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The occurrence of rhizobacteria from *Paspalum* genotypes and their effects on plant growth

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ABSTRACT: This work aimed to isolate and characterize plant growth promoting rhizobacteria (PGPR) from 10 *Paspalum* genotypes and evaluate the effect of their inoculation on *P. regnellii*, *P. atratum*, and *P. malacophyllum* genotypes. The bacterial population ranged from undetectable to 10^7 bacterial cells per gram of fresh matter in the *Paspalum* genotypes. Initially, we isolated 164 bacteria from rhizospheric soil and roots of the *Paspalum* genotypes using media N-free LG agar plate, semi-solid NFb, and LGI. The isolates were characterized genetically and physiologically. The sequencing of 16S rRNA showed the presence of many genera, and some are new in association with *Paspalum*. The most common was *Bacillus* followed by *Rhizobium*, *Paraburkholderia*, *Enterobacter*, *Cupriavidus*, *Pseudomonas*, *Dyadobacter* and *Acinetobacter*. Thirty-eight per cent of isolates produced siderophores, 25 % produced solubilized phosphate, and only 9 % produced indolic compounds. Three greenhouse experiments were performed in randomized blocks with six replicates using representative bacterial strains isolated from *P. regnellii*, *P. malacophyllum* and *P. atratum* cv. Pojuca. We also included strain Sp245 (*Azospirillum baldaniorum*), uninoculated control, and nitrogen control (150 kg N ha⁻¹). There was an increase of up to 53 % in shoot dry matter in *P. regnellii* inoculated with strain Sp245 and the shoots accumulated more N. In contrast, only small effects were observed for the other *Paspalum* genotypes inoculated with PGPR from the host genotypes. This study shows a high diversity of diazotrophic rhizosphere bacteria and suggests no strain specificity between the bacterial isolates and the *Paspalum* genotypes.

Keywords: PGPR, biofertilizer, tropical grasslands

Introduction

Brazil has the largest commercial cattle herd in the world, mainly pasture-based. There are about 160 million ha of pastureland in Brazil cultivated with 80 % of *Brachiaria* (syn. *Urochloa*) species, which might diminish genetic resistance to pests and diseases (USDA, 2020). On the other hand, the genus *Paspalum* is native to the Americas and is considered an alternative for diversification in Brazilian cultivated pastures (Manzur et al., 2020; Marques et al., 2020).

Soil bacteria benefit soil properties and plants and can be an alternative N source in tropical grasslands. Many bacterial genera associated to various managed grassland ecosystems are classified as diazotrophic bacteria, belonging to genera *Azospirillum*, *Azotobacter*, *Rhizobium*, *Bacillus*, *Pseudomonas*, and others (Antunes et al., 2019; Döbereiner, 1966; Ribeiro et al., 2020; Souza et al., 2017).

Plant Growth Promoting Rhizobacteria (PGPR) fix N, enhance production of different enzymes that stimulate plant development, aid solubilization of nutrients, and increase production of different phytohormones and other metabolites, such as siderophores, which are antagonistic to pathogens (Backer et al., 2018; Ribeiro et al., 2020).

However, few studies have investigated the PGPR occurrence associated to *Paspalum* genotypes and their potential contribution as biofertilizer to grass pastures

(Baldani and Baldani, 2005; Döbereiner, 1966; Prabha et al., 2019). This study, therefore, aimed to isolate and characterize rhizobacteria from 10 *Paspalum* genotypes and evaluate the effect of PGPR inoculation on *P. regnellii*, *P. atratum*, and *P. malacophyllum* genotypes.

Materials and Methods

Counts of culturable diazotrophic rhizobacterial population

The study analyzed 10 *Paspalum* genotypes: *Paspalum notatum* BGP 318, *Paspalum notatum* BGP 155, *Paspalum modestum* BGP 032, *Paspalum regnelliii* BGP 215, *Paspalum guenoarum* BGP 011, *Paspalum regnelliii* BGP 344, *Paspalum virgatum* BGP 343, *Paspalum plicatulum*. BGP 164, *Paspalum malacophyllum* BGP 293, and *P. atratum* cv. Pojuca BGP 098, which were collected from the Germplasm Active Bank-GAB (21°57′ S., 47°56′ W., altitude of 856 m), São Carlos, São Paulo (Brazil). The GAB was kept in a Typical Red Clay Latosol and fertilized annually with N, P, K, and S.

The plants containing rhizospheric soil were collected in polyethylene bags and transported to Seropédica, Rio de Janeiro, Brazil. The plants were conditioned in pots containing 5 kg of sterilized sand and vermiculite (1:1) and kept in a greenhouse. The aerial part was cut and after 15 days, a nutrient solution was added

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with 30 mL of KH_2PO_4 (35 g L^{-1}), 5 mL of $CaCl_2.2H_2O$ (150 g L^{-1}), and 5 mL of $MnSO_4.H_2O$ (5 g L^{-1}). The plants were kept in the solution until the bacterial isolating process, which occurred within two weeks.

The plants were collected and the rhizospheric soil was removed manually from air-dried roots. The roots were washed three times in sterile distilled water before processing. The rhizospheric soil and root samples (5 g) were homogenized with 45 mL of saline solution and then macerated with a pestle and a mortar. The samples were serially diluted up to 10^{-6} for root and rhizospheric soil. Then, an aliquot of 0.1 mL of dilutions 10^{-4} to 10^{-6} was used to inoculate, in triplicate, Petri dishes containing 25 mL of semi-selective solid LG medium for *Azotobacter* spp. Moreover, diluted samples (0.1 mL) were inoculated in flasks containing 5 mL of media of semi-selective semisolid NFb (0.5 % malic acid) and LGI (0.5 % sucrose) in order to count the diazotrophic bacteria population of genera *Azospirillum* and *Nitrospirillum*, respectively (Baldani et al., 2014).

