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Interactions between endophytic bacteria and their effects on poaceae growth performance in different inoculation and fertilization conditions

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Abstract

Plant growth-promoting bacteria (PGPB) are a broad group of microorganisms that offer a huge, unexplored potential. We assessed the genetic diversity of endophytic bacteria that were isolated from corn and wheat plants in 3 different types of soils: A1 (Red Argisol), A2 (Red Latosol) and A3 (Red Nitossol). Endophytic bacteria were isolated from seedlings using 7 solid culture media and were further analyzed by 16S gene sequencing. IAA (Indole-3-acetic acid) production and PSI (Phosphorus solubilization index) bacteria capacity were tested in a randomly scheme in triplicate. *In vitro* association was assayed with 6 randomly selected endophytic strains. The experiment design was arranged in a randomly scheme with 10 replications. *In vivo* assays were performed to evaluate plant growth promotion using a low-fertility soil in different inoculation and/or fertilization conditions. The experiment design was arranged in a factorial 3x4 scheme in triplicate. The following characteristics were evaluated in both experiments in triplicate: plant biomass, total nitrogen content (TN) and endophytic population. As results, a total of 136 isolates were collected, and from these isolates, 41 strains were sequenced and classified into 4 major phylogenetic categories. There was a 38-fold variation between the highest (*Ensifer adhaerens)* and the lowest (*Agrobacterium larrymoorei*) IAA producer, and only 14% of strains were high phosphate solubilizers. *In vitro* assays identified both positively (*Burkholderia ambifaria*) and negatively (*Pantoea ananatis*) associative strains. *In vivo* assays showed that plant genotype can limit or induce the endophytic microbiota and that plant microbiota are highly influenced by soil fertility.

Keywords: 16S rDNA sequencing, plant bacteria interaction, plant growth promotion, inoculant.
Abbreviations: BNF_biological nitrogen fixation; IAA_Indole-3-acetic acid; PGPB_Plant growth-promoting bacteria; PSI_Phosphorus solubilization index.

Introduction

Cereals such as corn and wheat are economically important due to their use in human diets. Both crops have several limiting factors in their development, including the requirement of fertilizers for essential metabolic functions that directly influence grain productivity (Hawkesford, 2014). Productivity increases are correlated with the use of appropriate management practices, including an appropriate nutritional supply.

In this context, the use of chemical fertilizers is essential for crop production. However, the cost of these fertilizers, their limited availability and the associated environmental issues create an urgent need to find alternative strategies that reduce their environmental impact and their production costs without incurring farmer losses (Reis 2007; Rojas-Tapias et al.,, 2012; Majeed et al.,, 2015). Plant-growth-promoting bacteria (PGPB) are free-living bacteria in the soil that aggressively colonize the rhizosphere of plants and directly enhance root and plant growth through biological nitrogen fixation (BNF) and phosphate solubilization. They indirectly impact root and plant growth through phytohormone stimulation (indole acetic acid, cytokinin, gibberellins) and by providing protection against pathogens and abiotic stress (Farrar et al., 2014; Prathap and Kumari 2015; Rolli et al., 2015).

Microorganisms represent the greatest wealth of biochemical and molecular diversity in nature. Countless studies have been conducted to study the isolation, characterization and identification of PGPB in grasses (Khalid et al., 2004; Roesch et al., 2007; Ilyas and Bano 2010; Arzanesh et al., 2011; Rashid et al., 2012; Arruda et al., 2013). Other studies have investigated plant-bacteria interactions (Weyens et al., 2009; Beneduzi et al., 2013; Hardoim et al., 2015) and have demonstrated their potential agricultural and environmental applications, such as the control of diseases and pests (Compant et al., 2005; Mendes et al., 2007), the promotion of plant growth (García

de Salamone et al., 2012; Rojas-Tapias et al., 2012), the biological fixation of nitrogen in plants, the production of metabolites of pharmacological and biotechnology interest (de-Bashan et al., 2004), the creation of vectors to introduce genes of interest into plants (Murray et al., 1992) and the production of organic products that reduce the need for fertilizers in nature (Sala et al., 2008; Singh et al., 2011), among others.

Farmers constantly demand technologies that reduce costs and achieve eco-sustainable alternatives. Thus, studies involving the isolation, characterization and identification of diazotrophs that associate with cereals are necessary and promising. Currently, molecular techniques can be used for taxonomic classification, enabling the assessment of phylogenetic relationships among strains and the evaluation of bacterial community composition (Lagos et al., 2015). The 16S ribosomal gene has been used to build an outline of the bacterial evolutionary tree. Several molecular analyses have been conducted from PCR products. The technique used to analyze fragment gene sequences enables direct evaluation of DNA polymorphisms, which can be used to determine relatedness between individuals and populations. These data provide phylogenetic inferences and enable assessment of the endophytic communities (Ahmad et al., 2009; Gaiero et al., 2013).

