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ORIGINAL PAPER

Bacteria isolated from soils of the western Amazon and from rehabilitated bauxite-mining areas have potential as plant growth promoters

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Abstract Several processes that promote plant growth were investigated in endophytic and symbiotic bacteria isolated from cowpea and siratro nodules and also in bacterial strains recommended for the inoculation of cowpea beans. The processes verified in 31 strains were: antagonism against phytopathogenic fungi, free-living biological nitrogen fixation, solubilization of insoluble phosphates and indole acetic acid (IAA) production. The resistance to antibiotics was also assessed. Sequencing of the partial 16S rRNA gene was performed and the strains were identified as belonging to different genera. Eight strains, including some identified as *Burkholderia fungorum*, fixed nitrogen in the free-living state. Eighteen strains exhibited potential to solubilize calcium phosphate, and 13 strains could solubilize aluminum phosphate. High levels of IAA production were recorded with L-tryptophan addition for the strain UFLA04-321 (42.3 μg mL^{-1}). Strains highly efficient in symbiosis with cowpea bean, including strains already approved as inoculants showed the ability to perform other processes that promote plant growth. Besides, these strains exhibited resistance to several antibiotics. The ability of the nitrogen-fixing bacteria to perform other processes and their adaptation to environmental conditions add value to these strains, which could lead to improved inoculants for plant growth and environmental quality.

Keywords Biological nitrogen fixation · Phosphate solubilization · Plant growth hormones · Resistance to antibiotics

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Introduction

Plant growth promoting bacteria represent a fraction of prokaryotes that compose the wide microbial diversity of soils. The growth promotion mechanisms used by these bacteria include the ones that act directly on plant development such as biological nitrogen fixation (BNF), solubilization of insoluble inorganic phosphate, production of plant-growth-regulating substances, and mechanisms that act indirectly on plant development such as antagonism against phytopathogenic agents or antibiosis.

Some studies show that *Rhizobium* that belong to the nitrogen fixing nodule-forming bacterial genera can promote the solubilization of insoluble inorganic phosphate, which can then increase the production of corn (*Zea mays* L.) and lettuce (*Lactuca sativa* L.) (Chabot et al. 1996). *Mesorhizobium*, another nodule-forming bacterial genus that can solubilize insoluble inorganic phosphate, can promote an increase in the phosphorus content of chickpeas

(*Cicer arietinum* L.) and barley (*Hordeum vulgare* L.) (Peix et al. 2001a). These bacteria are also actively involved in the synthesis of plant growth hormones such as auxin; which was confirmed in culture medium (Boeiro et al. 2007) and they promoted growth of radish plants (*Raphanus sativus* L.) (Antoun et al. 1998). A high positive correlation between the in vitro production of indol-3-acetic acid (IAA) derived from L-tryptophan and the yield of mustard seeds (*Brassica juncea* L.) has also been observed (Asghar et al. 2002).

BNF is relevant to agricultural systems and natural ecosystems because it increases the nitrogen level in these environments. Among the nitrogen-fixing bacteria, the strains that fix nitrogen through symbiosis and nodule formation in leguminous plants are highlighted; they are currently restricted to 15 genera in addition to diverse species that live in association with plants or that live in a free-living state in the soil. Among the 15 nodule-forming genera, only *Burkholderia* and *Azorhizobium* were reported as being also able to fix nitrogen in a free-living state (Dreyfus et al. 1988, 1983; Elliott et al. 2007; Moreira et al. 2006; Oliveira-Longatti et al. 2013; Silva et al. 2012).

Several studies have been performed to select bacterial strains that, when in symbiosis with cowpea, exhibit high BNF and increase their productivity. These studies reveal a wide diversity of strains with the potential for use as inoculants (Lacerda et al. 2004; Soares et al. 2006; Soares et al. unpublished results). Indeed, some *Bradyrhizobium* strains (UFLA 3-84, INPA 3-11B, BR3267) are already approved by the Brazilian Ministry of Agriculture, Livestock and Supply as cowpea inoculants.

There is agronomic interest in identify other important processes in these bacteria that might directly or indirectly promote the growth of leguminous and non-leguminous plants. Because most studies reporting on promoters for plant growth usually focus on the associative and endophytic rhizosphere bacteria such as *Pseudomonas*, *Azospirillum*, *Pantoea, Paenibacillus* and *Acinetobacter* (Collavino et al. 2010; Ikeda et al. 2013; Lugtenberg and Kamilova 2009; Monteiro et al. 2009; Ogut et al. 2010) there is a need for further research on symbiotic bacteria.

To perform these processes in the soil, the bacteria have to compete for resources with the huge microbial diversity and overcome the presence of a widely varying scope of antibiotics. Thus, the aim of this study was to verify the in vitro ability to perform different biotechnological processes and to display antibiotic resistance of the three strains currently approved as inoculants for cowpea, besides other 26 symbiotic strains and 2 non-symbiotic bacterial strains. All these 31 strains, including the inoculant ones, were isolated from nodules of the trap species cowpea and siratro inoculated with soils from Amazonian or from rehabilitated bauxite-extraction areas in Minas Gerais state. We also identified these strains by 16S rRNA sequencing.