The flasks inoculated with penicillin were incubated at 30 °C for five days and the pellicle formation on the medium subsurface was observed, characteristic of associative diazotrophic bacteria. After three successive culturing in the semisolid media, the pellicles were streaked out on solid agar plate media (NFb and LGI) added with 40 mg L^{-1} of yeast extract and incubated at 30 °C for five days. Colonies were inoculated into a fresh semisolid medium to check again the formation of the subsurface pellicle, followed by purification in a medium of Batata-Dextrose Agar (BDA) (Baldani et al., 2014).

The diazotrophic bacteria were quantified using the Most Probable Number (MPN) technique applying the McCrady table (Baldani et al., 2014) and by counting the colony-forming units (CFU).

Molecular characterization

We selected 74 bacterial isolates based on morpho-cultural characteristics and used for molecular characterization. Genomic DNA extraction was performed using the Genomic DNA purification Kit according to specifications of the manufacturer. The polymerase chain reaction (PCR) technique was used for amplification of the conserved 16S rRNA gene sequence. The PCR mix reaction with a final volume of 50 µL consisted of 50 ng of DNA template, 10X Taq DNA polymerase reaction buffer (10 mmol L^{-1} of Tris-HCl pH 9 and 50 mmol L^{-1} of KCl), 1.5 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} dNTP with equal concentrations of dATP, dTTP, dCTP and dGTP, 2 U of Taq DNA polymerase and 25 pmoL of each primer AMP–F [5'- GAG AGT TTG ATY CTG GCT CAG -3 '] and AMP-R [5'- AAG GAG GTG ATC CAR CCG CA - 3 '] (Wang et al., 1996). The amplification reaction was carried out in the PTC 100 thermocycler (MJ Research) of initial denaturation at 95 °C for 5 min, followed by 34 cycles of denaturation

at 94 °C for 15 sec, annealing at a temperature of 60 °C for 45 sec with an extension at 72 °C for 2 min, and a final extension at 72 °C for 30 min. At the end of the reaction, the products were analyzed by electrophoresis in agarose gel (1 %).

For the identification and phylogenetic analysis, PCR products of each isolate were purified using the DNA purification kit and then sequenced. The sequences of these 74 strains were submitted to the Genbank database.

The phylogenetic tree with 16 S rRNA ($\sim$1300 bp) was built using the Neighbor-Joining method. The bootstrap method with 1000 repetitions was used to evaluate the topology of the sequences (Felsenstein, 1985). The evolutionary distance was computed using the Tamura-3-parameter method and the phylogenetic analysis was carried out in the program MEGA 6 (Tamura et al., 2013).

The functional capacity of the PGPR strains

We selected 55 strains representing 10 *Paspalum* genotypes to assess the functional ability of the strains.

We used the National Botanical Research Institute's phosphate growth medium (NBRIP) agar culture medium containing $Ca_3 (PO_4)_2$ to evaluate the capacity of phosphate to solubilize the isolates, according to Nautiyal (1999). The bacteria were previously cultured in a DYGS medium for 24 h, and 10 µL (OD_{600} = 0.9-1.0) was placed onto the culture medium surface in the NBRIP plate, in triplicate. The P solubilization halo (mm) was quantified by calculating the solubilization index (SI) according to Kumar and Narula (1999) after 7 and 14 days of incubation. *G. diazotrophicus* PAL5 was used as a reference strain for inorganic P solubilization.

Isolates were also characterized by the production of indole compounds, as described by Sarwar and Kremer (1995). Bacterial culture (1 mL), previously cultured for 24 h in DYGS medium, was inoculated into 5 mL of DYGS medium supplemented with L-tryptophan (100 µg mL^{-1}) and incubated in the dark with shaking at 150 rpm at 30 °C for 48 h. Aliquots of 1 mL were removed and centrifuged at 5000 × g for 15 min. In a microplate 96-well, an aliquot of 150 µL of supernatant was mixed with 100 µL of the Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 49 ml of 35 % perchloric acid), which had been previously prepared. The quantification of indole compounds was evaluated by using a calibration curve prepared with serial dilutions of synthetic IAA standards (5-100 µg tryptophan mL^{-1}). The results were expressed in µg mL^{-1} of IAA per unit of optical density. *A. baldaniorum* Sp245 was used as reference for indolic compound production.

Siderophore production was evaluated according to Tortora et al. (2011). The isolates had been previously grown in test tubes containing 5 mL of the bacterial growth media NFB, LGI, and LG (iron-free, supplemented with 0.1 % NH_4Cl and without

bromothymol blue) and incubated for 72 h at 30 °C with constant stirring at 150 rpm. Then, four aliquots of 10 μL of the bacterial suspension (OD_{560} = 0.2) were placed onto Petri dishes containing solid LG, NFB, and LGI media without bromothymol blue, supplemented with 0.1 % NH_4Cl and Chromoazurol S (CAS) and incubated at 30 °C for 14 days. The production of siderophores was detected according to halo production. The strain ZAE94 *Herbaspirillum seropedicae* was used as positive reference.

The functional information was used to calculate the Principal Component Analysis (PCA) and showed the similarity of the isolates in growth-promoting traits. The PCA was calculated with the correlation matrix since the variables were expressed in different units and presented as arrows in the PCA plot. All analyses were performed using PAST 4.3 program.