To recommend an inoculant for commercial purposes, trials that transition from laboratory (*in vitro*) to field conditions are necessary to select bacterial strains with consistent plant-growth-promoting abilities. Therefore, this paper aims to isolate and characterize bacterial strains that present significant enhancements in plant growth promotion in cereals.

Results

Phenotypic characterization of bacterial isolates

Bacterial strains were isolated based on morphology and were distributed as follows: for wheat roots, 17 isolates were collected from A1 (Red Ultisol), 21 from A2 (Red Latosol) and 17 from A3 (Red Nitossol); for corn roots, 26 different bacterial strains were isolated from A1, 28 from A2 and 27 from A3 (Table 1). The soil with the highest number of isolates was A2 (36%). The soil types were not significantly different in terms of bacterial adherence, as the average number of isolates was 45 in all soil types. Based on the culture medium, 11% of the isolates were obtained in DYGS, 11% in NFB, 18% in JNFb, 12% in LG, 19% in LGD, 14% in LGI and 15% in LGI-P. All bacterial isolates grew between 28-30°C except for those in LGI medium (35-37°C). Among the isolates, 49 were classified as bacilli, one as streptococcus, 2 as diplococci and 84 as other cocci. Colonies were colorless (66 isolates), white (41), cream (1) and yellow (28). Most of the isolated colonies were gram negative (91%), while only 12 strains (9%) were gram positive.

Out of the 136 isolates, 41 had 16S rDNA sequences. The genera identified through 16S rDNA analysis grouped in 4 bacterial divisions (Fig.1). The gammaproteobacteria division was composed of *Pantoea* (12), *Pseudomonas* (1), and *Acinetobacter* (1). The alphaproteobacteria division included *Agrobacterium* (15) and *Ensifer* (1). *Burkholderia* (5) was the prominent genus found in Betaproteobacteria. The *Firmicutes* division included *Bacillus* (3) and *Brevibacillus* (1).

Bacterial phosphate solubilization and IAA production

Sequenced isolates differed in their capacity for phosphate solubilization and IAA production. For phosphate solubilization, regardless of the area and culture, 25 isolates (63%) showed no phosphate solubilizing capacity. Furthermore, 14% had a low solubility index (SI <2), 9.3% had a medium index (IS <4), and 14% had a high index (IS≥4), with a high potential for biofertilization (Table 1). Red Ultisol soil (A1) showed the highest number of good solubilizing strains, which is likely due to its low pH (pH 4.8). The isolates with a high capacity for phosphate solubilization belong to the genera *Burkholderia*, *Bacillus* and *Agrobacterium*. Bacterial controls used in this experiment were *H. seropedicae* and *A. brasilense*, which have no phosphate solubilizing capacity.

Bacterial isolates had IAA values ranging from 30.31 µg mg protein^{-1} (*Agrobacterium larrymoorei*) to 1170.98 µg mg protein^{-1} (*Ensifer adhaerens*), a 38-fold variation. Many of the isolates were better IAA producers than *A. brasilense* and *H. seropedicae*, which demonstrates the great diversity of IAA production across bacteria.

In vitro plant growth promoting assay

The results of the *in vitro* associative ability of isolates with wheat plants are shown in Table 2. When associated with wheat, strain M 3-87 (*Burkholderia ambifaria*) promoted a significant increase in root length. The high fresh weight of the shoot was likely due to its high IAA production capacity, which corresponds to an increased root length and improved interaction with plants. This isolate contained the largest number of endophytic cells (CFU) (1.36 10^6 CFU mL^{-1}).

While strain T 1-14 (*Pantoea ananatis*) presented an epiphytic and endophytic population, it had limited ability to increase root biomass and seemed to have a deleterious effect in inoculated seedlings. Strain M 2-77 (*Enterobacter asburiae*) failed to interact with host plants, and T1-1 (*Agrobacterium tumefaciens*) presented an intermediate performance in terms of plant growth. *A. brasilense* (control strain) presented relatively high epiphytic colonization, and *H. seropedicae* showed a relatively high endophytic colonization in wheat seedlings, increasing TN, but there was no effect on plant growth.

In vivo plant growth promoting assay

Wheat and corn belong to the same botanical family, but they respond differently to the presence of inoculation and/or fertilization in pots (Table 3). Strains M3-87 and T1-14 were chosen by their prior positive and negative responses. In both plant species, the presence of these strains showed a positive effect on TN content. In wheat, the results were collected from three groups (I, II and III) in which various bacterial genera and/or fertilization conditions were applied. *A. brasilense* increased TN by 69%, while this strain plus NPK and ammonium sulfate produced a 154% increase in TN compared to the control. The same effect was observed in T 1-14, where the TN increased by 58% and 154%, respectively, in the above-

described conditions. Strain M 3-87 did not respond with an increase in TN content, although M 3-87 was isolated from corn plantlets and showed a better affinity with wheat *in vitro* than did T 1-14.

