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Interrelationship of *Bradyrhizobium* sp. and Plant Growth-Promoting Bacteria in Cowpea: Survival and Symbiotic Performance

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The objective of this study was to evaluate the survival of cowpea during bacterial colonization and evaluate the interrelationship of the *Bradyrhizobium* sp. and plant growth-promoting bacteria (PGPB) as a potential method for optimizing symbiotic performance and cowpea development. Two experiments using the model legume cowpea cv. "IPA 206" were conducted. In the first experiment, cowpea seeds were disinfected, germinated and transferred to sterilized Gibson tubes containing a nitrogen-free nutritive solution. The experimental design was randomized blocks with 24 treatments [*Bradyrhizobium* sp. (BR 3267); 22 PGPB; absolute control (AC)] with three replicates. In the second experiment, seeds were disinfected, inoculated according to their specific treatment and grown in Leonard jars containing washed and autoclaved sand. The experimental design was randomized blocks with 24 treatments [BR 3267; 22 BR 3267 + PGPB; AC] with three replicates. Scanning electron microscopy demonstrated satisfactory colonization of the roots of inoculated plants. Additionally, synergism between BR 3267 and PGPB in cowpeas was observed, particularly in the BR 3267 + *Paenibacillus graminis* (MC 04.21) and BR 3267 + *P. durus* (C 04.50), which showed greater symbiotic performance and promotion of cowpea development.

Keywords: PGPB, synergism, BNF, co-inoculation, colonization, *Vigna unguiculata* (L.) Walp.

Introduction

The cowpea, which is grown in arid and semiarid regions of NE Brazil, is a legume that has socio-economic importance due to its high tolerance for adverse environmental conditions, such as low rainfall and high-salinity soil (Almeida *et al*., 2010). Due to the unique nutritional requirements of the cowpea, this legume has also been considered as an alternative to chemical fertilizers, which can negatively impact the environment (Moreira and Siqueira, 2006). In this regard, the use of nitrogen-fixing bacteria in symbiosis with plant species has been shown to be a viable method for supplying nitrogen and increasing plant productivity (Almeida *et al*., 2010). Biological nitrogen fixation (BNF) is known to be effective in well-nodulated cowpeas, which can dispense additional nitrogen and achieve high levels of productivity (Zilli *et al*., 2009).

The use of microorganisms that enhance nodulation and BNF is of fundamental importance, as this practice may contribute to increased plant productivity. Indeed, symbiotic microorganisms are of great importance to the agricultural industry because of their ecologically beneficial effects (Compant *et al*., 2010). Among these microorganisms are plant growth-promoting bacteria (PGPB), which stimulate plant growth, increase plant productivity, reduce the incidence of pathogens and mitigate the deleterious effects of biotic and abiotic stresses (Lugtenberg and Kamilova, 2009). The association of PGPB with nitrogen-fixing bacteria can also result in increased BNF, which requires a specific combination of strains and compatibility between the strains for increased productivity (Spaepen *et al*., 2009; Figueiredo *et al*., 2010).

The application of PGPB in conjunction with nitrogen-fixing bacteria has been the focus of numerous studies in the search for strategies to increase agricultural productivity, including cowpea productivity (Lima *et al*., 2011). However, the effect of co-inoculating *Bradyrhizobium* sp. and PGPB on promoting nodulation and BNF has not been examined previously. The present study was performed to evaluate cowpea survival during bacterial colonization and to examine the interrelationship of *Bradyrhizobium* sp. and PGPB as a method to optimize symbiotic performance and promote cowpea development.

Material and Methods

Multiplication and preparation of the inoculants

The following strains were used in this study: *Bradyrhizobium* sp. BR 3267, the standard strain for cowpea inoculation, obtained from the National Center of Agrobiology Research (CNPAB, RJ-Brazil), and the plant growth-promoting bacteria (PGPB) *Bacillus*, *Paenibacillus*, and *Brevibacillus*, obtained from the Federal University of Pernambuco (UFPE,

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Antibiotic Department) and the Federal University of Rio de Janeiro (UFRJ, Microbiology Institute). Prior to inoculation, strain BR 3267 was grown in Yeast-Mannitol (YM) culture medium using a rotator shaker (200 rpm, 28°C) for 96 h, while PGPB strains were grown in Trypticase Soy Broth (TSB) culture medium using a rotator shaker (200 rpm, 32°C) for 24 h or 48 h depending on the bacterial strain.