Materials and methods

Strains studied

This study investigated 28 strains and UFLA 03-84, INPA 03-11B and BR3267 strains. These last three strains are approved by MAPA (Ministério da Agricultura, Pecuária e Abastecimento—Ministry of Agriculture, Livestock and Supply) as inoculant for cowpea bean cultures based in published results (Lacerda et al. 2004; Martins et al. 2003; Soares et al. 2006). Table 1 shows the origin and symbiotic and cultural characteristics of these strains. The majority of these strains are able to establish symbiosis with the cowpea bean (Soares et al. unpublished results) (Table 1). In addition to these strains, other strains were used as positive controls for some of the tests: BR5401^T and ORS571^T served as positive controls for free-living biological nitrogen fixation, and BR11001^T and BR11080^T served as positive controls for auxin production.

Genetic identification of strains

The DNA of each strain was isolated using the ZR Fungal/Bacterial DNA Kit (Zymo Research Corp.). The 16S rRNA of the strains was amplified by PCR using the primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). A 2 μL aliquot of extracted DNA was used in polymerase chain reaction (PCR) with a final volume of 50 μL per reaction. The final concentrations of the reagents, per reaction, were 0.2 μM of each primer 27F and 1492R, 2.5 μM of magnesium chloride, PCR buffer at 1×, 0.2 μM of each dNTP, and 0.02 U Taq DNA polymerase (Taq DNA polymerase, Invitrogen). The amplification reaction was carried out in an Eppendorf Mastercycle® thermocycler under the following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation (94 °C for 40 s), annealing (55 °C for 40 s), extension (72 °C for 1.5 min), and one final extension at 72 °C for 7 min. An aliquot of each PCR reaction (20 μL) was analyzed using a 1 % (w/v) agarose gel with TAE buffer and ethidium bromide staining (5 μg mL^{-1}). PCR products purification and DNA sequencing (with the 27F primer) was performed by Macrogen with a 37 30 × 1 sequencer.

Sequences were compared to GenBank database to obtain most similar accessions and their similarities.

Free-living nitrogen fixation

To evaluate free-living nitrogen fixation, the strains and positive controls BR 5401^T and ORS 571^T, which are *Azorhizobium doebereinerae* and *Azorhizobium caulinodans* strains (Dreyfus et al. 1988; Moreira et al. 2006), respectively, were inoculated in the centre of vials (total

Table 1 Origin, symbiotic efficiency, identification and accession number of the strains UFLA 03-84, INPA 03-11B and BR 3267, which are currently approved as inoculants for the cowpea bean (*Vigna unguiculata* (L.) Walp) and of the 28 other strains

Strain	Origin location/ SUT	Trap species[a]	Symbiotic efficiency: fixation of N_2 in cowpea[b]	Growth characteristics in 79 medium[c]		Number of base pairs	Identification based in the most similar sequence found in GenBank			Accession number in GenBank (NCBI) of the strain
				G.R.	pH		Species	Similarity %	Accession number	
UFLA 03-84	Rondônia State/P	Cowpea	Efficient	S	Alkaline	–	*Bradyrhizobium* sp.	–	–	AF384136-
INPA 03-11B	Manaus-Amazonas/ Terra firme	–	Efficient	S	–	–	*Bradyrhizobium elkanii*	–	–	AF208510-
BR 3267	–	–	Efficient	I	Alkaline	–	*Bradyrhizobium Japonicum*	–	–	AY649439
UFLA 03-05	Benjamim Constant, AM/SF	Cowpea	No nodules	S	Alkaline	–	Not identified	–	–	–
UFLA 03-14	Benjamim Constant, AM/SF	Cowpea	Innefficient	S	Neutral	680	*Enterobacte*r sp.	99 %	GQ478275	KC879694
UFLA 03-15	Benjamim Constant, AM/SF	Cowpea	Innefficient	S	Acidic	633	*Burkholderia fungorum*	100 %	NR025058	KC879695
UFLA 03-16	Benjamim Constant, AM/SF	Cowpea	Not determined	S	Alkaline	647	*Enterobacter* sp.	100 %	DQ855282	KC879696
UFLA 03-17	Benjamim Constant, AM/SF	Cowpea	Innefficient	I	Acidic	640	Burkholderia fungorum	100 %	NR025058	KC879716
UFLA 03-18	Benjamim Constant, AM/SF	Cowpea	Innefficient	S	Acidic	440	*Pseudomonas* sp.	98 %	FJ482104	KC879697
UFLA 03-19	Benjamim Constant, AM/SF	Cowpea	Innefficient	F	Neutral	793	*Burkholderia fungorum*	100 %	NR025058	KC879698
UFLA 03-20	Benjamim Constant, AM/SF	Cowpea	Innefficient	S	Neutral	769	*Burkholderia fungorum*	100 %	NR025058	KC879699
UFLA 03-21	Benjamim Constant, AM/P	Cowpea	Efficient	S	Neutral	671	Not identified	–	–	–
UFLA 03-22	Benjamim Constant, AM/P	Cowpea	Innefficient	S	Acidic	778	*Pseudomonas koreensis*	100 %	NR025228	KC879700
UFLA 03-23	Benjamim Constant, AM/P	Cowpea	Innefficient	S	Neutral	656	*Pseudomonas koreensis*	99 %	NR025228	KC879701
UFLA 03-26	Benjamim Constant, AM/P	Cowpea	Innefficient	F	Acidic	643	*Pseudomonas* sp.	100 %	EU693553	KC879702
UFLA 03-27	Benjamim Constant, AM/P	Cowpea	Innefficient	S	Acidic	613	*Enterobacter* sp.	99 %	GQ478275	KC879717
UFLA 03-153	Poços de Caldas, MG/MR	Cowpea	Efficient	S	Alkaline	564	*Bradyrhizobium* sp.	98 %	FN600560	KC879718
UFLA 03-154	Poços de Caldas, MG/MR	Cowpea	Efficient	S	Alkaline	679	*Burkholderia fungorum*	100 %	NR025058	KC879703