In corn, the best response in height, fresh and dry weight was found when NPK and/or ammonium sulfate was applied to the plants (Table 3). *A. brasilense* T 1-14 and M 3-87 increased TN by 43%, 46% and 52%, respectively, compared to the control plants. The results indicate a decreasing epiphytic and endophytic microbial population due to chemical fertilizers. However, T 1-14 was an exception, as ammonium sulfate produced a higher CFU (5.7 x epiphytic and 8.7 x endophytic) compared to the control. Numerically, this reduction was greater when NPK and ammonium sulfate were applied, which demonstrates the bacterium's sensitivity to fertilizers. Re-inoculated T 1-14 (*Pantoea ananatis*) did not demonstrate significant differences *in vitro*, but it had a positive effect in pots.

Discussion

Isolating and characterizing a prodigious strain for use as biofertilizer is a difficult task; the strain must have the capacity to associate with different plants by overcoming the plant's immune system to establish an association. Additionally, the ideal strain should be an IAA producer and a macro- and micronutrient solubilizer, among other features. To colonize plants, bacteria should be able to recognize, adhere, invade and grow as a population. These characteristics could reduce the diversity of PGPB for biotechnological purposes due to plant and bacterial genotype dependence (Berg 2009).

Gammaproteobacteria and alphaproteobacteria were the major poaceae endophytic bacterial groups observed. Betaproteobacteria and firmicutes were also found. Donn et al., (2015) in a cross-year analysis of the soil microbiome in an intensive wheat cropping system, revealed a microbiota dominated by *Proteobacteria*, *Actinobacteria* and *Bacteroidetes.* Romero et al., (2014) and Chaturvedi and Singh (2016) reported that the most common genera of endophytes are *Pseudomonas*, *Burkholderia*, *Stenotrophomonas, Micrococcus, Pantoea, Microbacterium*, and *Bacillus.* The results noted a prevalence of *Rhizobia* strains, which was also observed by Romero et al., (2014). Majeed et al., (2015) observed that *Stenotrophomonas* spp. seem to prefer wheat plants as a host, which is similar to what we observed in this study (Table 1). Moreira et al., (2016) observed that the most abundant genera in cultivated wheat were *Pseudomonas*, *Burkholderia* and *Enterobacter* in 346 isolates.

There was not a clear association between endophytic genera and the soil or plant type used as bait (Table 1). The 41 sequenced isolates may not represent all of the diversity in the evaluated soils. In the literature, several authors reported that soil type is the major determinant in the structure of microbial communities. Additionally, the organic matter content, texture and structure, microaggregate stability, pH and the presence of essential nutrients such as N, P and Fe can determine microbial niches. Furthermore, other factors such as soil management, crop rotation, and the use of herbicides, fertilizers and irrigation must be considered (Dey et al., 2012; Arruda et al., 2013; Moreira et al., 2016). Although the sampled soils correspond to three distinct types (Red Ultisol, Red Latosol and Nitossol), they do not differ greatly in their physical and chemical characteristics. All soils had a high sand content and a pH of approximately 5.0 (Table 4), which would explain the presence of similar microbiota. Red Argisol type (A1), which was sandier and had a lower OM content than the others, presented the highest species diversity, with 21 distinct isolates (Table 2).

Strain T 2-32 (*Ensifer adhaerens*) would be an interesting strain to test in further experiments because of its stunting IAA production (1170.98 µg mg protein^{-1}) compared to *A. brasilense* AbV5 (commercial inoculant) or *H. seropedicae* (positive control) (Table 1). Among the isolates, *Agrobacterium, Bacillus* and *Burkholderia* were considered the strongest P solubilizers; this result has been confirmed by others (Rodríguez & Fraga 1999; Castanheira et al., 2016; Moreira et al., 2016).

In vitro strain selection may contribute to a better screening of potential biofertilizer strains, especially in gnotobiotic conditions, where environmental aspects could mask plant and bacterial interactions (Khalid et al., 2004; Fürnkranz et al., 2009). The results demonstrate the outstanding performance of M 3-87 in promoting root and aerial growth, qualifying this species as a PGPB (Compant et al., 2008). T 1-14 (*Pantoea ananatis*) had a negative effect on plantlet growth (Table 2). This observation is compatible with the fact that the plant might perceive this species as a pathogen; it could also be the result of phytotoxin production inhibiting plant growth (Compant et al., 2008).