Cowpea survival during bacterial colonization

To evaluate cowpea survival during bacterial colonization, cowpea cv. "IPA 206" seeds were disinfected as previously described (Hungria and Araújo, 1994), seeded in Petri dishes containing Germitest© paper and kept in a moist chamber until radicle protrusion. Following germination, seeds were transferred to sterilized Gibson tubes containing Hoagland and Arnon (1950) nutritive solution, modified according to Silveira *et al.* (1998) and free of nitrogen. For the inoculations, 1.0 ml of culture medium containing BR 3267 (10^8 CFU/ml) or PGPB (10^7 CFU/ml) was added. Uninoculated plants were used as an absolute control (AC). After 15 days, the cowpeas were harvested, and root length (RL) and root thickness (RT) were measured. To evaluate the efficiency of bacterial colonization of cowpea, root fragments (approximately 1–2 cm long) were fixed in modified Karnovksy solution and analyzed using scanning electron microscopy (SEM). The experimental design was randomized blocks with 24 treatments, one BR 3267, 22 PGPB and one AC, with three replicates. Data were then subjected to analysis of variance (ANOVA) using the statistical program ASSISTAT version 7.6 beta with 5% significance levels by F test, and the means were compared using Tukey's test ($p<0.05$).

Co-inoculation of *Bradyrhizobium* sp. and PGPB in cowpea

To evaluate the compatibility and efficiency of the interrelation of BR 3267 and PGPB, seeds of cowpea cv. "IPA 206" were disinfected (Hungria and Araújo, 1994), seeded and either inoculated with 1.0 mL of culture medium containing BR 3267 (10^8 CFU/ml) or co-inoculated with 1.0 mL of culture medium containing PGPB (10^7 CFU/ml) and 1.0 mL of culture medium containing BR 3267 in Leonard jars containing washed (pH 6.5) and autoclaved (120°C, 101 KPa, 1 h) sand as substrate. After thinning the cowpeas at seven days, two plants were kept in each Leonard jar. The experiment was conducted in a greenhouse at the Agronomical Institute of Pernambuco (IPA) that was maintained at 27–36°C and 50–70% relative humidity. During the experimental period, plants were irrigated by capillary action with Hoagland and Arnon (1950) nutritive solution modified according to Silveira *et al.* (1998) and free of nitrogen. Uninoculated plants were used as an absolute control (AC). Cowpeas were harvested 36 days after sowing, at which time the following variables were evaluated: root length, nodule number, shoot dry matter (SDM), root dry matter (RDM), nodule dry matter, nodule size, nitrogen accumulated in the SDM, SDM/RDM ratio, absolute growth rate, nitrogen content in the SDM, nitrogen fixation efficiency and specific nodulation (Bremner, 1965; Gulden and Vessey, 1998; Benincasa, 2003).

Statistical design and analysis

The experimental design included randomized blocks with 24 different treatments: one BR 3267, 22 combinations between BR 3267 and PGPB and one AC, with three replicates. Each variable studied was subjected to analysis of variance (ANOVA) using the statistical program ASSISTAT version 7.6 beta at 5% significance levels by F test, and means were compared using Tukey's test ($p<0.05$).

Results and Discussion

The effects of bacterial colonization on cowpea survival

Bacterial colonization in the roots of plants is an extremely complex process modulated by numerous biotic and abiotic factors, such as root exudation, humidity and luminosity (Compant *et al.*, 2010). To examine the ability of bacteria to colonize the roots of cowpeas, roots were inoculated with various bacterial species (as described in the 'Materials and Methods'), and the root fragments were subjected to scanning electron microscopy (Fig. 1). All of the strains tested were able to colonize cowpea roots; however, plants inoculated with *Brevibacillus brevis* (447) showed very low levels of colonization (Fig. 1B).

Although the bacterial species tested effectively colonized cowpea roots, the effect of these species on cowpea root growth and thickness had not yet been examined. It has been shown that PGPB can promote nutrient mineralization and hormone production (including auxins and gib-

