) (Albareda et al., 2008; Fernandes Júnior et al., 2012; Bashan et al., 2014). EPS are polymers synthesized and released into the extracellular medium and may represent viable alternatives as inoculants to replace peat and even reduce production costs (Vu et al., 2009; Freitas et al., 2011).

Microbial EPS have found a wide range of applications in industry, such as emulsifiers, stabilizers, binders, coagulants, and suspending agents (Donot et al., 2012), and are also used as gelling agents to improve food quality and texture (Poli et al., 2010). Biologically, EPS can prevent environmental insults, such as desiccation, predation by protozoans and phage attack and maintain the viability of rhizobia in the field (Staudt et al., 2012). Rhizobia are among the well-known EPS producers and excrete large amounts of these polysaccharides into the rhizosphere (Albareda et al., 2008; Serrato et al., 2008; Nwodo et al., 2012). EPS can protect the nitrogenase enzyme that is present in nitrogen-fixing nodules against high oxygen concentration (Vu et al., 2009). The formation of nitrogen-fixing nodules in legumes requires the infection of roots with rhizobia species (Oldroyd et al., 2011), and EPS can act as molecular signals during the process of pre-infection and infection that occurs in the rhizosphere (Vu et al., 2009).

In nitrogen-fixing nodules, biological nitrogen fixation (BNF) occurs, which is a process that represents an efficient source of nitrogen for the plant (Palacios et al., 2014) and that can positively contribute to crop production (Rinnofner et al., 2008; Krapp et al., 2011). For that, the inoculation of legume seeds with fixing bacteria is a well-established practice and contributes significantly to increased yields in grains production (Oldroyd et al., 2011; Rodrigues et al., 2013c). The extreme sensitivity of BNF to environmental oscillations limits the yield of legumes (Masson-Boivin et al., 2009; Voisin et al., 2010) and directly or indirectly regulates the photosynthesis and plant carbon status (Larrainzar et al., 2009). BNF and photosynthesis are the most important physiological processes that maintain life on earth (Rinnofner et al., 2008), and the use of microorganisms to enhance nodulation and BNF is of fundamental importance to increase plant productivity (Figueiredo et al., 2008; Rodrigues et al., 2013b).

Some microbial combinations can increase the nutrient availability to plants, the quality of the plant root system, plant productivity and the BNF (Antolín et al., 2010;

Rodrigues et al., 2013b). Legume-rhizobia symbiosis plays an important environmental role, reducing the use of mineral nitrogen fertilizers, which have high costs and minimize environmental risks, such as the eutrophication of water resources (Krapp et al., 2011).

In addition to rhizobia, plant growth-promoting bacteria (PGPB) positively affects plants when associated with plants in the rhizosphere (Bhattacharyya and Jha, 2012). PGPB are a group of microorganisms that naturally occur around plant roots and positively affect the host plant, inducing plant resistance to a variety of environmental stresses (Dimkpa et al., 2009; Hayat et al., 2010; Palacios et al., 2014).

Additionally, several PGPB enhance nodulation and nitrogen fixation in cowpea in symbiosis with rhizobia (Rodrigues et al., 2013a) and may stimulate root growth and the formation of lateral roots and root hairs (Rodrigues et al., 2013c).

We hypothesized that the EPS of *R. tropici* (EI-6 strain) is a good vehicle for inoculation and improves the symbiosis between cowpea, *Bradyrhizobium* sp. (nitrogen-fixing bacteria) and *Paenibacillus* sp. (PGPB). In order to test our hypothesis, EPS that are produced by *R. tropici* were initially characterized and evaluated as vehicles for the inoculation of cowpea with *Bradyrhizobium* compared to peat in sterile sand. Then, the effectiveness of EPS as carriers for cowpea inoculation with *Bradyrhizobium* and *Paenibacillus* (*P. graminis* and *P. durus*) on unsterile soil was evaluated, mimicking field conditions. We concluded that the use of EPS as carriers and *Paenibacillus* species represents an emerging technology for the improvement of cowpea-rhizobia symbiosis due to the positive effects on BNF and crop productivity. Thus, the combination of PGPB and rhizobia using EPS as inoculant vehicles represents the future of modern agriculture and, therefore, an environmental friendly alternative that is economically important in the use of chemical fertilizers and pesticides.

MATERIALS AND METHODS

Extraction of *R. tropici* exopolysaccharides

Exopolysaccharides (EPS) of *R. tropici* (EI-6 strain) isolated from root nodules of *Vigna unguiculata* (L.) Walp. (Figueiredo et al., 1999; Oliveira et al., 2012) were extracted. The EI-6 strain was multiplied in YMA culture medium for 72 h, and then grown in YM culture medium for another 72 h under constant agitation (200 rpm; 28°C). Then, the mixture was placed in flasks and maintained in a water bath (80°C; 20 min). EPS were precipitated with ethanol after cooling, and then collected and dried at 30°C for 72 h to remove residual ethanol. Finally, EPS were manually ground and sieved, placed in glass flasks and stored in a cool dry environment until

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characterization and use.

Characterization of EPS produced by *R. tropici*

EPS synthesized by EI-6 strain of *R. tropici* were rheologically and chemically analyzed (Borges et al., 2009). For rheological analyses, 1% (w/v) EPS solutions were prepared in distilled water with the addition of preservatives 0.1% methyl paraben and 0.05% propyl paraben (Borges et al., 2009). The EPS solution was agitated for 2 h at 24°C, heated at 60°C for 20 min, and finally maintained at room temperature for 24 h before testing. The viscosity of the EPS solution was measured in a rheometer (Haake® RS 150, Germany) in the rotary and oscillatory modes coupled with a plate-plate system with a PP35Ti sensor. A shear rate of 0.01-1000 s⁻¹ was used to analyze the elastic (G') and viscous modulus (G'') as a function of frequency (0.1-15 Hz).

For the thermal properties of EPS, differential scanning calorimetry (DSC) was performed, and the melting temperature (T_m) was determined (Borges et al., 2009). DSC was performed with a Perkin Elmer® (model Pyris 6) apparatus using a nitrogen atmosphere at a rate of 20 mL min⁻¹. The samples were subjected to the following test conditions: isotherm 20°C (1 min); first heating cycle of 20-420°C; isotherm 420°C (5 min); cooling cycle 420-20°C; isotherm of 20°C (5 min); and second heating cycle of 20-420°C. A DSC thermogram with a heating or cooling rate of 10°C min⁻¹ was recorded with an empty pan as reference. The degrees of crystallinity and recrystallization were not determined due to loss of sample when the temperature exceeded 300°C. The influence of the thermal history of the sample was not observed, even when a second heating cycle was carried out.

For chemical composition analysis, the EPS samples were hydrolyzed using 2.0 N HCl (80°C; 16 h) in water bath and submitted to comparative thin-layer chromatography (TLC) on silica gel 60 F254 aluminum sheets (Merck, Germany). Then, 5.0 µL of hydrolyzed samples and standards was applied in aluminum sheets together with an chloroform:methanol:acetic acid:water eluent (40:40:10:10; v/v). Samples of arabinose, fucose, glucose, galactose, mannose, rhamnose, xylose, and uronic acids (glucuronic and galacturonic acids) were used as standards. The chemical composition of EPS was determined by comparing with samples of standards, and the retention factor (R_f) was calculated for the monosaccharides as identified in EPS. A sulfuric-anisaldehyde reagent was used for detection (Borges et al., 2009).