Table 1 continued

Strain	Origin location/ SUT	Trap species[a]	Symbiotic efficiency: fixation of N_2 in cowpea[b]	Growth characteristics in 79 medium[c]		Number of base pairs	Identification based in the most similar sequence found in GenBank			Accession number in GenBank (NCBI) of the strain
				G.R.	pH		Species	Similarity %	Accession number	
UFLA 03-163	Poços de Caldas, MG/MR	Cowpea	Innefficient	S	Alkaline	692	*Bradyrhizobium elkanii*	99 %	FJ534721	KC879704
UFLA 03-164	Poços de Caldas, MG/MR	Cowpea	Efficient	S	Alkaline	699	*Bradyrhizobium elkanii*	99 %	FJ534721	KC879705
UFLA 03-165	Poços de Caldas, MG/MR	Cowpea	Efficient	S	Alkaline	798	*Acinetobacter genomosp.3*	100 %	FJ694758	KC879706
UFLA 03-170	Poços de Caldas, MG/MR	Cowpea	Innefficient	S	Alkaline	575	*Bradyrhizobium* sp.	100 %	FN600560	KC879707
UFLA 03-172	Poços de Caldas, MG/MR	Cowpea	Innefficient	S	Neutral	725	*Bradyrhizobium elkanii*	99 %	FJ534721	KC879719
UFLA 04-0110	Theobroma, Rondônia/AGRI	Siratro	Innefficient	S	Alkaline	788	*Burkholderia fungorum*	100 %	NR025058	KC879708
UFLA 04-1309	Pedro Peixoto, AC/F	Siratro	Innefficient	S	Alkaline	782	*Burkholderia fungorum*	100 %	NR025058	KC879709
UFLA 04-1020	RECA AC/AGRO	Siratro	Innefficient	S	Alkaline	742	*Burkholderia fungorum*	100 %	NR025058	KC879710
UFLA 04-885	Jí-Paraná, Rondônia/P	Siratro	Efficient	S	Alkaline	639	*Pseudomonas koreensis*	100 %	NR025228	KC879711
UFLA 04-0314	Theobroma, Rondônia/F	Siratro	Innefficient	S	Alkaline	791	*Burkholderia fungorum*	100 %	NR025058	KC879712
UFLA 04-321	Theobroma, Rondônia/F	Siratro	Innefficient	S	Alkaline	602	*Bradyrhizobium japonicum*	99 %	AY904765	KC879713
UFLA 04-546	Theobroma, Rondônia/P	Siratro	Innefficient	S	Alkaline	706	*Burkholderia fungorum*	100 %	NR025058	KC879714
UFLA 04-559	Theobroma, Rondônia/P	Siratro	Innefficient	S	Alkaline	789	*Burkholderia fungorum*	100 %	NR025058	KC879715

AM Amazônia state, *MG* Minas Gerais state, *AC* Acre state, *SF* Secondary forest, *P* Pasture, *AGRO* Agroforestry, *AGRI* Agriculture, *F* Fallow, *MR* Bauxite mining areas after recovery of vegetation, *BC* beans crop

[a] These promiscuous plant species were used to trap rhizobia from soil samples in these origins

[b] Based on shoot dry matter of inoculated plants compared to controls without inoculation: with and without mineral N. Experiments in Leonard jars. Soares et al. unpublished results

[c] Growth characteristics in 79 medium: G.R.: growth rate, F: fast (2–3 days), I: intermediate (4–5 days), S: slow (6–10 days); pH of culture medium after growth

volume 10 mL) containing 5 mL of semi-solid, nitrogen-free LO culture medium (Dreyfus et al. 1983), which composition L^{-1} was: 10 g sodium lactate, 1.67 g K_2HPO_4, 0.87 g KH_2PO_4, 0.05 g NaCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 40 mg $CaCl_2$, 4 mg $FeCl_3$, 5 mg $MoO_4Na{\cdot}2H_2O$, 10 mg biotin, 20 mg nicotinic acid, 10 mg pantothenic acid, 2 mL micronutrient solution (0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.235 g $MnSO_4{\cdot}H_2O$, 0.28 g H_3BO_3, 0.008 g $CuSO_4{\cdot}5H_2O$ and 0.024 g $ZnSO_4{\cdot}7H_2O$ dissolved in 200 mL of distilled water), 5 mL bromothymol blue (0.5 % in 0.2 N KOH), pH 7.0. Mannitol was also tested as sole carbon source by substituting sodium lactate in the LO medium by it. Each strain was tested in triplicate (3 vials/strain). The flasks were incubated for 3–7 days in the dark at 28 °C until a typical pellicle was formed near the surface of the medium. The inoculated samples were compared to the positive controls; inoculating strains that lead to pellicle formation during this period were considered free-living nitrogen-fixing bacteria, whereas those that did not form a pellicle were not considered free-living nitrogen-fixing bacteria.