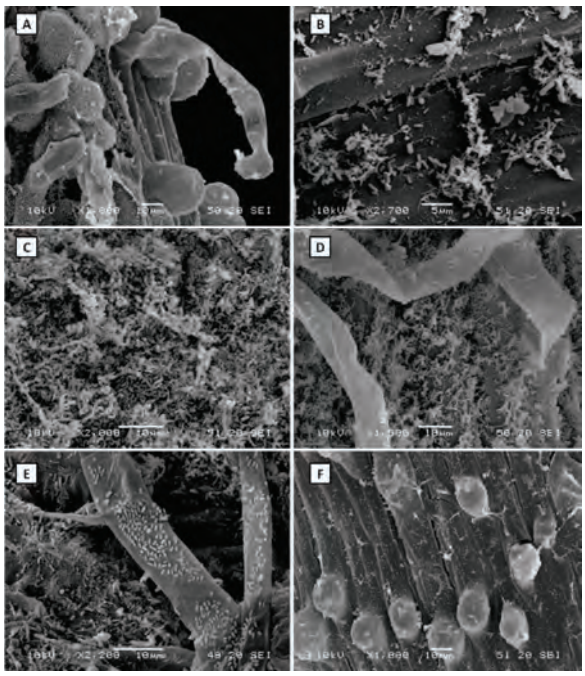

Fig. 1. Images of the root fragments of the cowpea submitted to inoculation with *Bradyrhizobium* sp. and PGPB compared to absolute control obtained by scanning electron microscopy. (A) *Bradyrhizobium* sp. (BR 3267), (B) *Brevibacillus brevis* (447), (C) *Paenibacillus polymyxa* (LMD 24.16), (D) *Bacillus pumilus* (445), (E) *Paenibacillus polymyxa* (Loutit), (F) Absolute control (AC).

Table 1. Root length (RL) and root thickness (RT) of the cowpea plants inoculated with *Bradyrhizobium* sp. or plant growth-promoting bacteria (PGPB)

Treatments		RL (cm)	RT (mm)
Bradyrhizobium sp.	BR 3267	23.50 a	2.51 ab
Bacillus sp.	ANBE 31	5.00 b	2.37 ab
B. aubitilis	441	7.66 ab	2.47 ab
B. cereus	440	11.00 ab	2.72 ab
B. pumilus	444	14.00 ab	2.16 ab
B. pumilus	445	15.33 ab	2.50 ab
B. pumilus	448	18.50 ab	2.68 ab
Brevibacillus brevis	447	17.16 ab	2.51 ab
B. megaterium	462	17.16 ab	2.55 ab
B. subtilis	455	16.00 ab	2.37 ab
B. subtilis	458	13.66 ab	2.40 ab
Paenibacillus brasilensis	24	11.50 ab	2.79 a
P. durus	CRIP 105	18.33 ab	2.33 ab
P. durus	V 22.32	17.50 ab	2.47 ab
P. durus	CRIL 156	15.66 ab	2.39 ab
P. durus	C 04.50	9.66 ab	2.55 ab
P. graminis	MC 22.13	10.16 ab	2.30 ab
P. graminis	MC 04.21	18.66 ab	2.24 ab
P. kribbensis	POC 115	23.26 a	2.64 ab
P. macerans	LMD 24.10	8.16 ab	2.58 ab
P. polymyxa	LMD 24.16	9.33 ab	2.05 b
P. polymyxa	PM 04.01	11.50 ab	2.52 ab
P. polymyxa	Loutit	11.00 ab	2.39 ab
Absolute control	AC	21.16 ab	2.39 ab
CV[a] (%)	-	18.59	8.79

In each column, means (three replicates) followed by same letter do not differ statistically from each other at $p<0.05$ according to the Tukey' test.
[a] Coefficient of variation.

berellins), effectively augmenting plant growth (Dobbelaere *et al*., 2003; Spaepen *et al*., 2009). In the present study significant differences in growth were observed in plants that had been colonized by different bacterial strains (Table 1). In particular, the colonization of cowpea plants with BR 3267 or *Paenibacillus kribbensis* (POC 115) resulted in greater root length (RL) compared to other treatments.

The greater RL observed in cowpea plants colonized with BR 3267 may be a response to an increase in nutrient availability resulting from greater nodulation. Plants that acquire nodules on their roots in response to nitrogen-fixing bacteria have greater influxes of nitrogen compounds due to the nitrogen fixation that occurs in bacteroids (Moreira and Siqueira, 2006; Zilli *et al*., 2009). During nitrogen fixation, atmospheric nitrogen is converted into ammonia, which is exported to the plant to be used in several metabolic reactions that can lead to increased plant growth (Figueiredo *et al*., 2010; Chianu *et al*., 2011).