The acetyl residue composition in EPS that were synthesized by *R. tropici* was determined by the hydroxamic acid reaction in accordance with McComb and McCready (1957), while the pyruvate residue contents were measured by the 2,4-dinitrophenylhydrazone method using pyruvic acid as the standard (Sloneker and Orentas, 1962). Data from acetyl and pyruvate residues present in EPS that were synthesized by EI-6 strain of *R. tropici* are shown in percentages (%; m/m). Sodium and potassium ions were measured by flame photometry (Micronal, Brazil) after the hydrolysis of the EPS samples with 2.0 N chloride acid (3:100; w/v) at 80°C in a water bath for 16 h in accordance with Klaić et al. (2011). Data on the sodium and potassium in EPS samples are displayed as percentages (%).

Use of EPS produced by *Rhizobium* as carriers: Preliminary experiment

A preliminary experiment was implemented to evaluate the efficiency of cowpea inoculation using EPS produced by *R. tropici* (EI-6 strain) as alternative carriers. In this pretesting, peat (traditional vehicle) was used as a carrier, uninoculated plants were used as controls, and both were tested in an axenic environment.

Initially, 1.0 g of the dried and sieved EPS was mixed with 1.5 mL of the *Bradyrhizobium* inoculum and 1.5 mL of sterile water to perform the EPS inoculant. For the peat inoculant, peat was autoclaved (120 °C; 101 kPa; 1h) for three consecutive days at intervals of 24 h prior to use, 1.0 g of which was mixed with 1.5 mL of the *Bradyrhizobium* inoculum and 1.5 mL of sterile water. The *Bradyrhizobium* inoculum was produced as described by Rodrigues et al. (2013b, c) and maintained at 10⁸ CFU mL⁻¹ for use. After maturation for two days at 24°C, EPS and peat inoculants were dissolved in 0.85% sodium chloride and agitated (300 rpm; 28°C) until the solution was homogeneous. Plate counts of the inoculants were performed using the drop plate method of dilutions (10⁵ to 10⁷) in YMA culture medium with 0.25% Congo red.

After preparing the EPS and peat inoculants, the experiments were performed under greenhouse conditions. 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. In each seed-sown Leonard jar, 1.0 mL of the EPS or peat inoculant was added. Uninoculated plants were used as an absolute control. Thinning was performed at seven days, and two plants were maintained in each Leonard jar. During the experimental period, cowpeas were irrigated by capillary with nitrogen-free Hoagland and Arnon nutritive solution modified by Silveira et al. (1998). The absolute growth rate was calculated based on the plants height as evaluated every seven days until collected at 35 days. In this experiment, the shoot, root and nodules dry weight; number of nodules; specific nodulation (Gulden and Vessey, 1998); and efficiency of nitrogen fixation (Bremner, 1965) were evaluated. Data obtained were subjected to analysis of variance (ANOVA) and means were compared by the Tukey's test at 5% probability.

Inoculation of cowpea with *Bradyrhizobium* and *Paenibacillus* using EPS as carriers

EPS were used as carriers for cowpea inoculation with *Bradyrhizobium* sp. (10⁸ CFU mL⁻¹) and/or *Paenibacillus* species (each at 10⁷ CFU mL⁻¹). The bacterial inoculum of each bacteria used here was prepared as described in Rodrigues et al. (2013b,c). For inoculant preparation, 1.0 g of the dried and sieved EPS was mixed with aliquots of *Bradyrhizobium* or *Paenibacillus* inoculum. The inoculant that contained only *Bradyrhizobium* sp. was produced with a mixture of 1.5 mL of *Bradyrhizobium* inoculum and 1.5 mL of sterile water. The inoculant of *Bradyrhizobium* sp. and *P. graminis* was a blend of 1.5 mL of *Bradyrhizobium* inoculum with 1.5 mL of *P. graminis* inoculum, while the inoculant of *Bradyrhizobium* sp. and *P. durus* was a blend of 1.5 mL of *Bradyrhizobium* inoculum with 1.5 mL of *P. durus* inoculum. The inoculant of *Bradyrhizobium* sp., *P. graminis* and *P. durus* was a blend of 1.5 mL of *Bradyrhizobium* inoculum with 0.75 mL of each *Paenibacillus* inoculum.

After production, the inoculants were matured at 24°C for 48 h, dissolved in 0.85% in saline solution, and agitated (300 rpm; 28°C; 30 min) until homogeneous. Drop plate of dilutions (10⁵ to 10⁷) in YMA culture medium with 0.25% Congo red was performed by plate counts of the inoculants. In a greenhouse of the Agronomy Institute of Pernambuco (IPA; Recife/PE), an experiment was conducted with inoculants that were prepared with EPS produced by *R. tropici*. Disinfected cowpea seeds were sown in pots containing 6.0 kg of non-sterile Spodosol (Table 1). This soil was submitted to liming with calcium carbonate (CaCO₃) and supplementation with phosphorus (P₂O₅) and potassium (K₂O) following the recommendations of culture.

To the surface of each cowpea-seed-sown pot, 2.0 mL of *Bradyrhizobium* inoculant, *Bradyrhizobium* and *P. graminis* inoculant, *Bradyrhizobium* sp. and *P. durus* inoculant, or *Bradyrhizobium* sp., *P. graminis* and *P. durus* inoculant produced as described above was added, and all of the inoculants drained into the substrate. Nitrogen-supplied and uninoculated plants were used as nitrogen and absolute controls, respectively. For nitrogen-

Table 1. Chemistry characterization of the Spodosol (0-20 cm) utilized in the experiment.

pH (water)	P (mg dm ⁻³)	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	Al ²⁺	H ⁺	S	CTC	V (%)
		(cmolc dm ⁻³)								
6.2	10.0	2.15	0.45	0.01	0.02	0.00	1.65	2.60	4.30	61

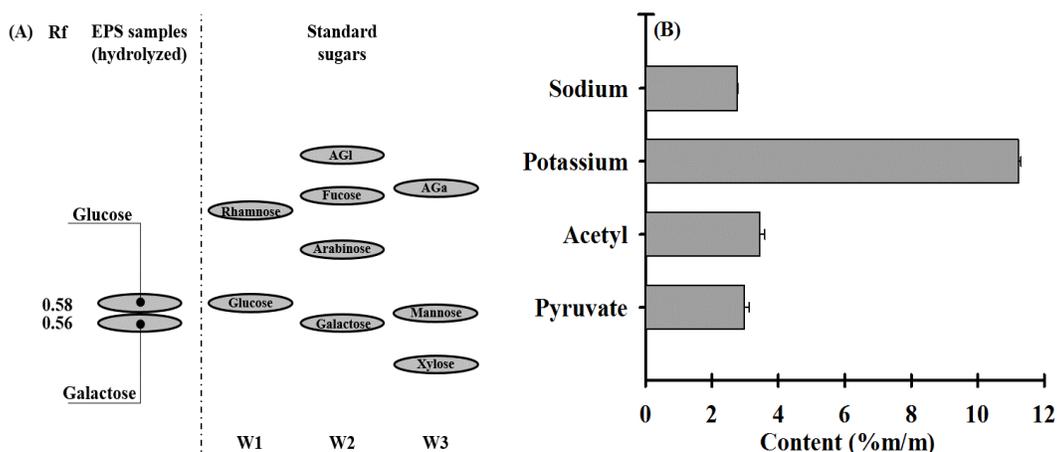


Figure 1. Chemical characteristics of exopolysaccharide (EPS) produced by EI-6 strain of *Rhizobium tropici*. **(A)** Schematic representation of the chromatographic profiles obtained by comparative thin-layer chromatography (TLC) technique on silica gel aluminum sheets. **(B)** Content of non-carbohydrate residues present in EPS produced by EI-6 strain of *R. tropici*. AGI and AGa are glucuronic and galacturonic acid, respectively. The retention factor (Rf) was calculated for the monosaccharides identified in EPS samples. W1, W2 and W3 represent wells where standards were applied.

supplied plants, nitrogen supplementation (0.06 g per pot of ammonium sulfate) was performed following recommendations of soil analysis seven, 14 and 21 days after thinning. After thinning at seven days, two cowpea seedlings were retained in each pot (experimental plot).