Greenhouse experiments

We selected three *Paspalum* genotypes based on forage features and seed availability to evaluate the best plant-bacteria interaction in greenhouse conditions. Three greenhouse experiments were carried out at the same time with the *Paspalum* genotypes (*P. regnellii*, *P. atratum*, and *P. malacophylum*) at Seropédica, Rio de Janeiro, Brazil. Three trains belonging to each genotype were selected based on functional characterization. The inoculation treatments consisted of strains: PR21 (*Rhizobium* sp.), PR23 (*Bacillus* sp.), PR8 (*Acinetobacter* sp.) for *P. regnellii*; PA121 (*Bacillus* sp.), PA122 (unknown genera) and PA123 (*Bacillus* sp.) for *P. atratum* cv. Pojuca genotype; PMA112 (*Pseudomonas* sp.), PMA115 (*Bacillus* sp.) and PMO18 (*Bacillus* sp.) for *P. malacophyllum genotype*. In addition to uninoculated and nitrogen (150 kg N ha^{-1}) controls, strain Sp245 (*A. baldaniorum*) was used as a positive reference because it promotes growth in different grasses and on previous study in our laboratory. N was applied as ammonium sulfate and split into two doses at 20 (50 kg N ha^{-1}) and 32 days (100 kg N ha^{-1}). The strains had been previously grown in a DYGS medium at 30 °C, at 150 rpm for 24 h followed by mixing 15 mL of the bacterial suspension in polypropylene bags containing 35 g of sterile peat. In the experiment, we used planosol soil with the following characteristics pH 5.84, Al 0.00 cmolc dm^{-3}, Ca 1.50 cmolc dm^{-3}, H + Al 2.49 cmolc dm^{-3}, Mg 0.75 mg L^{-1}, K 33.11 mg L^{-1}, P 5.84 %, and N 0.06 %. Two fertilizations were carried out with nutrient solution (Franco and Döbereiner, 1967) at 29 and 50 DAP to restore the micronutrients and macronutrients, except for N.

The experimental design was in randomized blocks with six replicates. Ten seeds were planted per 12 kg soil box. Seed inoculation was performed at a ratio of 10 g of peat kg per seed and sugar solution (10 %) was added at the proportion of 6 mL kg for better adhesion of the peat. Peat inoculants had approximately 10^9 bacterial cells g^{-1}.

The height of the plants was measured with a millimeter ruler (a representative main tiller was selected in each box at soil level measurement up to the young leaf ligule) and the number of tillers determined at 60 DAP as well as shoot dry matter accumulated at 38, 68 and 98 DAP. The plant material was harvested on three occasions by cutting shoots at 10 cm soil height at 38 days and 68 DAP and at the end of the experiment (98 DAP), the roots were also harvested and evaluated.

Fresh shoot and root matter was dried at 65 °C for 48 h and then weighed. The shoot samples were ground in a mill and N content was analyzed according to the Kjedhal method. Crude protein in the shoots was determined by multiplying the N concentration by 6.25.

The data from genotypes were analyzed independently. Software R version 3.3.2 and the analysis of variance (ANOVA) were used to test normality and homogeneity of the data. Tukey test was used ($p < 0.05$) to compare the means.

Results and Discussion

Quantification of the culturable rhizobacterial population

The number of PGPR ranged from not-detected to 10^7 bacterial cells per gram of fresh matter among the 10 *Paspalum* genotypes. The highest bacterial values were observed in roots (semisolid LGI medium and LG agar plate medium) and in the rhizospheric soil (semisolid NFb medium). The greatest number of rhizobacteria was found in roots of *P. notatum* genotype (BGP 318); however, the largest number of bacterial isolates was detected in *P. regnellii* genotype (BGP 215) (Table 1). Ribeiro et al. (2020) observed similar results evaluating 20 genotypes of *Brachiaria* in semisolid NFb and LGI media, where a great bacterial population did not correlate with a larger number of isolates.

We successfully isolated 164 bacterial strains associated to the 10 *Paspalum* genotypes, mostly from roots (54 %), in contrast to the rhizospheric soil (46 %) (Table 1). We isolated 126 isolates from the LG agar plate medium, 17 from the semisolid NFb and 17 from the semisolid LGI media. However, only 130 isolates remained viable after storage in the appropriate medium. The PGPR population was also variable for many grass genotypes, including elephant grass (Videira et al., 2012), Brachiaria (Ribeiro et al., 2020), sugarcane (Reis Jr. et al., 2000), and Bahia grass (Dobereiner, 1966).

Functional capacity of strains

We analyzed 55 strains and, out of this total, 21 isolates showed siderophore production (SI), 14 strains solubilized inorganic phosphate (PP), and only nine strains showed the production of indole compounds (IC) (Table 2). Eleven strains failed to grow in CAS agar medium. The other strains grew in CAS agar medium;

Table 1 – Most Probable Number (MPN) and Unit Forming Colony (UFC) of diazotrophic bacteria (DB) associated to roots and rhizosphere soil of 10 *Paspalum* genotypes kept at the Germoplasm Active Bank at Embrapa Pecuária Sudeste, São Carlos, São Paulo, Brazil.

Paspalum species	Genotypes	NFb culture medium				LGI culture medium				LG culture medium				Total number of isolates in both media/genotype
		Log_{10} n° cells or UFC g fresh roots or per soil		Number of isolates		Log_{10} n° cells or UFC g fresh roots or per soil		Number of isolates		Log_{10} n° cells or UFC g fresh roots or per soil		Number of isolates		
		R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	
P. notatum	BGP 318	3.48	3.40	0	1	8.04	3.04	0	2	8.49	6.63	7	4	14
P. notatum	BGP 155	3.40	3.18	1	2	2.60	2.85	3	2	7.68	7.82	6	4	18
P. modestum	BGP 032	*-	2.60	5	2	8.40	2.95	0	0	*-	7.11	0	10	17
P. regnelliii	BGP 215	3.44	4.25	2	3	4.60	3.73	4	0	7.61	7.18	33	13	55
P. guenoarum	BGP 011	*-	*-	0	0	*-	2.60	0	2	7.83	7.24	5	7	14
P. regnelliii	BGP 344	2.60	2.95	0	0	*-	4.40	0	0	8.28	7.56	2	4	6
P. virgatum	BGP 343	*-	*-	0	0	9.15	3.40	0	1	8.24	7.14	4	8	13
P. plicatulum	BGP 164	*-	*-	0	0	5.40	*-	1	0	8.29	7.32	3	2	6
P. malacophyllum	BGP 293	2.60	3.98	0	1	5.40	5.15	0	2	7.66	6.97	3	4	10
P. atratum	BGP 098	2.95	3.40	0	0	4.60	2.95	0	0	7.55	7.62	7	4	11
Total number of isolates from each niche/culture medium				8	9			8	9			72	54	164

*- = Below the minimum detection level method; R = Roots; RS = Rhizospheric soil. Isolation was processed with 2 *Paspalum* specimens from each genotype analyzed using the three highest dilution flasks that presented positive pellicle in the semi-solid medium or colonies in Petri dishes.