In vivo experiments are useful to check interactions between inoculate and natural soil microbiota. The microbial population linked to the corn plant was larger than that linked to wheat in the control plants, which is likely due to more abundant and diverse exudates in corn than in wheat. Several authors reported the effect of plant genotype on bacterial communities due to the stimulation of secondary compounds (Doornbos et al., 2012; Bouffaud et al., 2012; Hardoim et al., 2016; Kirzinger & Stavrinides 2016). Dey et al., (2012) reported that plants exude a wide variety of compounds, such as ethylene, sugars, and amino acids. These compounds are determined by plant genotype and directly influence the rhizospheric community. These compounds or signals can generate a specificity in plant and bacteria due to genotype and strain dependence (Drogue et al., 2012). Other authors also report that the amount and composition of exudates varies along the root length, with soil fertility and in biotic and abiotic stresses (Neumann 2007; Marschner et al., 2011). In corn, exudates are composed of 65% sugar, 33% organic acids and 2% amino acids (Baudoin et al., 2003; Aira et al., 2010). However, few studies have addressed the characterization of exudates in various wheat cultivars. It is important to note that soil microbiota could have an important role in increasing soil fertility, but our results showed that low fertility almost failed to promote the natural bacterial population that was observed in the control wheat plants. On the other hand, Egamberdiyeva (2007) reported that a positive bacterial associative capacity in wheat was correlated with the nutritional status of the soil, where the best results were obtained in low fertility soils.

Table 1. Number of phosphate solubilizers and IAA producers by bacterial isolates in each sampling site.

Sample Number	Isolate	Related bacteria	PSI	IAA Content (µg.mg^{-1})
1	T 1-1*	*Agrobacterium tumefaciens*	-	60.22e
2	T 1-2	*Agrobacterium tumefaciens*	-	97.00e
3	T 1-3	*Agrobacterium tumefaciens*	-	99.6e
4	T 1-4	*Agrobacterium tumefaciens*	-	187.68d
5	T 1-5	*Shigella flexneri*	-	192.06d
6	T 1-6	*Agrobacterium tumefaciens*	-	59.97e
7	T 1-8	*Shigella flexneri*	-	82.43e
8	T 1-10	*Burkholderia unamae*	-	106.10e
9	T 1-11	*Agrobacterium tumefaciens*	-	193.90d
10	T 1-13	*Stenotrophomonas rhizophila*	-	72.56e
11	T 1-14	*Pantoea ananatis*	3.13d	81.65e
12	T 1-16	*Agrobacterium tumefaciens*	-	161.43d
13	T 2-21**	*Pseudomonas chlororaphis*	-	292.42c
14	T 2-23	*Shigella sonnei*	-	446.54b
15	T 2-26	*Escherichia fergusonii*	-	268.97c
16	T 2-28	*Shigella flexneri*	2.91d	73.81e
17	T 2-32	*Ensifer adhaerens*	-	1170.98a
18	T 2-36	*Escherichia fergusonii*	-	234.54c
19	T 2-37	*Acinetobacter johnsonii*	-	100.13e
20	T 3-45	*Escherichia fergusonii*	-	382.67b
21	T 3-46	*Pantoea vagans*	-	84.83e
22	T 3-51	*Agrobacterium tumefaciens*	-	80.22e
23	M 3-56***	*Agrobacterium radiobacter*	1.31e	115.89e
24	M 3-72	*Agrobacterium radiobacter*	1.38e	234.21c
25	M 2-77	*Enterobacter asburiae*	-	180.38d
26	M 3-80	*Acinetobacter johnsonii*	-	46.67e
27	M 3-87	*Burkholderia ambifaria*	4.48c	340.93b
28	M 2-91	*Enterobacter asburiae*	3.05d	69.13e
29	M 2-93	*Bacillus safensis*	3.32d	97.05e
30	M 2-95	*Agrobacterium fabrum*	6.60a	223.68c
31	M 3-101	*Burkholderia unamae*	-	166.70d
32	M 3-103	*Bacillus thuringiensis*	6.98a	88.24e
33	M 1-118	*Burkholderia cepacia*	5.01b	71.61e
34	M 1-119	*Agrobacterium fabrum*	1.17e	134.77d
35	M 1-120	*Agrobacterium fabrum*	1.16e	238.79c
36	M 1-121	*Agrobacterium fabrum*	6.78a	62.49e
37	M 1-122	*Burkholderia sp.*	5.34b	158.19d
38	M 1-127	*Escherichia fergusonii*	1.31e	82.11e
39	M 1-128	*Bacillus megaterium*	1.19ef	164.94d
40	M 1-133	*Brevibacillus agri*	-	126.08d
41	M 1-135	*Agrobacterium larrymoorei*	-	30.31e
Control 1	SMR1	*Herbaspirillum seropedicae*	-	135.76d
Control 2	AbV5	*Azospirillum brasiliense*	-	90.08e

*T 1-1:T means isolated from wheat, 1-isolated in A1 (Red Ultisol – Iporã/PR), 1- the 1st isolated