Solubilisation of insoluble calcium and aluminium inorganic phosphates

Two experiments were carried out to establish whether the strains could solubilise calcium phosphate (P–Ca) or aluminium phosphate (P–Al). Solubilising activity (solubilisation ability and potential) was evaluated in GES medium, which composition L^{-1} was: 10 g glucose, 0.1 g KNO_3, 100 mL soil extract (The filtered supernatant of 1 kg soil in 1 L of distilled water, autoclaved and allowed to stand 48 h), 2 mL $MgSO_4$ (10 %), 2 mL $CaCl_2$ (1 %), 1 mL NaCl (10 %), 2 mL micronutrient solution (the same used in LO medium), 4 mL Fe-EDTA (1.64 %), and 15 g agar (Sylvester-Bradley et al. 1982). In the first experiment, P–Ca was obtained by adding 50 mL of a 10 % K_2HPO_4 solution and 100 mL of a 10 % $CaCl_2$ solution in 850 mL of culture medium with nutrient composition equivalent 1 L (all autoclaved separately) to produce an insoluble phosphate precipitate. In the second experiment, 3.04 g L^{-1} of $AlH_6O_{12}P_3$ was added. In the treatment containing P–Ca, the pH was adjusted to 6.8, whereas the pH was adjusted to 4.5 in the treatment with P–Al.

To obtain each inoculum, the strains were grown in liquid culture medium 79 (Fred and Waksman 1928), which composition L^{-1} was: 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 10.0 g mannitol and 0.4 g yeast extract, at a pH of 6.8. Saline solution (0.85 %) was added to the cultures to adjust the concentration of cells to an optical density at 600 nm (OD_{600}) of 0.5. Twenty microlitres of cell suspension was spotted at three equidistant points on a plate containing medium with the phosphate precipitate, thus resulting in three colonies per plate, with the experiment being repeated in triplicate for each strain (three plates). The diameter of the solubilisation halo (translucent area around the colony) was measured using a digital calliper daily during 18 days. These measurements were used to obtain the solubilisation index (SI), which was determined by the following equation: S.I = Halo diameter (mm)/Colony diameter (mm) (Berraquero et al. 1976). Based on the S.I, the strains were classified as having a low (S.I $<$ 2.00), medium (2.00 $\leq$ S.I $<$ 4.00) or high (S.I $\geq$ 4.00) solubilisation ability. Based to the onset of solubilisation, the strains were also classified as early (when the onset of solubilisation occurred until the third day), late (when the onset of solubilisation occurred after the third day) or non-solubilising (when solubilisation was not visible within 18 days).

Production of the growth hormone auxin

To determine whether the bacteria could produce indole-3-acetic acid (IAA), the experimental strains and the positive controls *Azospirillum brasilense* (BR 11001^T) and *A. lipoferum* (BR 11080^T) (Radwan et al. 2002; Tarrand et al. 1978) were grown in Dygs medium, which contains L^{-1}: 2 g glucose, 2 g malic acid, 1.5 g bacteriological peptone, 2 g yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$ and 1.5 g glutamic acid. After growth, the cultures were centrifuged, resuspended and adjusted to an OD_{600} of 0.5 using saline solution (0.85 %), as described before. Aliquots of the bacterial solutions (500 µL) were inoculated in 20 mL of Dygs medium (without L-tryptophan or supplemented with 100 mg L^{-1} of L-tryptophan) and incubated for 72 h at 30 °C with constant stirring. To quantify the indolic compounds produced after this period, the cultures were centrifuged at 17,792*g* for 10 min and 3 mL of supernatant was removed and mixed with 2 mL of Salkowski reagent (Sarwar and Kremer 1995). This mixture was placed in the dark for 30 min to develop a pink colour, which is indicative of IAA production. The colour intensity was determined in a spectrophotometer at 535 nm, following the methods described by Asghar et al. (2002). The concentration of IAA was estimated using a standard curve previously prepared with 0, 25, 50, 100, 150, 200 and 300 µg IAA mL^{-1} (Sigma-Aldrich) in sterilised, uninoculated culture media (Radwan et al. 2002).

Antifungal activity

The antagonistic activity of the strains against *Fusarium oxysporum* f. sp. *phaseoli*, a common bean pathogen, was studied following the methods of Peix et al. (2001b) with some modifications. Fungal mycelia grown on 5 mm discs in PDA (Potato Dextrose Agar, Difco) with pH 6.8 were placed in the centre of a Petri dish containing the same culture medium. Isolated colonies from three bacterial strains were streaked onto three areas of the plate around the disc. Each

strain was tested in triplicate (3 plates/strain). Mycelia growth in the absence of bacteria served as the control. The plates were incubated for 3–7 days at 28 °C depending on the genus. The results were evaluated by looking for a zone of inhibition in fungal growth in the presence of bacteria.