During the experimental period, the plants were irrigated by capillary with nitrogen-free Hoagland and Arnon nutritive solution modified by Silveira et al. (1998). The plants were collected at two stages: (1) flowering, a period of greater N₂-fixation (at 36 days), and (2) grain filling, during which all of the pods were full and mature (at 103 days). The following variables were evaluated: root length, nodule dry weight, shoot and root dry weight, relative efficiency and effectiveness of N₂ fixation, nitrogen accumulation and nitrogen content in shoot dry weight and the efficiency of nitrogen fixation (Bremner, 1965), and specific nodulation (Gulden and Vessey, 1998).

The absolute growth rate was calculated based on the plant height as evaluated every seven days until the first collection. After the first collection, the measurement of plant height was discontinued due to the start of pod formation, which diverts energy flow to grain filling with a concomitant reduction in growth rate. The nodule number was measured only in flowering plants due to the presence of senescent nodules in plant roots at grain filling. The variables that were related to the production pod number, pod length, pod weight, seed number and total weight of seeds were also obtained.

The experimental design was randomized blocks, with four replications using a 4 x 2 + 2 factorial arrangement: four treatments (inoculation with *Bradyrhizobium* sp.; co-inoculation with *Bradyrhizobium* and *P. graminis*; co-inoculation with

Bradyrhizobium sp. and *P. durus*; and co-inoculation with *Bradyrhizobium* sp., *P. graminis* and *P. durus*), two harvest periods (flowering and grain filling) and two controls (absolute and nitrogen controls). Data were subjected to analysis of variance by the F test and means were compared by the Tukey's test, both at 5% probability, using the statistical software ASSISTAT.

RESULTS

Characterization of *R. tropici* exopolysaccharides

The chemical analysis indicated that the exopolysaccharides (EPS) that were produced by EI-6 strain of *R. tropici* are heteropolysaccharides consisting of glucose and galactose monosaccharides with the absence of the glucuronic and galacturonic acids (Figure 1A). The retention factor (Rf) was calculated for glucose and galactose in the EPS samples at 0.58 and 0.56, respectively (Figure 1A). Moreover, the presence of acetyl and pyruvate residues was also detected in EPS that were produced by *R. tropici* at 3.44% and 2.97%, respectively (Figure 1B). Sodium and potassium, which were measured by flame photometry and subsequent acid hydrolysis, in EPS samples were 2.77% and 11.22%, respectively (Figure 1B). The absence of the uronic and glucuronic acids, together with the occurrence

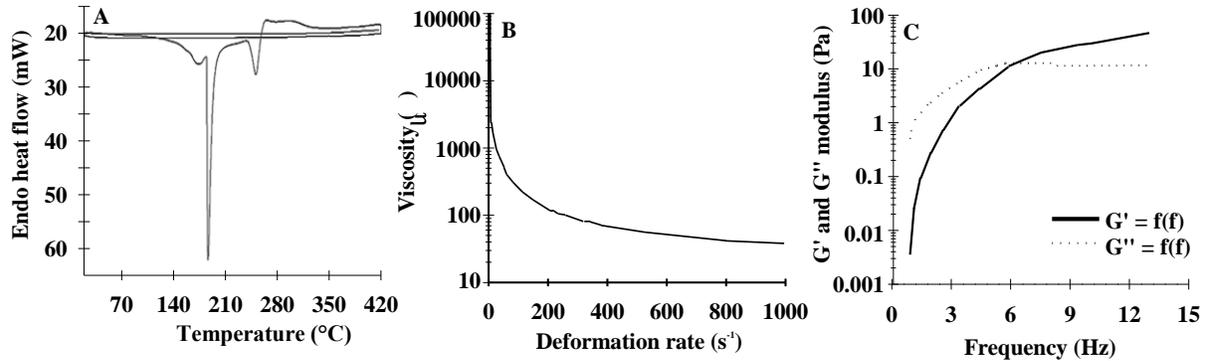


Figure 2. Characterization of exopolysaccharide (EPS) produced by EI-6 strain of *Rhizobium tropici*. **(A)** DSC thermogram. **(B)** Viscosity in function of the deformation rate variation (0.01 to 1000 s⁻¹). **(C)** Elastic (G') and viscous (G'') modulus or viscoelasticity in function of the frequency (0.1 to 15 Hz).

of the glucose and galactose units and acetyl, pyruvate, sodium and potassium substituents, indicate that EPS produced by EI-6 strain of *R. tropici* are polyanionic heteropolysaccharides.

The analysis of EPS produced by EI-6 strain of *R. tropici* showed a well-defined endothermic peak from which it was possible to determine the melting point of the sample as 178°C (Figure 2A). The existence of “shoulders” on the DSC thermogram before the melting point may be related to some sub-product originating from the complex production process of EPS. The EPS produced by *R. tropici* were defined as non-Newtonian fluids that were slightly viscous and pseudoplastic (Figure 2B). The viscoelastic analyses of the EPS defined them as viscous until 6.0 Hz and without true gel behavior due to elastic behavior over 6.0 Hz (Figure 2C).

Effectiveness of the EPS produced by *R. tropici* as carriers

A preliminary experiment was performed with the objective of evaluating the EPS synthesized by EI-6 strain of *R. tropici* as carriers compared to peat, a traditional vehicle that is used for cowpea inoculation. All of the variables analyzed in this preliminary experiment were modulated positively by the use of EPS as carriers and exhibited significant differences in relation to peat inoculation (Tukey's test; $P < 0.05$). The absolute growth rate of the cowpeas inoculated with *Bradyrhizobium* sp. using EPS as carriers increased 8% compared to that of cowpeas inoculated with *Bradyrhizobium* sp. using peat as a carrier (Figure 3A). Increases of up to 40% were registered for the shoot dry weight of cowpeas inoculated with *Bradyrhizobium* using EPS as carriers compared to cowpeas inoculated with *Bradyrhizobium* using peat as a carrier (Figure 3B).

Based on the fact that the preliminary experiment was

conducted with sterile sand, the uninoculated plants did not present nodules in their roots and therefore were not used as controls for variables related to nodule development. When the EPS were used as carriers for *Bradyrhizobium* inoculation, there was an increase of 59% in nodule dry weight compared to that registered when peat was used as a carrier (Figure 3C). For the number of nodules shown in the Figure 3D, the cowpeas inoculated with *Bradyrhizobium* using EPS as carriers showed an increase in this parameter of approximately 60% compared to cowpeas inoculated with *Bradyrhizobium* using peat as a carrier. The specific nodulation of cowpeas inoculated with *Bradyrhizobium* by EPS increased by more than 40% compared to that recorded when peat was used as a carrier (Figure 3E). Finally, the nitrogen-fixing efficiency was also observed and increased by 10% when EPS and peat inoculations were compared.