This study demonstrates that plant growth can be augmented by inoculation either with diazotrophic bacteria, as observed during *Bradyrhizobium* sp./cowpea association, or with certain PGPB, as observed for cowpeas that had been inoculated with *P. kribbensis* (POC 115), which increased RL compared to treatments with other PGPB (Table 1). POC 115 was previously isolated from the rhizosphere of maize sown in Cerrado soil in Brazil (Cotta *et al*., 2011) and was described by von der Weid *et al*. (2000). POC 115 produces an antimicrobial substance related to the iturin family of compounds, which increase cell membrane permeability in roots (Maget-Dana and Peypoux, 1994) and can promote plant growth due to the resulting increase in the nutrient absorption rate. In addition, species of the *Paenibacillus* genus secrete extracellular compounds into the rhizosphere, such as amino acids and secondary metabolites, which can result in a favorable environment for plant growth (Yoon *et al*., 2003).

Plants inoculated with the ANBE 31 strain of *Bacillus* demonstrated a shorter RL compared to plants colonized with other strains (Table 1). This finding may indicate that the interaction between this strain and cowpea plants had little effect on cowpea roots compared to other strains. Indeed, interactions in the plant rhizosphere play an important role in the transformation, mobilization, solubilization and uptake of nutrients by growing plants (Dey *et al*., 2004). Because there was apparently less interaction between this strain and cowpea plants, nutrient mobilization may be committed, leading to the restriction in root growth observed in these plants.

Thicker roots and the presence of mucilaginous substances, which act as lubricants, on the root surface can each result in an enhanced ability to acquire nutrients and water from the rhizosphere, effectively promoting root growth (Bengough *et al*., 2006). In this study, plants inoculated with different bacterial strains demonstrated significant changes in root thickness (Table 1). Plants inoculated with *P. brasilensis* (24) exhibited greater root thickness, whereas those inoculated with *P. polymyxa* strain LMD 24.16 showed minor increases in root thickness compared to plants inoculated with other strains (Table 1).

The 27% reduction observed in the root thickness of plants inoculated with *P. polymyxa* (LMD 24.16) compared to plants inoculated with *P. brasilensis* (24) may represent a smaller response by plant to the compounds synthesized by the microorganisms present in the rhizosphere. The PGPB can colonize internal and external plant organs, inducing beneficial or harmful effects on plant growth by synthesizing phytohormones in the root zone, which can impair root development in high concentrations (Spaepen *et al*., 2009).

Co-inoculation *Bradyrhizobium* sp. and PGPB in cowpea

The synergistic responses of the plant-rhizobia-PGPB association can vary considerably depending on numerous factors, such as bacterial strains, plant species, inoculum density and environmental conditions (Moreira and Siqueira, 2006). In the present study, cowpea plants were co-inoculated with BR 3267 and different PGPB to identify possible synergistic responses among these microorganisms. Therefore, the absolute growth rate (AGR), the root length (CR), shoot (SDM) and root (RDM) dry matter and the SDM/RDM ratio were evaluated in cowpea plants treated with different combinations of bacteria (Table 2). This growth analysis enabled the growth of each plant as a whole and the contribution of different organs to overall plant growth to be evaluated (Benincasa, 2003).

The growth rate of plants is a genetically controlled attribute, and the determinant factors that act during the early phases of plant growth affect only the exponential phase of growth and thereafter become less effective when the plant enters

Table 2. **Absolute growth rate (AGT), root length (RL), shoots (SDM) and roots (RDM) dry matter and SDM/RDM ratio in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with BR 3267 and plant growth-promoting bacteria (PGPB)**