Bacterial antibiotic resistance

Bacterial resistance to different antibiotics was evaluated by saturated disc diffusion technique in Petri dishes containing 79 solid medium (15 g agar L^{-1}, pH 6.8). The antibiotics studied were azithromycin (15 μg), streptomycin (10 μg), erythromycin (15 μg), ampicillin (10 μg), chloramphenicol (30 μg), rifamycin (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), clarithromycin (15 μg), amoxicillin (10 μg), gentamicin (10 μg) and vancomycin (30 μg) (Cecon™, Brazil).

Bacteria were grown in 79 liquid medium for 3 days with constant stirring. After the incubation, 100 μL of each bacterial culture was spread onto Petri dishes containing the 79 solid medium. Subsequently, using sterile forceps, three discs saturated with different antibiotics were added to each plate. Each strain was tested in triplicate (3 plates/strain). The discs were lightly pressed and kept equidistant from one another to prevent the inhibition zones from overlapping. The plates were inverted and incubated for 3–7 days at 28 °C. After this period, the diameter of the growth inhibition halo (a translucent area around the disc) was measured using a digital calliper.

Statistical analysis

The statistical analyses were performed using the Sisvar program, version 5.3 (Ferreira 2008) and the Scott-Knott test (5 % probability) to compare mean values. All tests were completely randomised.

Results

Genetic identification of strains

The 16S rRNA sequences from the 26 bacterial isolates evaluated were aligned against sequences available from GenBank and revealed the presence of five different genera of bacteria: *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, and *Bradyrhizobium*. Of these genera, only the last two were previously known as nodule-forming genera. Two strains (UFLA 03-05, UFLA 03-21) were not identified because the amplification of its 16S rRNA gene was not possible. The similarity among the sequences studied through GenBank access was between 98 and 100 % (Table 1).

Free-living nitrogen fixation

The strains UFLA 03-14 (*Enterobacter* sp.), UFLA 03-17, UFLA 04-0110, UFLA 04-242, UFLA 04-243, UFLA 04-0314, UFLA 04-546, and UFLA 04-559 (*Burkholderia fungorum*) were able to fix nitrogen while in their free-living state. The nitrogen-fixing strains formed a pellicle close to the LO culture medium surface in the presence of the two carbon sources; lactate and mannitol. The positive controls BR 5401^T and ORS 571^T did not form a pellicle when sodium lactate was replaced by mannitol.

Solubilization of insoluble calcium and aluminum inorganic phosphates

The ability to solubilize calcium and aluminum phosphates varied among strains in each genus (Tables 2, 3). Eighteen of the 31 strains (58 %), which belonged to all the five genera, could solubilize Calcium phosphate (P–Ca) in solid culture medium, and UFLA 03-15 and UFLA 04-0314 (*B. fungorum*) exhibited the highest solubilization indexes (SI) (2.75 and 2.25, respectively) (Table 2). All of the P–Ca solubilizing strains showed early solubilization behavior except for UFLA 03-20 and UFLA 04-242 (*B. fungorum*), which showed late solubilization behavior. The ability to solubilize aluminum phosphate (P–Al) was seen in 13 (42 %) strains belonging to all the five genera; all of the strains had low solubilization ability, with indexes varying between 1.00 and 1.81 mm and showed late solubilization behavior (Table 3) except for UFLA 04-559 (*B. fungorum*), which showed early solubilization behavior. Strains BR 3267 (*B. japonicum*), UFLA 03-165 (*Acinetobacter genomosp.3*), and UFLA 03-154, UFLA 03-20, UFLA 03-17, UFLA 04-559, and UFLA 04-242 (*B. fungorum*) solubilized both P–Ca and P–Al.

Production of the growth hormone auxin

Among the 31 strains studied, 29 (94 %) showed the ability to synthesize indole-3-acetic acid (IAA) when L-tryptophan was added to the culture medium (Fig. 1). However, 23 (74 %) of the strains and both of the control strains (BR 11080^T and BR 11001^T) synthesized IAA in the absence of L-tryptophan. Strains UFLA 03-153 and UFLA 03-170 (*Bradyrhizobium* spp.) did not produce IAA either in the presence or the absence of L-tryptophan. In the absence of L-tryptophan, UFLA 03-163, UFLA 03-172, and INPA 03-11B (*Bradyrhizobium elkanii*) and UFLA 04-559 (*B. fungorum*), UFLA 03-21 and UFLA 03-05 (unidentified) did not produce IAA. Production of IAA among the species varied between 0.00 and 12.59 μg mL^{-1} in culture medium that was not supplemented with L-tryptophan; and between 0.00 and 42.28 in culture medium that was supplemented with L-tryptophan. The

Table 2 Onset of solubilisation (On. Sol.) and solubilisation index (S.I) of calcium phosphate by strains grown in GES medium