Considering the results that were obtained in the preliminary experiment, the effectiveness of the EPS synthesized by EI-6 strain of *R. tropici* as carriers for cowpea inoculation with *Bradyrhizobium* and also for co-inoculation with *Bradyrhizobium* and *Paenibacillus* (*P. graminis* and/or *P. durus*) was tested, and data were collected during two periods (flowering and grain filling). The plants co-inoculated simultaneously with *Bradyrhizobium* sp. and two *Paenibacillus* species (*P. graminis* and *P. durus*) stood out from the others in many variables, mainly in grain filling, when an increase in senescence minimized by the simultaneous association occurs. The root development (in terms of root length, nodulation and root dry mass) in cowpeas associated with *Bradyrhizobium* and *Paenibacillus* was significantly different (Tukey's test; $P < 0.05$) compared to uninoculated or nitrogen-supplied plants (Figure 4).

The root length was measured in the flowering and grain filling periods; however, a significant difference between treatments in flowering was observed (Figure 4A). The root length at flowering was higher in plants co-

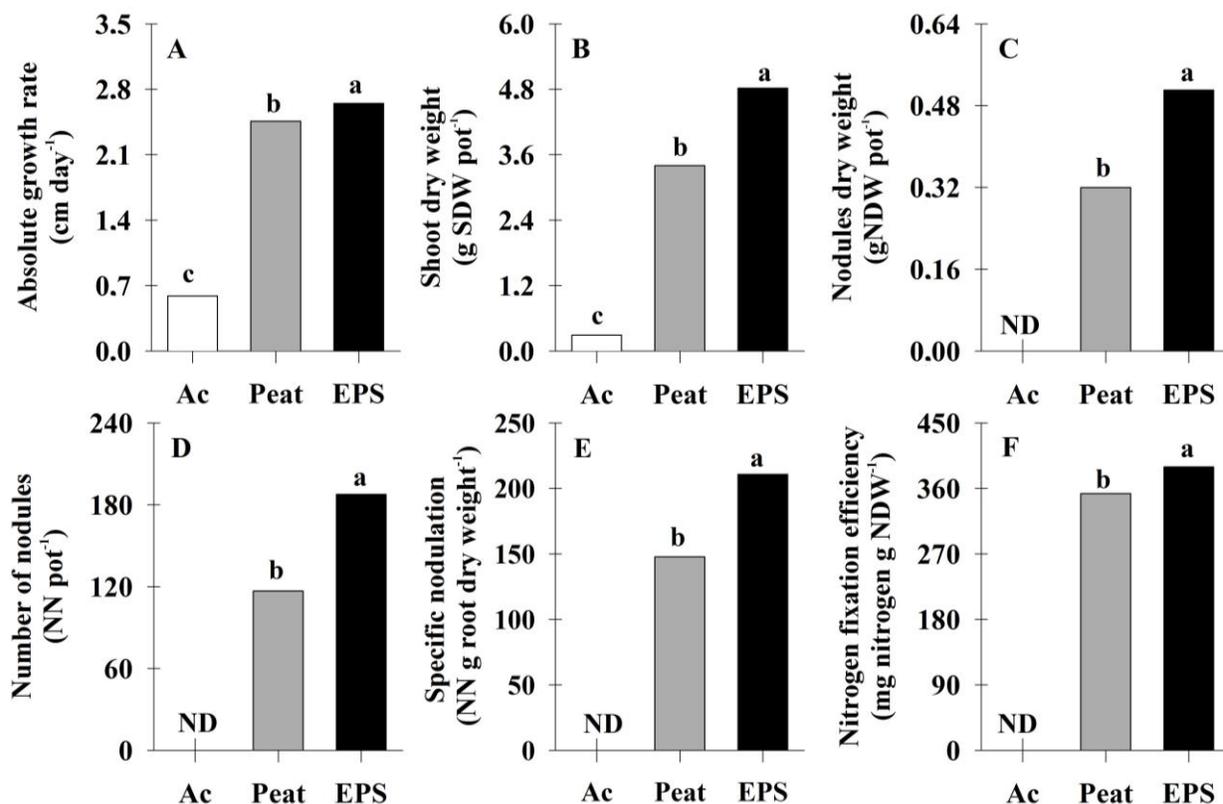


Figure 3. Growth parameters of cowpea inoculated with *Bradyrhizobium* sp. using peat or exopolysaccharide (EPS) produced by EI-6 strain of *Rhizobium tropici* as inoculant carrier. (A) absolute growth rate; (B) shoot dry matter; (C) nodules dry weight; (D) number of nodules; (E) specific nodulation; (F) nitrogen fixation efficiency. Ac = absolute control (uninoculated plants). ND = values non-determinate due the nodules absence in absolute control. Different lowercase letters represent significant differences among the carries types by Tukey's test at confidence of 0.05. *Coefficient of variance.

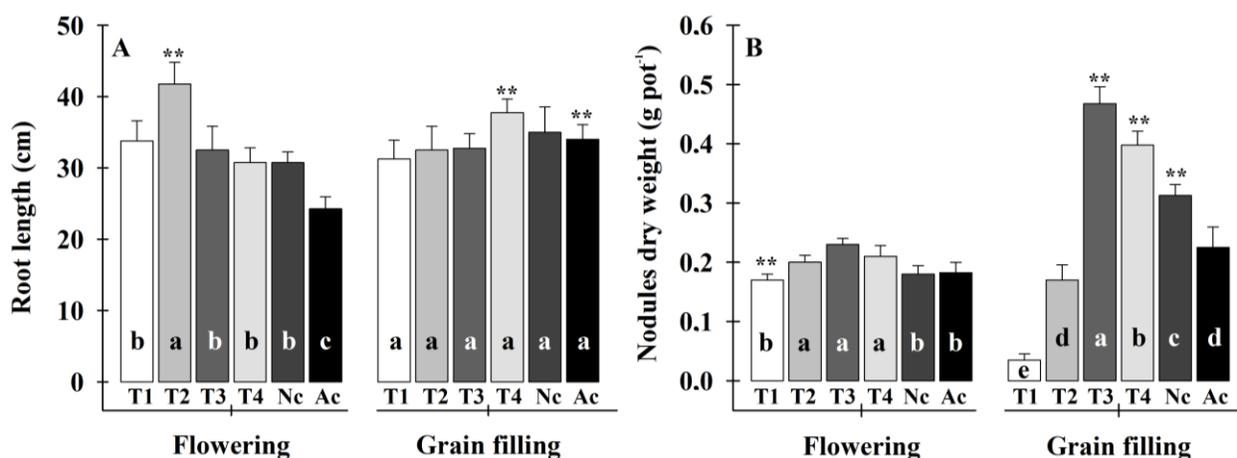


Figure 4. Characterization of cowpea roots. (A) Root length (CV*=10.39%). (B) nodules dry weight (CV*=28.19%) in cowpea inoculated only with *Bradyrhizobium* sp. (T1) or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. and *P. durus* (T3); or simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* (T4). Nitrogen-supplied and uninoculated plants are nitrogen control (Nc) and absolute control (Ac), respectively. Different lowercase letters represent significant differences among the treatments and asterisk (**) represent significant differences among the harvest periods, both at confidence of 0.05. Data are mean of four replicates and were compared by Tukey's test. *Coefficient of variance.