**T 2-21: T means isolated from wheat, 2-isolated in A2 (Red Latosol – Line La Salle-Palotina/PR) 21- the 21th isolated

***M 3-56: M isolated from corn, 3-isolated in A3 (Red Nitosol – Line Aparecidinha-Palotina/PR)., 56- the 56th isolated

PSI- phosphate solubilizing index

IAA-Indole acetic acid

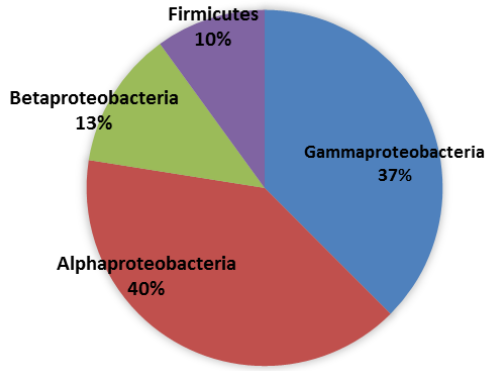

Fig 1. Taxonomic classification from isolated bacteria in 3 different types of soil through analysis of 16S rRNA sequences.

Table 2. *In vitro* morphophysiologic and biochemistry characterization of different strains inoculated in wheat plantlets.

		Biochemistry characterization of bacterial strains									
	Isolates	Root Length (cm)	Fresh Aerial Biomass (mg)	Dry Aerial Biomass (mg)	Fresh Root Biomass (mg)	Dry Root Biomass (mg)	Total N (g Kg^{-1})	Epiphytic cells (CFU $x10^6$ mL^{-1})	Endophytic cells (CFU $x10^6 mL^{-1}$)	PSI	IAA/Protein (µg mg^{-1})
T 1-1	*Agrobacterium tumefaciens*	12.50b	43.18c	6.01b	70.00ab	8.33a	59.50a	-	0.20	-	60.22b
T 1-14	*Pantoea ananatis*	10.00bc	22.96d	2.68c	27.00c	6.33ab	43.80f	1.90	0.13	3.13b	81.65b
M 2-77	*Enterobacter asburiae*	7.50cde	39.78c	6.61b	25.33c	4.66ab	45.40e	2.11	0.20	-	180.38b
M 3-87	*Burkholderia amfibaria*	15.50a	67.01a	21.50a	63.00ab	8.00ab	45.30e	0.31	1.36	4.49ab	340.93a
Ab-V5	*Azospirillum brasilense*	6.50de	53.28b	6.35b	64.66ab	8.33a	53.60b	1.88	0.66	-	135.76b
SMR1	*Herbaspirillum seropedicae*	6.00e	41.00c	5.81b	57.66b	8.33a	54.10b	0.93	1.60	-	90.08b
Control		9.00cd	53.00b	6.81b	79.33a	8.00ab	52.10c	-	-	-	-

IAA-Indole-3-acetic acid.
PSI-Phosphorus solubilization Index *P <0.05.

Table 3. Plant biomass and microbiota of wheat and corn plants in different fertilization and/or inoculation conditions.

Treatments	Wheat						Corn					
	Height (cm)	Fresh Shoot (g)	Dry Shoot (g)	Total N (g.Kg-1)	Epiphytic UFC.10^6	Endophytic UFC.10^6	Height (cm)	Fresh Shoot (g)	Dry Shoot (g)	Total N (g.Kg-1)	Epyphitic UFC.10^6	Endophytic UFC.10^6
Group 1												
Control	25.63b	0.29a	0.09a	23.60d	0	0	26.33c	1.26c	0.20b	39.00c	31	33
A. brasiliense	27.53b	0.40a	0.11a	39.90bc	7	1.65	24.33c	1.27c	0.22b	56.00bc	1	0.08
A. brasiliense + NPK	23.40b	0.25a	0.08a	31.20cd	43.3	33.3	58.33a	12.88a	1.48a	83.00b	25	5
A. brasiliense + $(NH_4)_2SO_4$	24.73b	0.35a	0.09a	47.50b	8.33	13.33	27.66c	1.74c	0.23b	156.30a	21	20
A. brasiliense +NPK+$(NH_4)_2SO_4$	36.10a	1.18a	0.32a	60.00a	38.3	23.33	48.00b	7.47b	1.31a	140.00a	1	20
Group 2												
Control	25.63a	0.29b	0.09a	23.60c	0	0	26.33b	1.26b	0.20b	39.00d	31	33
T 1-14	24.06a	0.40ab	0.11a	37.40b	23.3	15	24.33b	1.28b	0.22b	57.00cd	26	45
T 1-14 +NPK	26.73a	0.32ab	0.08a	41.90b	0.7	15	48.00a	6.85a	1.00a	93.00bc	8	0.05
T 1-14 +$(NH_4)_2SO4$	34.40a	0.78a	0.20a	35.90b	20	5	28.26b	1.45b	0.23b	156.30a	181	290
T 1-14 +NPK $(NH_4)_2SO_4$	29.80a	0.72ab	0.16a	60.10a	18.33	8.33	46.33a	5.36a	0.87a	140.00a	0.5	41
Group 3												
Control	25.63a	0.29a	0.09a	23.60a	0	0	26.33c	1.26c	0.20c	39.00c	31	33
M 3-87	25.53a	0.32a	0.11a	29.20a	3.33	3.33	25.33c	1.18c	0.23c	59.30c	51	71
M 3-87+NPK	27.76a	0.37a	0.10a	40.90a	16.6	21.6	52.00a	8.80a	1.43a	102.60b	5	8
M 3-87+$(NH4)_2SO_4$	30.10a	0.40a	0.10a	39.80a	16	19.5	29.83c	1.63bc	0.23c	159.00a	61	4
M 3-87+NPK+$(NH_4)_2SO_4$	31.76a	1.19a	0.24a	61.80a	1	0.4	43.00b	3.94b	0.65b	169.60a	35	27