Strains genus or species	On. Sol. (days)[a]	S.I (mm)		Strains genus or species	On. Sol. (days)[a]	S.I (mm)	
		Initial	End			Initial	End
Bradyrhizobium spp.				*Pseudomonas* spp.			
UFLA 03-84^T	NG	–	–	UFLA 03-18	NG	–	–
INPA 03-11B	3	1,21[b]	1,56	UFLA 03-22	3	1,67	1,89
BR 3267	3	1,94	1,73	UFLA 03-23	NG	–	–
UFLA 03-153	GNFH	–	–	UFLA 03-26	NG	–	–
UFLA 03-163	3	1,82	2,00	UFLA 04-244	NG	–	–
UFLA 03-164	NG	–	–	*B. fungorum*			
UFLA 03-170	NG	–	–	UFLA 03-15	6	1,38	1,38
UFLA 03-172	NG	–	–	UFLA 03-17	3	1,00	2,04
UFLA 04-321	NG			UFLA 03-19	3	2,13	2,03
Enterobacter sp.				UFLA 03-20	9	2,05	1,69
UFLA 03-14	NG	–	–	UFLA 03-154	3	2,11	2,01
UFLA 03-16	3	2,72	2,72	UFLA 04-0110	3	1,33	2,01
UFLA 03-27	3	1,57	1,57	UFLA 04-242	6	2,12	2,12
Acinetobacter spp.				UFLA 04-243	3	2,14	2,15
UFLA 03-165	3	2,36	2,41	UFLA 04-0314	3	2,81	2,25
Not identified				UFLA 04-546	12	2,08	2,37
UFLA 03-05	GNFH	–	–	UFLA 04-559	3	1,25	2,10
UFLA 03-21	NG	–	–				

[a] Onset of Solubilisation

[b] S.I = halo diameter (mm)/ colony diameter (mm). *NG* No growth. *GNFH* Grew but did not form a halo by the 18th day

Table 3 Onset of solubilisation (On. Sol.) and Solubilisation index (S.I) of aluminium phosphate by strains grown in GES medium

Strains genus or species	On. Sol. (days)[a]	S.I (mm)		Strains genus or species	On. Sol. (days)[a]	S.I (mm)	
		Initial	End			Initial	End
Bradyrhizobium spp.				*Pseudomonas* spp.			
UFLA03-84^T	6	1,27[b]	1,42	UFLA 03-18	6	1,00	1,16
INPA03-11B	GNFH	–	–	UFLA 03-22	GNFH	–	–
BR 3267	6	1,31	1,29	UFLA 03-23	NG	–	–
UFLA 03-153	6	1,30	1,22	UFLA 03-26	NG	–	–
UFLA 03-163	GNFH	–	–	UFLA 04-244	12	1,77	1,81
UFLA 03-164	NG	–	–	*B. fungorum*			
UFLA 03-170	NG	–	–	UFLA 03-15	GNFH	–	–
UFLA 03-172	CNFH	–	–	UFLA 03-17	6	1,43	1,40
UFLA 04-321	12	1,29	1,33	UFLA 03-19	GNFH	–	–
Enterobacter sp.				UFLA 03-20	6	1,31	1,31
UFLA 03-14	6	1,37	1,27	UFLA 03-154	6	1,00	1,09
UFLA 03-16	GNFH	–	–	UFLA 04-0110	CNFH	–	–
UFLA 03-27	GNFH	–	–	UFLA 04-242	12	1,42	1,35
Acinetobacter spp.				UFLA 04-243	GNFH	–	–
UFLA 03-165	6	1,77	1,78	UFLA 04-0314	GNFH	–	–
Not identified				UFLA 04-546	NG	–	–
UFLA 03-05	GNFH	–	–	UFLA 04-559	3	1,27	1,27
UFLA 03-21	GNFH	–	–				

[a] Onset of Solubilisation

[b] S.I = halo diameter (mm)/ colony diameter (mm). *NG* No growth. *GNFH* Grew but did not a form halo by the 18th day

maximum production of IAA was attained by the strain UFLA 03-14 (*Enterobacter* sp.), while UFLA 03-17 and UFLA 03-15 (*B. fungorum*) produced slightly less IAA than the positive control BR 11001^T (*Azospirillum brasilense*) in the absence of L-tryptophan. Greater significant differences in the production of IAA were observed when the culture medium was supplemented with L-tryptophan. In media supplemented with L-tryptophan, strain UFLA 04-321 (*Bradyrhizobium*