Treatments	AGT (cm/day)	RL (cm)	SDM (g/jar)	RDM (g/jar)	SDM/RDM
Bradyrhizobium sp. (BR 3267)	3.67 ab	13.40 ab	4.15 abc	0.89 abc	4.85 a
BR 3267 + *Bacillus* sp. (ANBE 31)	2.28 ab	14.56 ab	2.91 c	1.06 abc	2.92 ab
BR 3267 + *B. cereus* (440)	3.38 ab	13.50 ab	4.33 ab	1.48 abc	3.32 ab
BR 3267 + *B. aubitilis* (441)	2.77 ab	15.30 ab	3.38 bc	1.02 abc	3.54 ab
BR 3267 + *B. pumilus* (444)	4.68 a	14.23 ab	3.52 abc	0.70 bc	5.02 a
BR 3267 + *B. pumilus* (445)	2.24 ab	12.96 ab	4.01 abc	1.30 abc	3.53 ab
BR 3267 + *B. pumilus* (448)	2.12 ab	16.63 ab	4.02 abc	1.69 ab	2.41 ab
BR 3267 + *Brevibacillus brevis* (447)	3.16 ab	13.70 ab	4.31 ab	1.09 abc	4.15 a
BR 3267 + *B. megaterium* (462)	2.48 ab	13.56 ab	4.04 abc	0.90 abc	4.61 a
BR 3267 + *B. subtilis* (455)	4.56 a	13.66 ab	4.18 abc	0.93 abc	4.45 a
BR 3267 + *B. subtilis* (458)	2.59 ab	14.23 ab	3.57 abc	1.11 abc	3.73 ab
BR 3267 + *Paenibacillus brasilensis* (24)	3.88 a	16.63 ab	4.09 abc	1.07 abc	3.85 a
BR 3267 + *P. durus* (CRIP 105)	2.79 ab	14.80 ab	4.10 abc	1.03 abc	4.31 a
BR 3267 + *P. durus* (V 22.32)	3.88 a	13.70 ab	4.28 abc	1.27 abc	3.78 a
BR 3267 + *P. durus* (CRIL 156)	3.25 ab	13.26 ab	3.84 abc	1.06 abc	3.65 ab
BR 3267 + *P. durus* (C 04.50)	4.84 a	14.23 ab	4.41 ab	1.19 abc	3.73 ab
BR 3267 + *P. graminis* (MC 22.13)	3.19 ab	13.73 ab	3.57 abc	0.98 abc	4.02 a
BR 3267 + *P. graminis* (MC 04.21)	3.50 ab	13.83 ab	4.86 a	1.20 abc	4.15 a
BR 3267 + *P. kribbensis* (POC 115)	3.89 a	13.10 ab	4.37 ab	1.18 abc	3.78 a
BR 3267 + *P. macerans* (LMD 24.10)	2.97 ab	13.16 ab	4.68 ab	1.91 a	2.44 ab
BR 3267 + *P. polymyxa* (LMD 24.16)	3.52 ab	12.53 b	3.77 abc	1.08 abc	3.60 ab
BR 3267 + *P. polymyxa* (PM 04.01)	3.56 ab	15.66 ab	4.68 ab	1.54 abc	3.91 a
BR 3267 + *P. polymyxa* (Loutit)	3.00 ab	13.23 ab	4.20 abc	1.34 abc	3.14 ab
Absolute control (AC)	0.71 b	18.40 a	0.29 d	0.40 c	0.73 b
CV[a] (%)	31.17	12.19	11.25	32.02	26.19

In each column, means (three replicates) followed by same letter do not differ statistically from each other at $p<0.05$ according to the Tukey' test.
[a] Coefficient of variation.

the establishment phase (Benincasa, 2003). In this study, plants inoculated with BR 3267 and those co-inoculated with BR 3267 + PGPB demonstrated no significant differences in growth rate (Tukey's test at $p<0.05$), except for the AC and BR 3267 + *P. durus* (C 04.50); BR 3267 + *B. pumilus* (455); BR 3267 + *B. pumilus* (444); BR 3267 + *P. durus* (V 22.32); BR 3267 + *P. kribbensis* (POC 115) and BR 3267 + *P. brasilensis* (24) treatments. Plants co-inoculated with BR 3267 + C 04.50 showed a 5-fold increase and a 32% increase in AGR compared to AC plants and those inoculated only with BR 3267, respectively. These results reinforce previous suggestions that co-inoculation is not always effective at augmenting plant growth and that this inefficiency can result from an increased production of phytohormones by PGPB that are released into the plant root surface and lead to inhibition and/or delay of plant growth (Compant *et al.*, 2010).

Root development may indirectly contribute to the effective nodulation of roots and favor BNF (Vessey and Buss, 2002). Therefore, in this study, cowpea plants were analyzed for root length (RL) in response to different co-inoculations (Table 2). AC plants and plants co-inoculated with the symbiotic pairs BR 3267 + *P. brasilensis* (24) and BR 3267 + *B. pumilus* (448) exhibited greater RL in comparison to other treatments (Table 2). The greater root growth that was observed in AC plants may be due to nutrient scavenging by the plants, as these plants were grown in nitrogen-free conditions. According to Krapp *et al.* (2011), plants can stimulate root growth and alter root architecture with a concomitant reduction in shoot growth under conditions of nitrogen deprivation in an attempt acquire this nutrient.

PGPB can augment plant growth by inducing nutrient mineralization, making these nutrients available to the plant for their metabolic activities (Lugtenberg and Kamilova, 2009). Furthermore, PGPB strains can undergo asymbiotic nitrogen fixation, allowing nitrogen to be available to the plant and be used in metabolic reactions that induce plant growth or lead to variable responses, depending on how effectively the PGPB associates with the plant species (Spaepen *et al.*, 2009). This variability in plant growth was observed when comparing plants co-inoculated with either *P. brasilensis* (24) or *P. polymyxa* (LMD 24.16), which induced greater and smaller RLs, respectively (Table 2). This variability in responses suggests that differences in the specificity of each strain and the intrinsic characteristics of each strain alter the effectiveness of the bacteria-plant interaction. These differences may be influenced by various characteristics, such as the presence of organic acids and plant hormones synthesized by these bacteria or even by root exudates synthesized by the plants that have negative effects on the bacteria (Dobbelaere *et al.*, 2003; Compant *et al.*, 2010).