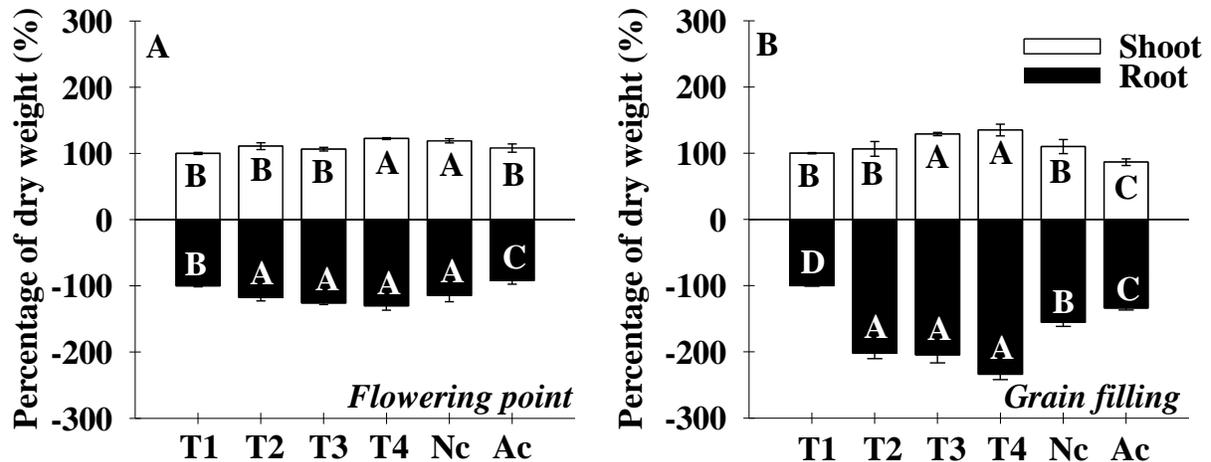


Figure 5. Distribution of dry weight between the shoot ($CV^*=15.48\%$) and root ($CV^*=16.08\%$) in cowpea inoculated only *Bradyrhizobium* sp. (T1) or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus graminis* (T2); with *Bradyrhizobium* sp. and *P. durus* (T3); or simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* (T4). Nitrogen-supplied and uninoculated plants are nitrogen control (Nc) and absolute control (Ac), respectively. Values presented are treatment means (four replicates) collected at the (A) flowering and (B) grain filling. Means followed by the same letters do not differ statistically ($p < 0.05$) from each other, according to Tukey's test. *Coefficient of variance.

inoculated with *Bradyrhizobium* sp. and *P. graminis* compared to other treatments; in particular, the uninoculated plants showed a decrease of 42% compared to this treatment. Although no significant difference was observed in grain filling, the plants co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* showed increased root length (35.75 cm) compared to the other treatments. Cowpeas co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* and uninoculated plants exhibited an increase in root length of 20 and 40%, respectively, in grain filling compared to flowering period.

In terms of nodule dry weight, plants that were co-inoculated with *Bradyrhizobium* and *Paenibacillus* stood out from others treatments during both the flowering and grain filling periods (Figure 4B). The nodule dry weight in cowpeas co-inoculated with *Bradyrhizobium* and *Paenibacillus* was greater than 0.20 g pot^{-1} in flowering, and the increase on average was 20% when *Paenibacillus* species were present. Numerically, plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* showed major nodule dry weight (0.23 g pot^{-1}) at flowering compared to the other treatments. In grain filling, plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* displayed a major nodule dry weight in grain filling (0.47 g pot^{-1}) and a possible increase of 103% when the periods of flowering and grain filling were compared. Intriguingly, plants inoculated only with *Bradyrhizobium* sp. exhibited a significant reduction of 79% in nodule dry weight when the periods of flowering and grain filling were compared (Figure 4B).

Figure 5 shows the dry matter formation of cowpeas inoculated or co-inoculated with *Bradyrhizobium* and

Paenibacillus at flowering and grain filling, with significant differences between the treatments (Tukey's test; $P < 0.05$). In general, the presence of *Paenibacillus* species was positive and stimulated an increase in dry weight in the shoots and roots. At flowering, the plants inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited more root (~30% upper) and shoot (~20% higher) dry matter than did the plants inoculated only with *Bradyrhizobium* sp. or uninoculated plants (Figure 5A). The uninoculated plants exhibited smaller size at flowering, mainly in roots, where the accumulation of dry mass was approximately 10% lower compared to that of other plants (Tukey's test; $P < 0.05$).

As observed in Figure 5B, the plants co-inoculated with the *Bradyrhizobium* sp. and *P. durus* and those co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited increases in shoot dry matter of 29 and 35%, respectively, compared to plants inoculated with *Bradyrhizobium* sp. during the period of grain filling (Figure 5B). Plants that were co-inoculated with *Bradyrhizobium* and *Paenibacillus* showed a large dry matter accumulation in roots during the grain filling period compared to the other plants, and these increases were as high as 100% compared to the plants inoculated only with *Bradyrhizobium* sp. (Tukey's test; $P < 0.05$). In general, the plants co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* stood out from the other treatments in terms of shoot and root dry matter in flowering as in grain filling (Figure 5). Uninoculated plants, similar to as noted in flowering, presented smaller shoots and roots than in the other

Table 2. Absolute growth rate, number of nodules, specific nodulation and nitrogen fixation efficiency in cowpea inoculated only *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* species and harvest at grain filling period. Nitrogen-supplied and uninoculated plants are nitrogen and absolute control, respectively.

Treatment	Absolute growth rate (cm day ⁻¹)	Number of nodules (pot ⁻¹)	Specific nodulation (NN g ⁻¹ RDM [§])	N-fixation efficiency (mg N g ⁻¹ NDM [†])
<i>Bradyrhizobium</i> sp.	2.65b	187.5c	139.8c	1036.6b
<i>Bradyrhizobium</i> sp. and <i>P. graminis</i>	1.83c	210.0b	140.6b	833.9b
<i>Bradyrhizobium</i> sp. and <i>P. durus</i>	2.28c	273.5a	180.9a	916.8b
<i>Bradyrhizobium</i> sp., <i>P. graminis</i> and <i>P. durus</i>	3.76a	302.5a	187.3a	1256.6a
Nitrogen control	3.88a	161.3d	119.3d	1033.1b
Absolute control	4.40a	157.8d	108.4d	792.2c
Coefficient of variance (%)	15.33	8.97	16.19	7.86

In each column the means followed by the same letters do not differ statistically ($P < 0.05$) from each other according to Tukey's test. [§]nodule number g⁻¹ root dry matter; [†]mg nitrogen g⁻¹ nodule dry matter. Values presented are treatment means (four replicates) collected at flowering.

treatments (Figure 5B).

Table 2 displays the absolute growth rate, number of nodules, specific nodulation and nitrogen-fixation efficiency of cowpeas inoculated or co-inoculated with *Bradyrhizobium* and *Paenibacillus*.