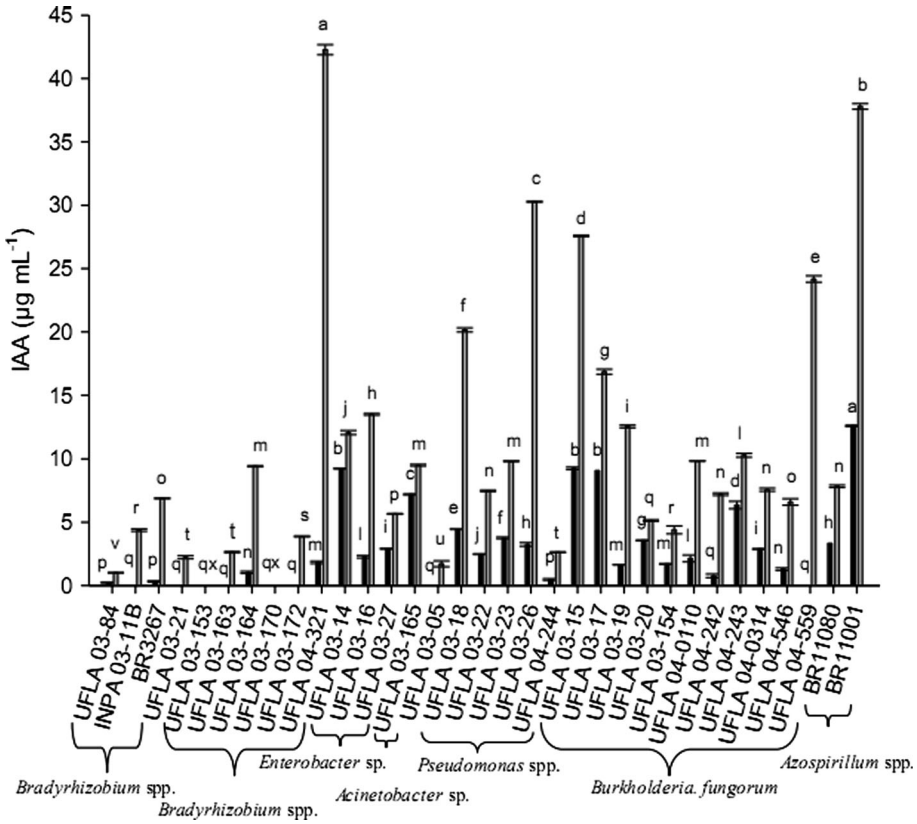

Fig. 1 Production of indole-3-acetic acid (IAA) by strains grown in Dygs medium in either the presence of 100 mg L^{-1} of L-tryptophan (grey bars) or in the absence of the amino acid (black bars). Values followed by the same letter under the same treatment were not significantly different according to the Scott-Knott test at 5 % probability

japonicum) exhibited a significantly larger production of IAA relative to the strain BR 11001^{T}.

Antifungal activity and bacterial antibiotic resistance

None of the strains exhibited the potential to inhibit the growth of *Fusarium oxysporum* f. sp. *phaseoli*.

Virtually all of the strains were resistant at least to four of the 12 antibiotics studied except for UFLA 04-0314 (*B. fungorum*), which was resistant only to three (Table 4). Four strains, UFLA 03-153, UFLA 03-164, UFLA 03-170, and UFLA 03-172 (*Bradyrhizobium*), were resistant to 11 of the 12 antibiotics studied. UFLA 03-84 (*Bradyrhizobium* sp.) exhibited resistance to all of the antibiotics studied. In general, the *Bradyrhizobium* genus was the most resistant to antibiotics. The strains were resistant most often to chloramphenicol and vancomycin, followed by ampicillin. Only UFLA 03-84 and UFLA 03-172 (*Bradyrhizobium* spp.) were resistant to rifamycin.

Discussion

The strains UFLA 03-153 and UFLA 03-164, which are highly efficient in BNF when grown in symbiosis with the cowpea bean (Soares et al. unpublished results) (Table 1), and the strains currently approved as inoculants, UFLA 03-84, INPA 03-11B, and BR 3267, exhibited the ability to perform additional plant-growth-promoting processes. Tables 1 and 2 show that the strain BR 3267 can solubilize P–Ca and produce IAA independently from the addition of L-tryptophan. UFLA 03-153 and UFLA 03-84 solubilized P–Al and synthesized IAA, whereas the former only synthesized IAA in the presence of L-tryptophan. UFLA 03-11B solubilized P–Ca and synthesized IAA independently with the addition of L-tryptophan. All of the strains that were efficient in BNF when grown in symbiosis with the cowpea exhibited an initial resistance to more than eight antibiotics and eventually to all 12 antibiotics studied. None of the BNF strains that grow efficiently in symbiosis with the cowpea fixed nitrogen in the free-living state. The strains UFLA 04-0110 and UFLA 04-0321, which exhibited the greatest potential to synthesize IAA in both the absence and presence of L-tryptophan, could both solubilize phosphates (P–Ca and P–Al). UFLA 04-0110 was resistant to four antibiotics, and UFLA 04-0321 was resistant to nine antibiotics.