PGPB colonize different plant organs and exert various beneficial effects on these various organs, such as increased seed germination and the development of roots and leaves (Dey *et al.*, 2004; Spaepen *et al.*, 2009). In this study, cowpea plants showed significant differences in SDM ($p<0.05$) among treatments compared to the AC. The co-inoculation of cow-

Table 3. Nodule number (NN), nodule size (NZ), nodule dry matter (NDM) and specific nodulation (SN) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with BR 3267 and plant growth-promoting bacteria (PGPB)

Treatments	NN (jar^{-1})	NZ (mg/nodule)	NDM (g/jar)	SN (NN/g RDM)
Bradyrhizobium sp. (BR 3267)	103 a	0.0045 a	0.46 a	122.64 a
BR 3267 + *Bacillus* sp. (ANBE 31)	101 a	0.0037 a	0.37 a	98.29 a
BR 3267 + *B. cereus* (440)	135 a	0.0043 a	0.59 a	105.44 a
BR 3267 + *B. aubitilis* (441)	135 a	0.0031 a	0.42 a	151.00 a
BR 3267 + *B. pumilus* (444)	119 a	0.0040 a	0.45 a	166.51 a
BR 3267 + *B. pumilus* (445)	137 a	0.0040 a	0.55 a	120.57 a
BR 3267 + *B. pumilus* (448)	105 a	0.0046 a	0.47 a	62. 39 a
BR 3267 + *Brevibacillus brevis* (447)	115 a	0.0039 a	0.45 a	111.13 a
BR 3267 + *B. megaterium* (462)	132 a	0.0039 a	0.52 a	148.63 a
BR 3267 + *B. subtilis* (455)	120 a	0.0043 a	0.52 a	129.53 a
BR 3267 + *B. subtilis* (458)	89 a	0.0052 a	0.44 a	100.09 a
BR 3267 + *Paenibacillus brasilensis* (24)	120 a	0.0041 a	0.48 a	113.23 a
BR 3267 + *P. durus* (CRIP 105)	116 a	0.0047 a	0.53 a	115.27 a
BR 3267 + *P. durus* (V 22.32)	106 a	0.0052 a	0.55 a	92.03 a
BR 3267 + *P. durus* (CRIL 156)	114 a	0.0045 a	0.51 a	110.73 a
BR 3267 + *P. durus* (C 04.50)	143 a	0.0038 a	0.54 a	121.46 a
BR 3267 + *P. graminis* (MC 22.13)	134 a	0.0035 a	0.45 a	156.42 a
BR 3267 + *P. graminis* (MC 04.21)	134 a	0.0041 a	0.54 a	118.24 a
BR 3267 + *P. kribbensis* (POC 115)	112 a	0.0050 a	0.56 a	95.73 a
BR 3267 + *P. macerans* (LMD 24.10)	121 a	0.0047 a	0.55 a	63.59 a
BR 3267 + *P. polymyxa* (LMD 24.16)	143 a	0.0034 a	0.47 a	131.37 a
BR 3267 + *P. polymyxa* (PM 04.01)	114 a	0.0051 a	0.54 a	102.95 a
BR 3267 + *P. polymyxa* (Loutit)	127 a	0.0043 a	0.54 a	94.09 a
CV[a] (%)	18.59	23.26	16.66	34.30

In each column, means (three replicates) followed by same letter do not differ statistically from each other at $p<0.05$ according to the Tukey' test.
[a] Coefficient of variation.

pea plants with the symbiotic pair BR 3267 + *P. graminis* (MC 04.21) resulted in greater amounts of SDM (Table 2). Additionally, the presence of PGPB influences plants to produce more biomass in the shoot, and this response can vary depending on the plant species and bacterial strain used (Araújo, 2008). This response also allows for the proper maintenance of photosynthesis, which produces carbon skeletons that can be used in BNF (Antolín *et al*., 2010).