Table 2 – Phosphate solubilization index (PP), Indolic compounds production (IC, µg IAA mL^{-1} OD^{-1}), and Siderophore production (SI) by rhizospheric soil (RS) and root bacterial strains isolated from ten *Paspalum* genotypes.

Strains	Genera	Genotype	Origin*	PP	IC	SA
Positive Control	-	-	-	2.84	36.62	+
PN4	Enterobacter	BRA 25020	R	Nd	16.83	Nd
PN10	Enterobacter	BRA 25020	R	Nd	9.41	Nd
PN11	Bacillus	BRA 25020	R	1.11	Nd	Nd
PN13	Rhizobium	BRA 12254	R	Nd	Nd	+
PN15	Bacillus	BRA 12254	R	Nd	Nd	+
PN18	Bacillus	BRA 12254	RS	1.45	Nd	Nd
PN21	Bacillus	BRA 12254	RS	Nd	Nd	+
PMO29	Rhizobium	BRA 6491	RS	2.27	Nd	Nd
PR4	Bacillus	BRA 19186	R	Nd	Nd	+
PR6	Bacillus	BRA 6491	RS	Nd	Nd	+
PR8	Acinetobacter	BRA 19186	R	2.11	17.78	+
PR19	Bacillus	BRA 19186	RS	1.75	Nd	+
PR21	Rhizobium	BRA 19186	RS	Nd	22.86	+
PR22	Unknown	BRA 19186	R	2.36	20.27	+
PGU79	Rhizobium	BRA 3824	R	Nd	Nd	+
PS88	Unknown	BRA 12424	RS	Nd	Nd	+
PGL96	Pseudomonas	BRA 11401	R	Nd	13.60	+
PGL101	Bacillus	BRA 11401	RS	2.24	9.26	Nd
PP105	Bacillus	BRA 12700	RS	Nd	Nd	+
PA118	Burkholderia	BRA 9610	R	1.05	2.79	+
PA121	Bacillus	BRA 9610	R	2.15	Nd	Nd
PA123	Bacillus	BRA 9610	RS	Nd	Nd	+
PMO1	Bacillus	BRA 6491	R	Nd	Nd	+
PMO31	Bacillus	BRA 6491	RS	1.78	22.9	+
PNN4	Bacillus	BRA 12254	RS	1.73	Nd	+
PR23	Bacillus	BRA 19186	RS	1.51	Nd	+
PMO18	Bacillus	BRA 6491	RS	Nd	Nd	+
PNL4	Unknown	BRA 12254	R	1.12	Nd	Nd
PNL13	Unknown	BRA 12254	RS	1.67	Nd	Nd
PNL14	Bacillus	BRA 12254	R	Nd	Nd	+

Mean values of 3 replicates. *Origin = bacterial isolates from roots (R) or rhizospheric soil (RS). Nd = Not detected; + = siderophore halo production.

nevertheless, they did not produce siderophores (no halo formation) until the 14th day of incubation. The best siderophore strain producers were PA123, PN21, PA118, PNL14, PMO31, PNN4, and PR23, most of which were isolated from rhizospheric soil and belonged to the genus *Bacillus* (Table 2).

The siderophore production was the main activity detected among the bacterial isolates from *Paspalum* genotypes. This trait could be an advantage against ineffective indigenous bacteria in rhizosphere. Siderophores are organic molecules with low molecular weight synthesized by microorganisms considered an alternative to chemical pesticides and biocontrol agents in the agriculture (Sasirekha and Srividya, 2016).

Bacterial strains isolated from roots (7) and rhizospheric soil (2) of *Paspalum* genotypes BGP 098, BGP 343, BGP 318, BGP 343, BGP 215, and BGP 032 produced IC ranged from 2.79 to 36.62 µg of IAA mL^{-1} OD^{-1}, all genotypes are closely related to genera *Paraburkholderia*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Acinetobacter*. The production of indolic compounds by PGPR agrees with reports of other authors (Cássan et al., 2013; Chaves et al., 2015; Ribeiro et al., 2020), who found values of indole production *in vitro* ranging from 10 to 50 IAA µg mL^{-1} OD^{-1} for PGPR isolated from sugarcane and Brachiaria. Exogenous indole compounds are known to play a role in root development (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996) and act during plant drought stress (Nabti et al., 2010).

Although P is present from 95 to 99 % in soils, it is unavailable to the plants, due to its fixation on soil mineral particles or precipitation by ions Al^{+3} and Fe^{+3}. Phosphate-solubilizing rhizobacteria increase solubilization of insoluble P compounds by lowering the pH through microbial production of organic acids (Anand et al., 2016; Sharma et al., 2013; Vassileva et al., 2000; Wu, 2005).

According to Madhaiyan et al. (2004), the P solubilization index (SI) can be classified as high (SI > 3), medium (2 < SI < 3), and low (SI < 2). The SI ranged from 1.11 to 2.36, which was considered medium-to-low and lower than that observed for *G. diazotrophicus* strain PAL5 (reference strain) with an SI from 2.84 to 3.07.