Nitrogen-supplied plants and those that were co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* exhibited a pronounced increase in absolute growth rate of greater than 40% at flowering (Table 2). Furthermore, the plants co-inoculated with *Bradyrhizobium* and *P. durus* and the plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited increases of 46 and 61% in the number of nodules, respectively, compared to plants inoculated only with *Bradyrhizobium* sp. (Table 2). The plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* exhibited an increase in the number of nodules of 12% compared to plants inoculated only with *Bradyrhizobium* sp. (Table 2).

There was a decrease in the number of viable nodules in plants collected at grain filling; thus, the specific nodulation and nitrogen fixation efficiency of cowpea were measured only at flowering. Plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* and those co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* showed increases of 29 and 34% in specific nodulation compared to the plants inoculated only with *Bradyrhizobium* sp. (Table 2).

Additionally, cowpeas co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) stood out in terms of nitrogen-fixation efficiency and exhibited increases of 59 and 21% compared to uninoculated plants and to plants inoculated with *Bradyrhizobium* sp., respectively (Table 2). The nitrogen-fixation efficiency in uninoculated plants was lower than that of the other treatments and showed a 24% reduction compared to plants inoculated only with

Bradyrhizobium sp. (Tukey's test at 5%).

The nitrogen content and nitrogen accumulated in shoot dry weight in cowpeas inoculated or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp. are shown in the Figure 6. Plants co-inoculated with *Bradyrhizobium* and *Paenibacillus* exhibited higher nitrogen content (Tukey's test; $P < 0.05$) than did the other treatments at flowering, mainly compared to uninoculated plants, and the values in these treatments were greater than 30% nitrogen per pot.

Plants co-inoculated with *Bradyrhizobium* sp. and *P. graminis* and those co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited increases in the nitrogen content at grain filling of 43 and 33%, respectively, compared to the plants inoculated only with *Bradyrhizobium* sp. (Figure 6A). There was a decrease of up to 40% in the nitrogen content at grain filling for all treatments compared to the data obtained at flowering; however, the plants inoculated with *Bradyrhizobium* and *Paenibacillus* showed a high nitrogen content compared to others treatments.

Cowpeas inoculated or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp. exhibited higher values of nitrogen accumulation compared to plants inoculated only with *Bradyrhizobium* sp. at both flowering and grain filling (Figure 6B). Plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited an increase of 49% in nitrogen accumulation compared to the plants inoculated only with *Bradyrhizobium* sp. at flowering.

For the plants co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp., average increases of 33% were observed. Uninoculated plants showed less nitrogen accumulation than in other treatments, and a 15% reduction was registered compared to plants inoculated only with *Bradyrhizobium* sp. (Tukey's test; $P < 0.05$).

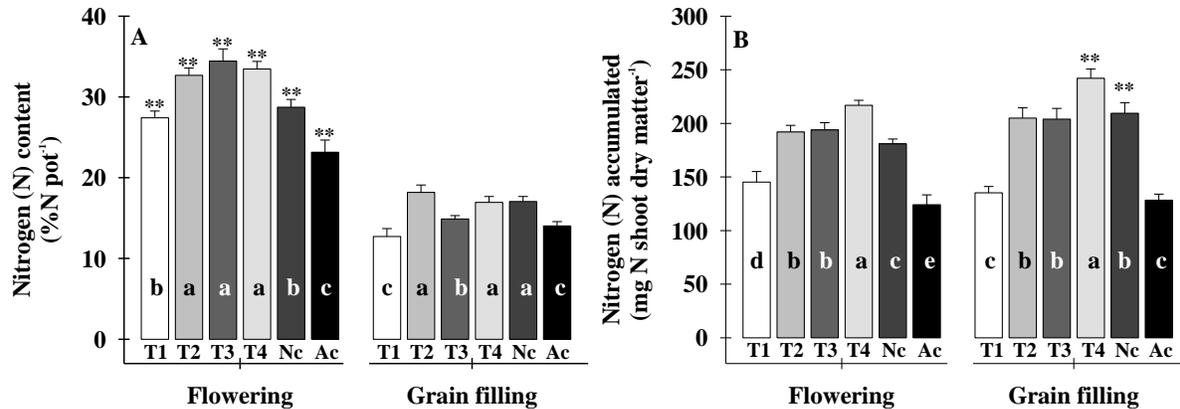


Figure 6. Nitrogen flow in cowpea plants. **(A)** Nitrogen content (CV*=11.15). **(B)** Nitrogen accumulated (CV*=18.47) in shoot dry weight of the cowpea inoculated only with *Bradyrhizobium* sp. (T1) or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus graminis* (T2); *Bradyrhizobium* sp. and *P. durus* (T3); or simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* (T4). Nitrogen-supplied and uninoculated plants are nitrogen control (Nc) and absolute control (Ac), respectively. Different lowercase letters represent significant differences among the treatments and asterisk (**) represent significant differences among the harvest periods, both at confidence of 0.05. Data are mean of four replicates and were compared by Tukey's test. *Coefficient of variance.

Table 3. Relative efficiency and efficacy in cowpea plants inoculated only with *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp. and harvest at the flowering and grain filling periods.

Treatment	Efficiency (%) ¹		Efficacy (%) ²	
	Flowering	Grain filling	Flowering	Grain filling
<i>Bradyrhizobium</i> sp.	101	116	84	87
<i>Bradyrhizobium</i> sp. and <i>P. graminis</i>	112	123	93	92
<i>Bradyrhizobium</i> sp. and <i>P. durus</i>	107	150	89	112
<i>Bradyrhizobium</i> sp., <i>P. graminis</i> and <i>P. durus</i>	123	156	103	120

¹Relative Efficiency = (shoot dry matter of inoculated treatments ÷ shoot dry matter of absolute control) × 100;

²Efficacy = shoot dry matter of inoculated treatments ÷ shoot dry matter of nitrogen control) × 100. Uninoculated and nitrogen-supplied plants are absolute and nitrogen control, respectively.

At grain filling, plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) presented an increase of 79% in nitrogen accumulation (Figure 6B).

Table 3 shows the relative efficiency and efficacy as calculated compared to uninoculated and nitrogen-supplied plants for two harvest periods. Plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited a great efficiency at flowering (~20% higher) and grain filling (~30%) periods compared to plants inoculated only with *Bradyrhizobium* sp. (Table 3). Additionally, at grain filling, plants co-inoculated simultaneously with *Bradyrhizobium* sp. and two *Paenibacillus* species (*P. graminis* and *P. durus*) and plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* maintained a high efficiency compared to other treatments (Table 3).

When the periods of flowering and grain filling were

compared, plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* and those co-inoculated simultaneously with *Bradyrhizobium* sp. and two *Paenibacillus* (*P. graminis* and *P. durus*) species displayed increases in efficiency of 43 and 33%, respectively.

Plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited a higher efficacy compared to other treatments during the flowering period, and when compared to plants inoculated only with *Bradyrhizobium* sp., there was an increase of 19% (Table 3).

In grain filling, plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* and those that were co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) presented increases in efficacy of 25 and 33%, respectively, compared to plants that were inoculated only with *Bradyrhizobium* sp. (Table 3). By comparing the two periods, flowering and grain filling, it was

Table 4. Pods number, pod length, pod weight, seeds number and total weight of seeds of cowpea inoculated only *Bradyrhizobium* sp. or co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* species and harvest at grain filling period. Nitrogen-supplied and uninoculated plants are nitrogen and absolute control, respectively.