The promotion of root growth is generally considered to be a beneficial feature for improving water uptake by plants (Reynolds and Tuberosa, 2008). The induction of this process by the presence of PGPB in roots is particularly important in field conditions because root growth results in effective maintenance of water retention, growth and plant productivity (Belimov *et al*., 2009). As shown in Table 2, cowpea plants co-inoculated with BR 3267 + *P. macerans* (LMD 10.24) exhibited greater amounts of RDM, although this difference was not significant ($p<0.05$). Furthermore, plants co-inoculated with BR 3267 + *B. pumilus* (444) showed higher SDM/RDM ratios, although this difference was also not statistically significant ($p<0.05$) according to Tukey's test.

Bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, which form part of the rhizobia group, can induce the formation of nodules in several legume species (Velázquez *et al*., 2010). Cell-cell contact between the rhizobia and the host plant is an important prerequisite for the formation of nodules, which are hypertrophic structures located in the roots, where rhizobia fix N_2 and convert it into ammonia (Zilli *et al*., 2009; Figueiredo *et al*., 2010). In the present study, inoculated and co-inoculated cowpea plants not showed significant differences ($p<0.05$) in the number, size and weight of dry matter in cowpea nodules (Table 3).

The efficiency of nitrogen metabolism resulting from BNF in bacteroids can be evaluated in terms of nitrogen content (N content), specific nodulation (SN), nitrogen accumulated (N_{ac}) and nitrogen fixation efficiency (N_2FE). Cowpea plants inoculated with BR 3267 and with BR 3267 + *P. graminis* (MC 04.21) showed higher N_{ac}, while AC plants exhibited the lowest N_{ac} in comparison to other plants (Fig. 2). It is possible that the association between plant-*Bradyrhizobium* sp. and PGPB induces an overall shift in the flow of fixed nitrogen in bacteroids for the synthesis of nitrogenous compounds such as proteins, which are produced in response to PGPB and remain in the nodules rather than being translocated into other plant tissues.

The symbiotic association of cowpea + BR 3267 + MC 04.21 displayed an N_{ac} similar to that observed for BR 3267; this finding may indicate that the flow of nitrogen from rhizobia to the plant was maintained or that BNF in the presence of PGPB was stimulated, ensuring additional fixed nitrogen to adequately supply the metabolism of the plant and rhizobia. The N_2FE in cowpea plants was not significantly different ($p<0.05$) between plants inoculated with BR 3267 and co-inoculated plants (Fig. 3), except for plants co-inoculated with BR 3267 + *P. macerans* (LMD 10.24), BR 3267 + *B. megaterium* (462), BR 3267 + *B. pumilus* (445) and BR 3267 + *Bacillus* sp. (ANBE 31). SN did not differ significantly according to Tukey's test ($p<0.05$) among any of the groups (Table 3).