Pseudomonas and *Bacillus* are considered the most frequent phosphate solubilizing bacteria; however, the occurrence of other P solubilizing species has also been reported (Sharma et al., 2013).

The strains differ in terms of their functional abilities and most were capable of producing siderophore, while *Bacillus* sp. strain PGL101 and *Acinetobacter* sp. strain PR8 produced indolic compounds and solubilized phosphate. PMO31 (unknown genus), *Pseudomonas* sp. strain PGL96 and *Rhizobium* sp. strain PR21produced indolic compounds and siderophores, while *Bacillus* sp. strains PR23 and PR19 solubilized P and produced siderophores. The siderophore production was the main activity detected among the bacterial isolates from *Paspalum* genotypes, which could be an advantage against ineffective indigenous bacteria in rhizosphere. Siderophores are organic molecules with low molecular weight synthesized by microorganisms considered as an alternative to chemical pesticides and biocontrol agents in the agriculture (Sasirekha and Srividya, 2016).

Only strains PA118 (*Paraburkholderia*), isolated from roots of *P. atratum* cv. *Pojuca* (BGP 098), and PR22 (unknown genus) from roots *of P. regnellii* (BGP 215), performed better and showed capacity to solubilize phosphate (SI) and produce indolic compounds (IC) and siderophore (SA).

The PCA explained 69 % of data variability in the first two components (Figure 1). The first component explained 41 % of the variation and could be represented for SA mainly in rhizospheric soil. The second component explained 27 % of the variation and could be represented for SI and followed by IC. These *in vitro* results allowed to select strains with multifunctional abilities, pointing to possibilities to assess PGPR inoculation in *Paspalum* forage grasses.

Molecular characterization

The taxonomical analysis based on 16S rRNA sequences of 74 strains isolated from the 10 *Paspalum* genotypes showed that the strains belonged to 11 genera of the *Proteobacteria classes* (*alpha*, *beta* and *gamma*) and *Firmicutes*. Most isolates belonged to the genus *Bacillus* (*N* = 55), *Rhizobium* (*N* = 7), *Paraburkholderia* (*N* = 2), *Enterobacter* (*N* = 2), *Cupriavidus* (*N* = 1), *Pseudomonas* (*N* = 2), *Dyadobacter* (*N* = 1), *Acinetobacter* (*N* = 2), *Microbacterium* (*N* = 1), and *Azospirillum* (*N* = 1) (Figures 2A and 2B). Interestingly, the genus *Bacillus* was found in roots and rhizospheric soil of all *Paspalum* genotypes, while *Rhizobium* was found mainly in roots of *Paspalum* genotypes (BGP 293, BGP 155, BGP 215, BGP 032, and BGP 011), except for *P. atratum* BGP 098 genotype, which was found in rhizospheric soil. The *P. notatum* (BGP 318
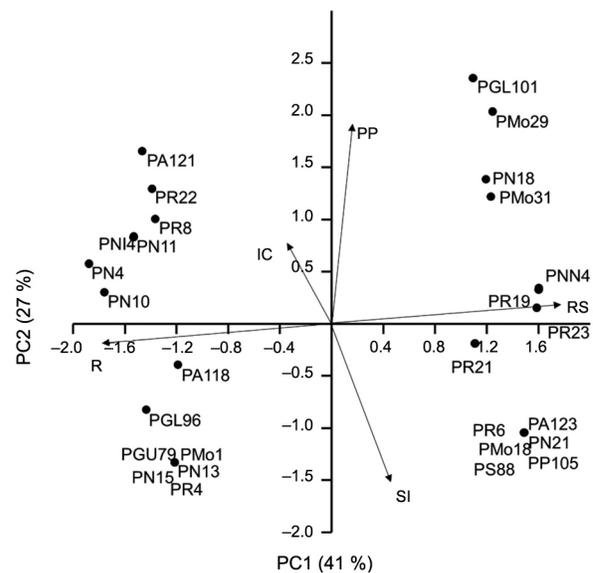

Figure 1 – Principal component analysis (PCA) of functional characterization by 28 bacterial strains from *Paspalum* genotypes. IC = Indolic compound production, PP = Phosphate solubilization index, SI = Siderophore production, RS = rhizosferic soil and R = fresh roots.
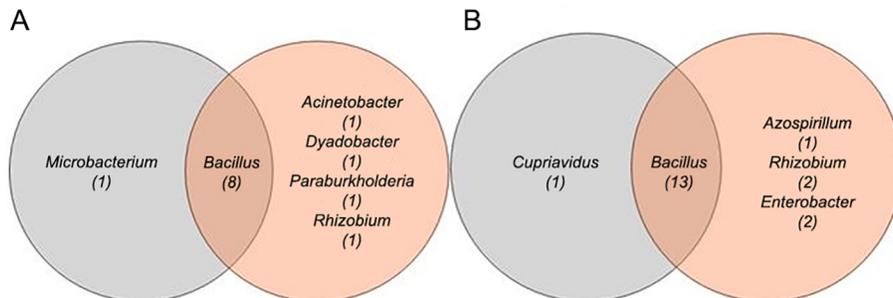

Figure 2 – Venn diagram demonstrating the number of Rhizobacteria genera isolated from rhizospheric soil (in blue) and root samples (in pink) in (A) *P. regnellii* and (B) *P. notatum*.

and BGP 155) and *P. regnellii* (BGP 215 and BGP 344) were colonized by different bacterial species, mainly in roots (Figure 3). In contrast, only strain 3LI was isolated from the soil of *P. notatum* (PGP 318) in the semi-selective NFb medium and was similar to *Azospirillum* sp.