Mediums followed by the same letter in the collumm did not differ statistically by Tukey test, at 5% probability.

Table 4. Physicochemical characteristics of the layer 0-20 cm soil from different collection sites.

Soil	pH	OM $g.dm^{-3}$	P $mg.dm^{-3}$	K	Ca^{+2}	Mg^{+2}	Al^{+3}	$H^{+}+Al^{+3}$	EB	V%	Clay%	Silt%	Sand%
				$cmol.dm^{-3}$									
A1[†]	4.8	8.4	2.7	0.13	1.13	0.33	2.4	2.5	1.6	38.5	9.0	4	87
A2[‡]	5.2	14.7	44.7	0.23	2.08	0.79	0	3.2	3.1	49.3	18.0	22	60
A3[§]	5.3	20.8	42.7	0.30	2.92	1.22	0	3.4	4.4	56.4	16.0	22	62

[†]A1- Red Ultisol (Iporã/PR);
[‡]A2- Red Latosol (Line La Salle-Palotina/PR);
[§]A3– Red Nitosol (Line Aparecidinha-Palotina/PR).
OM-Organic matter
EB : Exchange Bases
V%: Percentage of Base Saturation

Although the TN plant levels are an indication of biological nitrogen fixation, the fixation process is energy dependent, and it is influenced by the amount and the sources of C exuded by the host plant, as well as other available compounds, such as NO_3, O_2 and Mo (Bashan and De-Bashan 2010; Naher et al., 2011). A higher TN was mostly explained by fertilization (NPK and ammonium sulfate) rather than by a bacterial effect (Table 3). Re-inoculation effects were tested using strains M3-87 and T1-14, which were isolated from corn and wheat, respectively. Strain M 3-87 (*Burkholderia ambifaria*) was isolated from corn and showed a positive *in vitro* performance. However, in pots, it seemed to have a better capacity for escaping from the plant's defense system instead of increasing the root surface by IAA production (Zamioudis & Pieterse 2012). The presence of fertilization (NPK and ammonium sulfate) could have affected the M 3-87 population in wheat plants. The bacterium could use the chemical nutrient to promote its own growth. However, Aira et al., (2010) observed an inverse proportionality in the dosage of fertilizers and bacterial activity. Shaharoona et al., (2008) found that *Pseudomonas sp.* inoculated in wheat with fertilizer (NPK) had reduced efficiency in proportion to the fertilizer concentration. NPK could act as a stressor, decreasing the bacterial cell's osmotic potential, and leading to death. Low natural soil fertility could have reduced the nutritional sources (carbon) for this strain, restricting its growth inside the plant. Although *in vitro* and *in vivo* conditions had different inoculation periods (5 and 45 days, respectively), the epiphytic and endophytic microbiota were present in the same order of magnitude (10^6) (Table 2 and 3).
When T 1-14 (*Pantoea ananatis*) was reinoculated in pots, it also showed a positive response to fertilizer applications. The higher epiphytic and endophytic bacterial populations that were observed *in vivo* compared to *in vitro* could potentially be due to the effect of the inoculation period, as the bacteria had 45 days to achieve an association with its host plants.

Materials and Methods

Sampling and sample preparation

Corn (cv IAPAR 114) and wheat (cv CD 120) bait plants were sown in three native forest areas in the West Region of Paraná, Brazil: A1-Red Argisol (23°59'01.5"S 53°44'54.9"W), A2-Red Latosol (24°15'23.8"S 53°47'02.8"W) and A3-Red Nitossol (24°12'32.7"S 53°45'27.0"W), which were classified according to Bhering and Santos (2008). The areas differ in terms of their fertility and granulometry (Table 4).
Bait plants were harvested 60 days after germination, and 1 g of roots derived from several plants in each bait site were weighed in triplicate. The roots were washed with tap water and then sterilized in a laminar flow chamber by immersion in 70% ethanol for 30 s, then in sodium hypochlorite (commercial product) 0.2% for 60 s, followed by triple washing in distilled water. The roots were soaked using a mortar and pestle with 9 mL of saline solution (0.9% NaCl).
Subsequently, serial dilutions were performed (10^1 to 10^5). A 0.1 mL sample from a 10^5 dilution extract was plated in a solid medium of DYGS (Rodrigues Neto et al., 1986), NFb, JNFb, LG, LGD, LGI, or LGI-P (Döbereiner 1995). Petri dishes were placed in a 30°C growth chamber, except for those with LGI medium (35°C ± 2°C), for up to 7 days. The chiming of the bacteria was carried out every 7 days to obtain pure cultures. Pure cultures were stored in penicillin flasks containing a culture slant solid medium, and flasks were kept in an acclimatized dark room.