Treatment	Pods number (unit pot ⁻¹)	Pod length (cm pot ⁻¹)	Pod weight (g pot ⁻¹)	Seeds number (unit pot ⁻¹)	Total weight of seeds (g pot ⁻¹)
<i>Bradyrhizobium</i> sp.	5.75c	18.92ab	14.38b	56.25ab	10.76b
<i>Bradyrhizobium</i> sp. and <i>P. graminis</i>	5.50c	19.48ab	13.87c	49.50b	10.61b
<i>Bradyrhizobium</i> sp. and <i>P. durus</i>	6.00b	18.34b	14.10b	46.50b	10.50b
<i>Bradyrhizobium</i> sp., <i>P. graminis</i> and <i>P. durus</i>	7.00a	19.84a	17.59a	58.50a	12.06a
Nitrogen control	4.75c	17.17c	12.51d	48.25b	9.46c
Absolute control	3.75d	14.52d	10.08e	34.75c	8.08d
Coefficient of variance (%)	14.03	12.15	13.25	20.32	12.19

Values presented are treatment means (four replicates). In each column the means followed by the same letters do not differ statistically ($P < 0.05$) from each other, according to Tukey's test.

observed that the plants co-inoculated with *Bradyrhizobium* sp. and *P. durus* and those co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited increases in efficacy of 23 and 17%, respectively, and consequently stood out compared to other treatments (Table 3).

Features related to the production of cowpea when subjected to different combinations of *Bradyrhizobium* sp. and *Paenibacillus* species were evaluated, with significant differences in all of the analyzed variables (Tukey's test; $P < 0.05$). Plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) stood out from the other treatments. The pod numbers were 22 and 87% higher in plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) compared with plants uninoculated or inoculated only with *Bradyrhizobium* sp., respectively. Compared to nitrogen-supplied plants, the plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) exhibited an elevated pod number (47% upper). Moreover, these plants exhibited greater pod length than uninoculated and nitrogen-supplied plants by 37 and 16%, respectively (Table 4).

For pod weight, the uninoculated and nitrogen-supplied plants produced pods with a lower weight compared to plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*), at 43 and 29%, respectively (Table 4). Furthermore, the seed number was 68% greater in plants co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) compared to the uninoculated plants. In addition, these plants showed an increase in the seed number of 21% superior compared to nitrogen-supplied plants. In relation to the total weight of the seeds, the plants co-

inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) stood out in relation to the other treatments and exhibited increases of 49 and 27% compared to uninoculated and nitrogen-supplied plants, respectively (Table 4).

DISCUSSION

Characterization of *Rhizobium tropici* exopolysaccharides

The production of exopolysaccharides (EPS) by different *R. tropici* strains may be important not only for commercial use, but also for enhancing nodule formation in agriculture (Albareda et al., 2008; Serrato et al., 2008; Oliveira et al., 2012). In this study, the EPS produced by the EI-6 strain of *R. tropici* were defined as heteropolysaccharides (Figure 1) due to presence of repeating units of different monosaccharides of glucose and galactose (Freitas et al., 2011; Donot et al., 2012). In agreement with Janczarek (2011), rhizobial EPS are linear or branched hetero- and homopolymers that consist of repeating units containing common monosaccharides, such as glucose, galactose or mannose, substituted with non-carbohydrate residues. Moreover, the EPS produced by *R. tropici* vary with the type of monosaccharide constituents and the presence of non-carbohydrate decoration, such as acetyl, pyruvate, succinate, and phosphate residues (Freitas et al., 2011; Ghosh et al., 2011; Staudt et al., 2012). Indeed, in this study, acetyl and pyruvate groups were identified in samples of EPS that were synthesized by *R. tropici*.

The type and quantity of substituent groups in the polysaccharide chain depend on species, environmental conditions, and physiological factors (Ghosh et al., 2011). Moreover, the composition of polysaccharides and the presence of ionizable groups, such as acetate and pyruvate

groups, confer a polyelectrolyte behavior to the EPS produced by *R. tropici*, which greatly affects their physical properties (Vu et al., 2009). Due to its ability to produce large quantities of polysaccharide, *Rhizobium* may prove an excellent model bacterium for the development of biotechnology products (Staudt et al., 2012). According to our results, the EPS of *R. tropici* were considered inert to the bacterial strains used in this study and were therefore used as alternative vehicles for inoculation.

The commercial applicability of EPS mainly depends on the thermal and viscometric behavior (Poli et al., 2010; Freitas et al., 2011). In this study, the EPS synthesized by the EI-6 strain of *R. tropici* were submitted to DSC analysis from 20 to 420°C, and the DSC thermogram displayed an endothermic peak (melting point) at 178°C (Figure 2). The melting point of the EPS produced by *R. tropici* was similar to that of other bacterial polymers obtained from *Pseudomonas* and *Beijerinckia*, which ranged from 162 to 170°C, and *Xanthomonas*, which ranged from 120 to 185°C (Borges et al., 2009; Oliveira et al., 2012). The existence of “shoulders” on the DSC thermogram before the melting point can be related to some sub-product originating from the complex production process of EPS (Albareda et al., 2008; Staudt et al., 2012). The EPS that were synthesized by *R. tropici* could tolerate higher temperatures (above 100°C), which is a favorable feature that permits use on the industrial scale (Poli et al., 2010).

In the DSC thermogram of EPS produced by *R. tropici*, no exothermic peak was observed, which might be due to the conformational changes in sugar molecules at different temperatures (Ghosh et al., 2011). Indeed, there are relationships between the carbohydrate structure of an EPS and their physical properties (Nwodo et al., 2012). The flow characteristics of polymeric gel systems are complex and can be described in terms of viscosity and viscoelastic behavior (Serrato et al., 2008). These parameters determine the behavior of a fluid (Figueiredo et al., 1999; Vu et al., 2009). The evaluation of bacterial EPS viscosity is of fundamental importance to determine its quality and potential industrial and/or commercial applicability (Nwodo et al., 2012). Furthermore, the inherent biocompatibility and apparent non-toxic nature of some bacterial EPS increase their possibility for application, mainly in medicine and agriculture (Poli et al., 2010; Freitas et al., 2011).

In this study, an analysis of EPS synthesized by *R. tropici* defined them as having typical non-Newtonian pseudoplastic behavior without true gel behavior (Figure 1B and 1C). In general, non-Newtonian fluids are frequently utilized in different industrial processes, such as fruit juice clarification, and in food additives (Vu et al., 2009; Staudt et al., 2012). The pseudoplastic behavior is common in most non-Newtonian fluids, mainly in emulsions and gums (Freitas et al., 2011). Pseudoplastic fluids can have a wide range of use, such as applications in the food and drug industry and in agriculture (Albareda

et al., 2008; Nwodo et al., 2012).

Effectiveness of the EPS produced by *R. tropici* as carriers

The use of EPS carriers for cowpea inoculation with *Bradyrhizobium* induces positive alterations in plant growth and development compared to responses obtained with peat as a carrier (Figure 3). Moreover, changes in growth variables were also recorded in response to the inoculation and co-inoculation of cowpea with *Bradyrhizobium* sp. and *Paenibacillus* species using EPS as carriers (Figures 4 and 5; Table 2). The evaluation of plant growth and development is very complex due to the involvement of the effect of biotic and abiotic external factors on the physiological plant processes (Masson-Boivin et al., 2009; Hayat et al., 2010; Voisin et al., 2010). Growth parameters inform about the ability of plants to translocate photoassimilates that are produced by photosynthesis to fixation sites for plant organs that serve as growth and differentiation sites (Masson-Boivin et al., 2009). Thus, leaf area expansion is a strategy that allows the vegetable to expose the leaves to a higher light intensity, ensuring a better light use (Rinnofner et al., 2008; Voisin et al., 2010).