Döbereiner (1966) detected the presence of *Azorhizophilus paspali* (syn. *Azotobacter paspali*) in 98 % and 66 % of samples of *P. notatum* cv. *batatais and P. plicatum*, respectively, but not from the other *Paspalum* genotypes. Surprisingly, we were not able to isolate this genus from any of the *Paspalum* genotypes kept in the *Paspalum* GAB; nevertheless, we were able to isolate the *A. paspali* strain PN22 of *P. notatum* cv. *Batatais* grown in Seropédica, Rio de Janeiro, Brazil (unpublished data).

Most of our isolates belonged to the *Bacillus genus*, although many other new genera were also found, which are associated to the *Paspalum* genotypes. The genus *Bacillus* represents 95 % of Gram-positive bacterial species found in the soil (Goswami et al., 2016). It was also the most representative group in our study for *Paspalum* plants. Beule et al. (2019), however, studied bacterial soil communities and cultivar-dependence in pastureland in northwestern Florida (USA) and found *Candidatus udaeobacter*, *Bradyrhizobium* spp., and *Candidatus solibacter* as the most abundant genera across *Paspalum* cultivars.

Genera *Rhizobium*, *Paraburkholderia*, *Azospirillum*, *Enterobacter*, *Cupriavidus*, *Pseudomonas*, *Microbacterium* were associated to different grasses (Baldani and Baldani, 2005; Prabha et al., 2019; Ribeiro et al., 2020), while *Dyadobacter* and *Acinetobacter* were isolated from wheat rhizosphere and stems of maize and iceberg lettuce, respectively (Chelius and Triplett, 2000; Du et al., 2016; Rooney et al., 2016).

There is no clear explanation why A*zorhizophilus*. (syn *Azotobacter*) *paspali* was not isolated or more isolates belonged to *Azospirillum* spp. or *Nitrospirillum amazonense* of the 10 *Paspalum* genotypes. One possibility could be the N level (150 kg N ha^{-1}) applied every year to ensure plant nutrition in GAB. It is known that N interferes with the bacterial population associated to grasses e.g. sugarcane, rye-grass, maize and Bahia grass (Beule et al., 2019; Castanheira et al., 2014; Souza et al., 2016; Marques et al., 2017; Van Deynze et al., 2018). Another possibility could be the genotype specificity observed by Döbereiner (1966), although no specific chemo-attractant compound was detected associated to *P.notatum* cv. *Batatais* in other studies (Döbereiner et al., 1972; Machado and Döbereiner, 1969) that could be associated to the bacterial specificity observed by Döbereiner (1966).

PGPR inoculation effects on *P. regnellii* (BGP 215), *P. atratum* (BGP 098), and *P. malacophylum* (BGP 293)

Plants of *P. regnellii* inoculated with *Azospirillum baldaniorum* strain Sp245 performed better than other inoculated plants for most variables in the sum of the
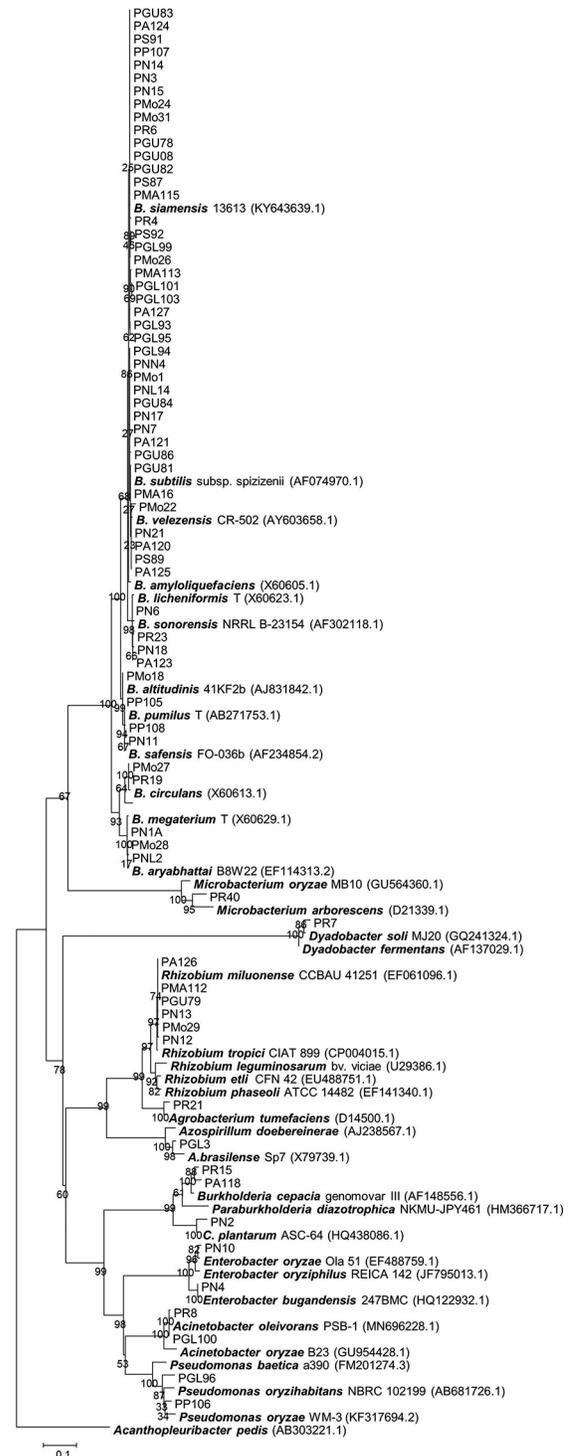

Figure 3 – Phylogenetic tree based on 16S rRNA gene sequences (~ 1450 bp), including the bacterial type strain deposited in the NCBI database. Phylogeny was based on clustering of sequences according to the Neighbor-joining algorithm and Kimura model using the MEGA6.1 program. Numbers in the branches indicate the percentage of 1000 sub-samples (bootstrap). The scale represents the number of mutations per nucleotide position. The *Acanthopleuribacter* pedis 16S rRNA gene sequence was used for tree rooting.