Phosphate solubilization

Bacterial phosphate solubilization was detected *in vitro* as the ability to solubilize phosphate by inoculation with NBRIP medium containing insoluble phosphate (Nautiyal et al., 2000). Briefly, a bacterial colony was collected with a toothpick, and each ¼ plate of NBRIP medium plate was inoculated. The presence of a halo around the colonies was considered to indicate phosphate solubilization. The plate was observed on different days for two weeks in a culture incubated at 28 °C. These colonies were stored at -80°C in DYGS medium supplemented with 20% glycerol. From these measurements in triplicate, the solubilization index (SI) was obtained using the formula: SI=Diameter of Halo (mm) / Diameter of colony (mm) (Berraquero et al., 1976). The isolates were classified as low (IS <2), medium (IS> 2) or high (IS≥4) potential for phosphate solubilization.

In vitro IAA production

In vitro indole acetic acid synthesis was measured by colorimetric quantitative methodology (Asghar et al., 2002). Bacterial cultures were grown in DYGS medium and incubated at 28°C for 24 h. The cultures were centrifuged at 10000 g for 10 min and the supernatant was quantified spectrophotometrically at 535 nm with Salkowski's reagent. The IAA level was estimated according to a standard curve (0.2 a 45 µg mL^{-1}) (Sarwar and Kremer 1995; Kuss et al., 2007). Total protein was performed following Lowry et al., (1951). All experiments were conducted in triplicate. The data were submitted to Analysis of Variance (ANOVA), and the means were grouped by the Scott-Knott test at 5% probability, with the help of the multimedia application GENES (Cruz 2006).

DNA extraction and PCR amplification

Bacterial genomic DNA was extracted following the protocol proposed by Cheng and Jiang (2006), where the bacterial strains were grown in liquid DYGS. After it was extracted, the purified DNA was stored in a freezer at -20°C. PCR reaction was performed using 3 µL of 10x Taq Buffer, 2.25 µL of 25 mM $MgCl_2$, 1 µL of 10 mM of each deoxyribonucleotide, 1 µL of each oligonucleotide primer Y1F (5´TGGCTCAGAACGAACGCTGGCGGC3´) for the 16S rDNA gene and the Y3R primer (3´TACCTGTTACGACTTCACCCCAGTC5´), generating an amplification product comprising almost the entire gene of approximately 1.5 Kb, 2 µL of Taq DNA polymerase, 2 µL of DNA (50 to 100 ng) to a final volume of 30 µL. Samples were amplified in a thermocycler according to the following program: 93°C for 2 min; 35 cycles of 93°C for 45s, 62°C for 30s, and 72°C for 2 min; and a final extension for 5 min at 72°C. Electrophoresis was carried at 70 V for approximately 1 h in an automated image capturer (Loccus Biotechnology L.PIX).

Partial sequencing of the 16 S rRNA gene

To sequence the Y1-Y3 PCR products, the fragments were purified with exo /SAP enzymes (protocol according to the manufacturer) and sequenced using dye terminator chemistry in a sequencer XL ABI3500 Genetic Analyzer (Applied Biosystems). Nucleotide sequence identities were determined by the Blast and Seqmatch programs and were used to taxonomically classify the sequences according to the bacterial taxonomy proposed by Bergey's Trust or were classified using the NCBI RDP site (Ribosomal Database Project - http://rdp.cme.msu.edu).