Plant species differ in growth dynamics according to the supply of nitrogen and carbon (Rinnofner et al., 2008; Krapp et al., 2011). As observed in this study, uninoculated plants altered the root growth, probably in response to low nitrogen concentrations in the soil solution (Rinnofner et al., 2008; Antolín et al., 2010). Additionally, cowpeas co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) showed better growth parameters, indicating that this co-inoculation was able to maintain a continuity of the root growth process. Moreover, plants co-inoculated with *Bradyrhizobium* and *P. durus* and simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) showed higher shoot, root and nodule dry weight compared to plants inoculated only with *Bradyrhizobium* during grain filling. These responses appear to be related to the presence of the C 04.50 strain of *P. durus*. The C 04.50 strain of *P. durus* can secrete extracellular compounds – unique to this bacterial species – in the rhizosphere, such as amino acids, phytohormones and secondary metabolites, promoting a favorable environment for plant development (Yoon et al., 2003).

Silva et al. (2007) reported that *P. macerans*, *P. durus* and *P. polymyxa* improved the symbiosis between *Bradyrhizobium* and cowpea, ameliorating the nitrogen-fixation efficiency. The higher shoot and root dry matter may be associated with the phytohormones produced and released by PGPB on the root surface that can utilize the photoassimilates produced in the shoot to sustain energy for cell division, ensuring its growth (Figueiredo et

al., 2008; Larrainzar et al., 2009; Hayat et al., 2010; Bhattacharyya and Jha, 2012). BNF in legume root nodules is fueled by carbon that is supplied from shoots, and the increased nodule supply with carbohydrates results in stimuli for BNF and plant growth (Larrainzar et al., 2009; Magori and Kawaguchi, 2009). In addition, the growth of nodules interacts strongly with the shoot due to competition for photoassimilates produced by plants (Voisin et al., 2010).

In this study, the plants co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus* sp. exhibited increases in root dry matter, which may represent a more effective partitioning of the carbon supply than plants inoculated only with *Bradyrhizobium*. This response reinforces the idea that the flow of photosynthetically fixed carbon was directed to support root and nodule growth (Larrainzar et al., 2009; Magori and Kawaguchi, 2009; Masson-Boivin et al., 2009; Voisin et al., 2010). During the vegetative stage, the photosynthetic rate has a greater effect on the total dry mass and decreases sharply as the pods begin to develop, which is probably related to the decreased total nitrogen content of Rubisco activity (enzyme responsible for fixing CO₂ during photosynthesis) and the integrity of the nodules (Antolín et al., 2010; Krapp et al., 2011). This affirmation is supported by the decrease in viable nodules in plants that were collected at grain filling, probably in response to the natural process of senescence.

Increased nodulation and plant growth promotion have been associated with the presence of PGPB in the root environment (Magori and Kawaguchi, 2009; Antolín et al., 2010). Indeed, the number of nodules, specific nodulation and nitrogen-fixation efficiency – all measured in the nodules – increased in response to the presence of PGPB, mainly in plants that were co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*). The host plants should tightly control the number of nodules, and nodule development is an energetically expensive process (Voisin et al., 2010; Krapp et al., 2011). According to Magori and Kawaguchi (2009), the nodule biomass is associated with the amount of symbiotically fixed nitrogen by the plant-*Rhizobium* association. Here, the increase in nodule-related parameters in the plants that were co-inoculated simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) indicates a better flow of photosynthetically fixed carbon that will supply the energy that is required for nodule organogenesis (Masson-Boivin et al., 2009). Thus, it is possible that better specific nodulation and nitrogen fixation efficiency in these plants is linked to an improvement of plant and *Bradyrhizobium* symbiosis due the addition of the *Paenibacillus* in rhizosphere. Hayat et al. (2010) reported that different types of substances that are released by bacteria diffuse into the rhizosphere and can positively stimulate microbial activity.

The variables that are related to the nitrogen flow from

rhizobia to plant – nitrogen content and nitrogen accumulation in the shoot dry weight – present significant differences (Tukey's test at 5%) in response to different treatments, mainly in response to the presence of PGPB (Table 2 and Figure 5). Furthermore, the relative efficiency and efficacy – calculated compared to uninoculated and nitrogen-supplied control plants, respectively – increased in response to treatments, mainly in cowpeas that were inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* (Table 3). The improvement of the relative efficiency and efficacy as recorded in plants that were co-inoculated simultaneously with *Bradyrhizobium* sp., *P. graminis* and *P. durus* indicate that the presence of PGPB induced a better ability to fix nitrogen and provide it to the plant vegetative development (Figueiredo et al., 2008; Bashan and De-Bashan, 2010; Rodrigues et al., 2013a,b,c). In fact, PGPB can promote better root development, resulting in the efficient uptake of water and nutrients, and consequently improve plant growth (Dimkpa et al., 2009; Bashan and De-Bashan, 2010; Palacios et al., 2014). Thus, the use of bacterial strains to improve the legume-rhizobia symbiosis with the aim of increasing the nitrogen in the soil, due to nitrogen's critical roles, is an extremely important aspect of crop production.

Features that are related to the production of plants when subjected to different combinations of *Bradyrhizobium* sp. and *Paenibacillus* species (*P. graminis* and *P. durus*) were evaluated, and significant differences in all of the different variables were found (Table 4). *Bradyrhizobium* was able to establish an efficient symbiosis with cowpea, resulting in a good yield compared to the nitrogen treatment (Figueiredo et al., 1999, 2008; Silva et al., 2007; Rodrigues et al., 2013a,b,c). Indeed, in this study, the data that were obtained for plants that were inoculated only with *Bradyrhizobium* sp. were statistically similar (Tukey's test; $P < 0.05$) to those that were obtained for N-supplied plants. Moreover, the co-inoculation of cowpea simultaneously with *Bradyrhizobium* sp. and the two *Paenibacillus* species (*P. graminis* and *P. durus*) promoted greater increases than in the other production variables that were analyzed. The use of PGPB promotes numerous beneficial effects both for the plant species as well as for the fixing bacteria, which also lead to increased plant productivity and BNF (Dimkpa et al., 2009; Hayat et al., 2010; Palacios et al., 2014). These results indicate the maintenance of fixation and transport of fixed nitrogen in this treatment during the pods formation; thus, such results highlight the importance of an ideal combination of microorganisms to promote increased productivity.

Conclusions

EPS produced by *R. tropici* (EI-6 strain) were considered

inert to bacterial species and were successfully utilized as alternative vehicles for inoculation because they promoted greater symbiotic efficiency, growth, and productivity in cowpeas, mainly in plants co-inoculated simultaneously with *Bradyrhizobium* sp., *Paenibacillus graminis* and *P. durus*. The use of EPS produced by *R. tropici* and *Paenibacillus* species represents an emerging technology for the improvement of cowpea-rhizobia symbiosis with positive effects on physiological and agronomic aspects.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The National Council of Technological and Scientific Development (CNPq) supported this work and we would like to acknowledge the Federal Agency for Support and Evaluation of Graduate Education (CAPES) for the scholarship.

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