three cuttings (Table 3). The *A. baldoniorum* species was recently created and included the old *A. brasilense* strain Sp245 and two other strains (Vi22 and BR12001) (Ferreira et al., 2020). Inoculation of strain Sp245 promoted a Shoot Dry Matter (SDM) yield 53 % higher than the control treatment, while total N in plants inoculated with strain PR23 was 26 % higher than the control, but 36 % lower than strain Sp245 and N (120 kg N ha⁻¹) treatments. There was an increase of 58 % in total N of *P. regnellii* shoots compared to the treatment without inoculation with strain Sp245. Interestingly, *P. regnellii* plants inoculated with Sp245 presented early seed emergence (seven days after planting) and accumulated 24 % more N than the treatment with N fertilizer (120 kg N ha⁻¹) in the first harvest (Figure 4), also greater plant height and number of tillers at 60 DAP. Inoculation of strain PR21 was similar to Sp245 for root dry matter (RDM), while inoculation of strain PR23 did not differ from the control (Table 3).

The response of *P. atratum* cv. *Pojuca* to strain inoculation showed that this genotype is highly responsive to N fertilization (120 kg N ha⁻¹) in the sum of the three cuttings (Table 3). Nevertheless, promising results were observed due to the inoculation of strains PA121 and PA123 regarding the number of tillers at 60 DAP (Table 3) and in the first SDM cutting (Figure 5),

respectively. However, negative or null effects were demonstrated with inoculation of strain Sp245 for most variables.

Inoculation of strain Sp245 also promoted a positive effect on *P. malacophylum* with outstanding results as a shoot growth-promoting bacterium, while strains PMA112 and PMO18 appear as root growth-promoting rhizobacteria (Table 3). The gain in SDM was higher in the second and third harvests with strain inoculation of Sp245; however, an increase in the shoot N content was not verified (Figure 6). Total crude protein was higher only in the N fertilizer treatment (Table 3).

The analyses of responses of the three *Paspalum* genotypes to inoculation with PGPR strains suggest a genotype effect associated to each inoculated strain. The best response was observed in *P. regnellii* inoculated with strain Sp245, which showed high shoot biomass and total N accumulated in plant tissues. Our initial hypothesis that PGPR strains isolated from the host plant, that is, "host genotype specificity", could be more efficient when inoculated in their host genotype was not confirmed. The host genotype strains showed reduced efficiency in all three *Paspalum* genotypes. Obviously, a greater number of host genotype strains need to be tested mainly in association with *P. regnellii*, which showed response to all agronomic traits. Many of these

Table 3 – Inoculation effect of PGPR strains on height (H), number of tillers (T), total shoot dry matter (SDM), total nitrogen uptake (N), total crude protein (CP), and root dry matter (RDM) of *P. regnellii*, *P. atratum* cv. *Pojuca* and *P. malacophyllum*.

	H	T	SDM	N	CP	RDM
				P. regnelliii		
	cm	units	g per plant	———————— mg per plant ————————		g per plant
Control	44.23 c	4.00 c	2.74 c	37.93 cd	237.08 c	2.52 b
Rhizobium spp. PR21	41.02 c	4.00 c	2.66 c	35.55 d	222.21 c	3.31 ab
Bacillus spp. PR23	43.93 c	5.83 b	3.09 bc	47.71 c	298.20 bc	2.44 b
Acinetobacter spp. PR8	44.75 c	4.67 c	2.58 c	36.26 d	226.64 c	2.56 b
150 kg de N	49.20 b	5.00 b	8.27 a	132.35 a	827.22 a	5.24 a
Sp245	55.75 a	7.67 a	4.19 b	60.07 b	375.43 b	3.11 ab
CV (%)	11.11	13.37	32.93	36.80	36.80	28.84
				P. atratum cv. *Pojuca*		
	H	T	SDM	N	CP	RDM
Control	45.72	4.33 b	3.38 b	42.23 b	263.94 b	2.38 b
Unknown spp. *PA122*	45.48	4.17 b	3.10 b	37.59 b	234.97 bc	2.77 b
Bacillus spp. PA121	48.42	5.50 a	3.16 b	39.34 b	245.87 b	2.82 b
Bacillus PA123	47.22	4.50 b	3.05 b	40.75 b	254.71 b	2.71 b
150 kg de N	46.62	3.83 b	6.22 a	88.84 a	555.27 a	5.05 a
Sp245	51.68	6.50 a	2.48 c	28.42 c	177.61 c	2.16 b
CV %	8.35	23.18	11.37	11.54	11.54	26.06
				P. malacophyllum		
	H	T	SDM	N	CP	RDM
Control	40.05 c	4.50 b	2.33 bc	32.44 bc	202.77 b	0.59 d
Pseudomonas spp. PMA112	36.79 c	4.83 b	2.34 bc	33.30 bc	208.14 b	2.77 a
Bacillus spp. PMO18	36.73 c	4.67 b	2.16 bc	32.17 bc	201.07 b	1.56 b
Bacillus spp. PMA115	39.33 c	4.83 b	2.06 c	29.25 c	182.85 b	0.90 cd
150 kg N	45.02 b	5.83 b	5.78 a	81.81 a	511.29 a	1.01 c
Sp245	53.28 a	11.17 a	2.67 b	34.89 b	218. 62 b	0.36 e
CV %	10.67	25.87	14.94	15.68	15.68	23.98