In vitro plant growth promoting assay

To determine the capacity of plant interaction among isolates, 4 bacterial strains (T 1-1, T 1-14, M 2-77, and M 3-87) varying in terms of IAA production, phosphate solubilization and type of bait plants were tested *in vitro*. The bacteria *Azospirillum brasilense* Ab-V5 and *Herbaspirillum seropedicae* SMR1 were used as positive controls, and the negative control was not inoculated. Fifty seeds of wheat genotype cv CD 120 (responsive to association) (Neiverth et al., 2014) were aseptically prepared using acidified hypochlorite solution and were pregerminated in agar-water medium for 24 h. The inoculum was prepared from a colony of each strain and was transferred to a preinoculum containing 5 mL medium DYGS liquid in a 50 mL conic tube and maintained at 28 °C in a shaker (120 rpm/overnight). One mL of the preinoculum was transferred to a 20 mL DYGS liquid medium until the log phase of growth was reached. The cell mass was measured by turbidimetry at 660 nm. For inoculation, the concentration used was 10^6 cells per pregerminated seed. The seeds were dried at room temperature for 2 h and then transferred to test tubes containing 20 mL of MS medium (Murashige and Skoog 1962) containing polypropylene balls, which formed a 5-cm support layer for seeds without the addition of nitrogen at pH 5.8. Each tube received a pregerminated seed and 10 replications were done per treatment in a complete randomized design. Tubes were disposed in a growth chamber under a photoperiod of 16 h light / 8 h dark with a temperature of 25 ± 2 ° C for 5 days. After 5 days of culture, three seedlings from each treatment were removed from the tubes and the shoot and roots were separated. Plant height, root length, fresh weight (mg) of shoot and root were measured. These samples were dried with air circulation at 65°C for 48 h to obtain the dry weight of shoot and root. The shoot dry matter was used to analyze the total nitrogen content (TN) (Kjeldahl) according to the method described by Bremner and Mulvaney (1982).

Counting of epiphytic and endophytic bacterial cells in roots

For assessment of the epiphytic bacterial cell count, 0.1 g from *in vitro* cultivated wheat roots was immersed in tubes containing 1 mL saline (0.9%) and taken to the sonicator for 1 min. Then, 100 µL sample aliquots were taken and inoculated into micro tubes with 900 µL of saline (0.9%). This procedure was performed until the 10^8 dilution. For endophytic bacterial cell counting, the same roots used for epiphytic counting were immersed in a solution of Chloramine - T 1% for 30 s, then rinsed with distilled water and autoclaved. Next, the roots were ground in 1 mL saline (0.9%) and placed in the sonicator for 1 min, and the 8 dilutions were performed as described above. The dilutions were allocated on a single Petri dish containing DYGS solid medium, where each of the 3 microdrops (5 µL) of dilution constituted the replicates (Romeiro 2001). The plates were placed in a growth chamber at 28 ° C and the bacterial cells were counted after 16 h.

In vivo plant growth promoting assay

In vivo experiments were conducted to evaluate the plant growth promotion in wheat and corn. Treatments with inoculation and/or fertilization in a low fertility (LF) (Red Argisol) soil were conducted: pH = 3.6, OM = 1.34 g dm^{-3}, P = 2.8 g dm^{-3}, Ca^{+2} = 0.87 cmol.dm^{-3}, Mg^{+2} = 0.14 cmol.dm^{-3}, Al^{+3} = 1 cmol.dm^{-3}, K^{+1} = 0.02 cmol.dm^{-3}, Fe = 26 mg dm^{-3}, CEC = 1.2, V = 25%, clay = 6.0%, sand = 76%. Five seeds of wheat (cv. CD120) and corn (Pioneer 30F53) were inoculated with strains T 1-14, M 3-87 and *Azospirillum brasilense* Ab-V5 composing 3 groups with 4 different treatments: 1- Strain (10^6 cells.$seed^{-1}$), 2- Strain +NPK (400 kg ha^{-1}) (4-20-20), 3-Strain+Ammonium sulfate (50 kg ha^{-1}) in coverage and 4- Strain + NPK + Ammonium sulfate in coverage in pots containing 4.5 kg of previously sifted soil. Strains and fertilizers were not added to the control treatments.

Thinning was done 15 days after sowing, leaving only three plants per pot. The experimental design was a 3 (strains) x 4 (fertilization/inoculation conditions) factorial completely randomized, with each group conducted in triplicate. After 30 days of sowing, ammonium sulfate was used in coverage. The final evaluation of growth promotion was held 45 days after sowing and was evaluated based on plant height, shoot fresh weight, dry matter of shoot and total nitrogen in the leaf tissue, as previously described. Bacterial cell counting was performed by collecting intact roots from pots, which were cut and washed in running water to remove excess soil. For each sample, 0.5 g was taken to a laminar flow hood, where previously proposed protocol was followed.

Statistical analysis

Data were submitted to Analysis of Variance (ANOVA) by F-test at 5% probability and the means were compared by a Tukey test at 5% probability using the GENES statistical program (Cruz 2006).

Conclusion

An understanding of bacterial endophytic strain diversity is important in ecological and biotechnical applications, as the soil's bacterial community continually interacts with plants that are competing for nutrients and water, thus forming a complex ecosystem. Not all of the isolates demonstrated IAA and PSI capacities, which are markers of promising biofertilizer strains. Isolates showed distinct performances under in vitro and in vivo conditions. In vivo experiments displayed a

prominent response by the endophytic population due to the presence of fertilizer, confirming that microbiota are highly influenced by soil fertility. Additionally, it was observed that plant genotype had a strong influence on limiting or promoting the endophytic bacterial population.

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