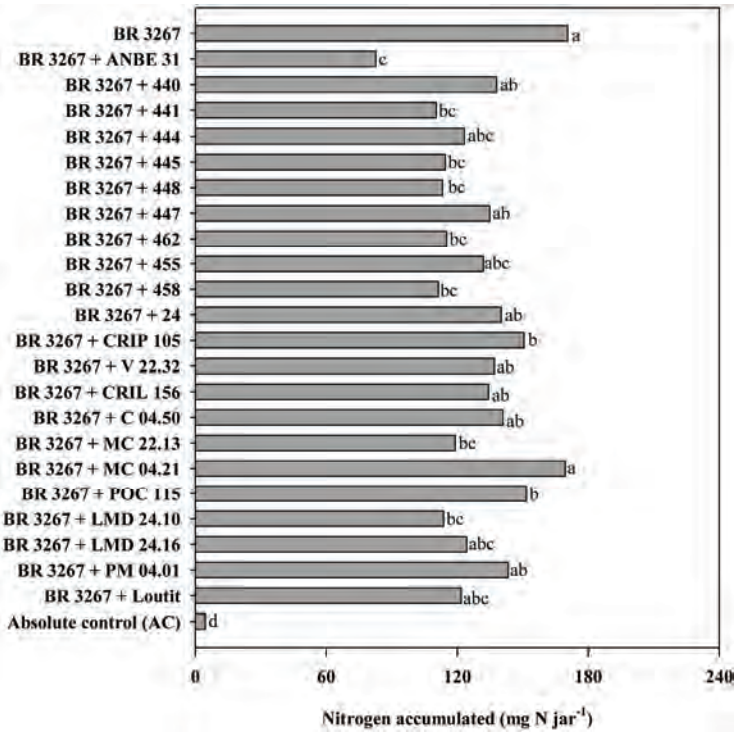

Fig. 2. Nitrogen accumulated (N_{ac}; CV*=12.54%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) isolated or co-inoculated with PGPB [*Bacillus* sp. (ANBE 31); *B. cereus* (440); *B. aubitilis* (441); *B. pumilus* (444, 445, 448); *Brevibacillus brevis* (447); *B. megaterium* (462); *B. subtilis* (455, 458); *Paenibacillus brasilensis* (24); *P. durus* (CRIP 105, V 22.32, CRIL 156, C 04.50); *P. graminis* (MC 22.13, MC 04.21); *P. kribbensis* (POC 115); *P. macerans* (LMD 24.10); *P. polymyxa* (LMD 24.16, PM 04.01, Loutit)]. Means (three replicates) followed by same letter do not differ statistically from each other at p<0.05 according to the Tukey' test. *Coefficient of variation.

Nitrogen, which is a limiting factor for growth and primary production in various plant species, is present in minute concentrations in most terrestrial ecosystems in its biologically available form (Robertson *et al.*, 2009). To minimize nitrogen limitation, some plant species can form symbiotic associations with nitrogen-fixing bacteria, which can effectively convert atmospheric nitrogen into ammonia in the bacteroids (Figueiredo *et al.*, 2010) and release it to the plants in exchange for carbon skeletons (Lugtenberg and Kamilova, 2009). Therefore, the nodulation process in root plants induced by rhizobia represents an efficient method for nitrogen acquisition and an alternative to the use of nitrogen-based fertilizers.

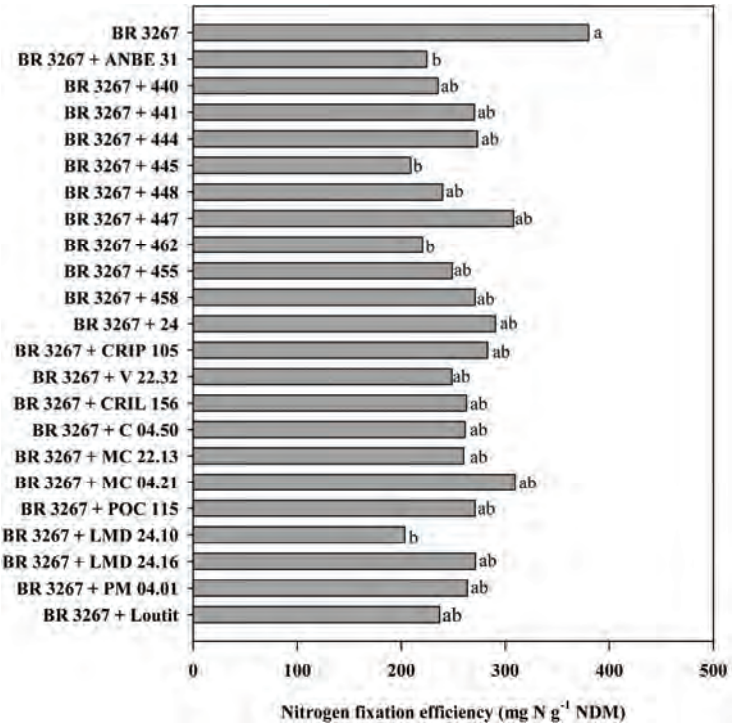

Fig. 3. Nitrogen fixation efficiency (N_2FE; CV* = 18.28%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) isolated or co-inoculated with PGPB [*Bacillus* sp. (ANBE 31); *B. cereus* (440); *B. aubitilis* (441); *B. pumilus* (444, 445, 448); *Brevibacillus brevis* (447); *B. megaterium* (462); *B. subtilis* (455, 458); *Paenibacillus brasilensis* (24); *P. durus* (CRIP 105, V 22.32, CRIL 156, C 04.50); *P. graminis* (MC 22.13, MC 04.21); *P. kribbensis* (POC 115); *P. macerans* (LMD 24.10); *P. polymyxa* (LMD 24.16, PM 04.01, Loutit)]. Means (three replicates) followed by same letter do not differ statistically from each other at p<0.05 according to the Tukey' test. *Coefficient of variation.

Conclusions

Scanning electron microscopy analysis demonstrated satisfactory bacterial colonization in inoculated cowpea roots. Additionally, synergism was observed among *Bradyrhizobium* sp. (BR 3267) and PGPB (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) that promoted plant growth and symbiotic performance in cowpeas, particularly in those plants co-inoculated with the symbiotic pairs BR 3267 + *P. graminis* (MC 04.21) and BR 3267 + *P. durus* (C 04.50).

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