Values of three harvests and means of six replicates. The same letters do not differ by the Tukey test ($p \leq 5$ %).
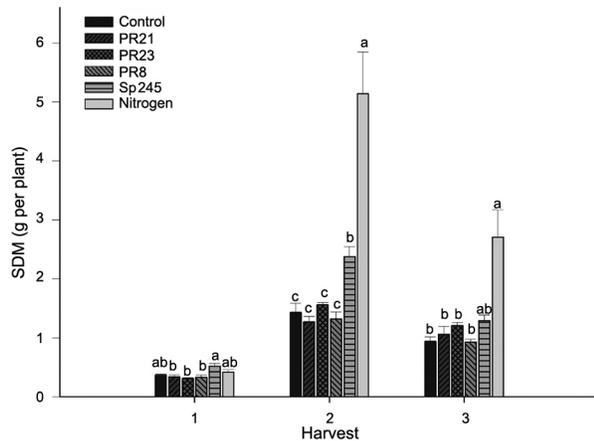
Figure 4 – Shoot dry matter (SDM) accumulation at 38 days after planting (1), 68 days after planting (2) and (3) 98 days after planting in *P. regnellii* plants inoculated with PGPR. Error bars represent the mean standard error (n = 6). The same letters do not differ by the Tukey test ($p \leq 5$ %).
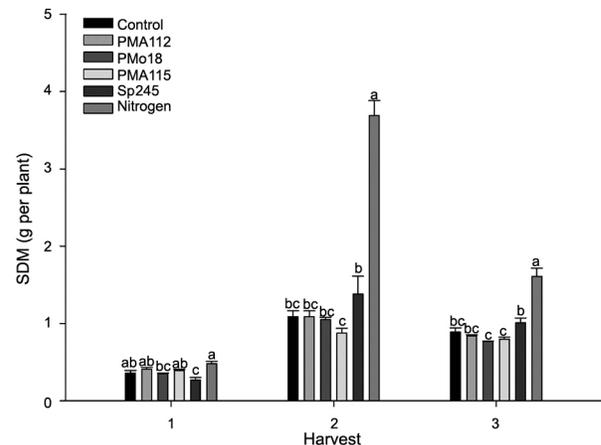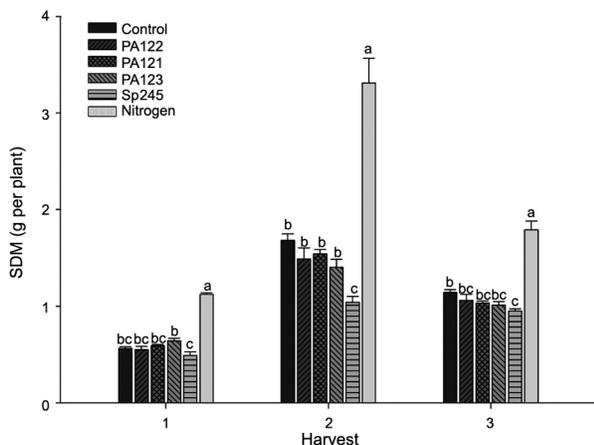

Figure 6 – Shoot dry matter (SDM) accumulation of *P. malacophylum* plants at 38 days after planting (1), 68 days after planting (2) and (3) 98 days after planting and inoculated with PGPR. Error bars represent the mean standard error (n = 6). The same letters do not differ by the Tukey test ($p \leq 5$ %).

Figure 5 – Shoot dry matter (SDM) accumulation of *P. atratum* cv. *Pojuca* plants collected at 38 days after planting (1), 68 days after planting (2) and (3) 98 days after planting and inoculated with PGPR. Error bars represent the mean standard error (n = 6). The same letters do not differ by the Tukey test ($p \leq 5$ %).

strains have not yet been tested in *Paspalum* genotypes and therefore should be explored in more detail.

The beneficial effect of *A. brasilense* on growth of different cereals or even different species may be associated to motility and the chemotaxis ability in different types of root exudates (Bashan and De-Bashan, 2015; Reinhold et al., 1985). *A. baldaniorum* (*old A. brasilense*) strain Sp245 normally establishes endophytically in plants, such as wheat (Schloter et al., 1994), promoting plant growth through the production of phytohormones, such as IAA, cytokine, gibberellin, and bacterial volatiles (Sharifi et al., 2018). These chemical substances enhance root development that, in turn, increase nutrient uptake and water use efficiency therefore promoting biomass gain (Cassán et al., 2013). These effects may have happened with Sp245 strain in

association with *P. regnellii* although it does not explain the partial response or absence of response to other *Paspalum* genotypes (*P. malacophylum* and *P. atratum* cv. Pojuca). This strain increased mainly the shoot biomass of *P. malacophylum*, while its inoculation had a deleterious effect on all agronomic variables evaluated in *P. atratum* cv. Pojuca. Lobo et al. (2019) found a similar result during the inoculation of *Bacillus subtilis* strain BS-263 in maize plants, although the strain also produced indolic compounds *in vitro*.

The plant immune system may be the cause of the deleterious effect of inoculation caused by some bacterial strains, which may not recognize the bacteria as beneficial thus making the plant-bacterial interaction incompatible (Teixeira et al., 2019). While *P. regnellii* inoculated with strain Sp245 showed increased biomass accumulation of the whole plant, promoting traits, inoculation with the host strains *Pseudomonas* sp. strain PMA112 and *Bacillus* sp. strain PMO18 promoted only root growth in *Paspalum malacophylum*.

The PGPR association promote favorable environmental conditions for the establishment of the plant, that is, the pH, humidity, and supply of photo assimilates in contrast to the N fixed by the microorganisms (Reis et al., 2018). Several studies indicate that the efficiency of associative diazotrophs depends on plant genotype and age, which generate various responses that must be previously tested to certify the inoculation technique. The strain/species also modulates the communication between the pre-existing microbial community and the strain introduced as an inoculant (Hungria et al., 2016; Ikeda et al., 2020; Reis et al., 2018).

In conclusion, our results showed a great diversity of PGPR associated to *Paspalum* genotypes and revealed that the plant response to bacterial inoculation has no relationship with the host *Paspalum* genotypes.

Authors' Contributions

Acknowledgments

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