Several authors have previously demonstrated that some strains of the genus *Burkholderia* can fix nitrogen when grown either symbiotically (Elliott et al. 2007; Vandamme et al. 2002) or in association with plants (Caballero-Mellado et al. 2004). However, reports on nitrogen fixation by *B. fungorum* species grown either in symbiosis with the common bean (Ferreira et al.2012) or in the free-living

Table 4 Resistance of strains to different antibiotics

Strains	AZI	STR	ERY	AMP	CHL	RFM	KAN	NAL	CLA	AMO	GEN	VAN	Σ(R)
Genera or species	Diameter of inhibition halo (mm)												
Bradyrhizobium spp.													
UFLA03-84^T	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	12
INPA03-11B	R(a)	R(a)	R(a)	R(a)	R(a)	14,41(c)	21,69(d)	R(a)	R(a)	7,35(b)	R(a)	R(a)	9
BR 3267	R(a)	13,14(b)	R(a)	20,08(c)	R(a)	13,19(b)	R(a)	R(a)	R(a)	19,21(c)	R(a)	R(a)	8
UFLA 03-153	R(a)	R(a)	R(a)	R(a)	R(a)	15,38(b)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	11
UFLA 03-163	R(a)	R(a)	R(a)	R(a)	R(a)	11,87(b)	22,15(c)	R(a)	R(a)	R(a)	R(a)	R(a)	10
UFLA 03-164	R(a)	R(a)	R(a)	R(a)	R(a)	13,19(b)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	11
UFLA 03-170	R(a)	R(a)	R(a)	R(a)	R(a)	9,91(b)	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	11
UFLA 03-172	R(a)	R(a)	R(a)	R(a)	R(a)	R(a)	12,94(e)	R(a)	R(a)	R(a)	R(a)	R(a)	11
UFLA 04-321	R(a)	16,52(b)	R(a)	R(a)	R(a)	42,49(d)	18,85(c)	R(a)	R(a)	R(a)	R(a)	R(a)	9
Enterobacter sp.													
UFLA 03-14	R(a)	11,72(b)	R(a)	R(a)	R(a)	16,22(c)	20,32(d)	14,86(c)	R(a)	R(a)	10,52(b)	R(a)	7
UFLA 03-16	R(a)	15,20(c)	R(a)	R(a)	29,04(d)	14,90(c)	15,17(c)	32,27(e)	R(a)	R(a)	10,67(b)	10,45(b)	5
UFLA 03-27	R(a)	13,56(c)	R(a)	R(a)	R(a)	13,19(c)	14,05(c)	22,51(d)	R(a)	14,86(c)	9,30(b)	9,41(b)	5
Acinetobacter spp.													
UFLA 03-165	R(a)	10,40(c)	10,60(c)	11,74(c)	R(a)	18,13(e)	14,80(d)	20,53(f)	R(a)	11,66(c)	7,85(b)	R(a)	4
Not identified													
UFLA 03-05	R(a)	12,79(d)	R(a)	R(a)	27,61(f)	12,49(d)	16,20(e)	28,79(f)	R(a)	R(a)	9,69(c)	6,52(b)	5
UFLA 03-21	R(a)	R(a)	R(a)	15,78(b)	R(a)	21,15(c)	R(a)	R(a)	R(a)	20,16(c)	R(a)	R(a)	9
Pseudomonas spp.													
UFLA 03-18	R(a)	8,72(b)	R(a)	R(a)	R(a)	22,10(d)	21,38(d)	11,11(c)	R(a)	R(a)	10,66(c)	R(a)	7
UFLA 03-22	R(a)	13,25(b)	R(a)	R(a)	R(a)	15,81(c)	21,01(c)	21,32(d)	R(a)	R(a)	12,69(b)	R(a)	7
UFLA 03-23	R(a)	13,43(c)	R(a)	R(a)	R(a)	14,62(c)	20,76(d)	19,26(d)	R(a)	R(a)	9,41(b)	R(a)	7
UFLA 03-26	R(a)	R(a)	R(a)	R(a)	R(a)	16,44(d)	13,96(c)	19,49(e)	R(a)	R(a)	9,45(b)	R(a)	8
UFLA 04-244	R(a)	19,41(c)	19,83(c)	R(a)	R(a)	25,10(d)	24,60(d)	18,04(c)	R(a)	19,49(c)	9,56(b)	R(a)	5
B. fungorum													
UFLA 03-15	R(a)	9,13(b)	R(a)	R(a)	R(a)	12,56(c)	13,23(c)	23,79(d)	R(a)	R(a)	7,62(b)	R(a)	7
UFLA 03-17	26,19(f)	19,59(d)	19,07(d)	R(a)	R(a)	21,97(e)	25,13(f)	33,65(g)	16,73(c)	R(a)	11,28(b)	R(a)	4
UFLA 03-19	27,04(g)	20,17(d)	18,82(c)	R(a)	R(a)	22,16(e)	24,21(f)	34,26(h)	17,78(c)	R(a)	11,13(b)	R(a)	4
UFLA 03-20	R(a)	16,56 (c)	R(a)	R(a)	26,06(e)	17,95 (d)	15,92(c)	35,94 (f)	R(a)	R(a)	12,18 (b)	R(a)	6
UFLA 03-154	27,95(g)	11,89(c)	R(a)	R(a)	R(a)	18,90(f)	14,40(d)	32,59(h)	16,63(e)	R(a)	9,57(b)	R(a)	5
UFLA 04-0110	27,62(f)	20,40(d)	18,98(c)	R(a)	R(a)	24,74(e)	25,50(e)	30,91(g)	18,44(c)	R(a)	9,38(b)	R(a)	4
UFLA 04-242	23,92(g)	19,81(e)	15,54(d)	R(a)	R(a)	21,62(f)	24,85(g)	30,09(h)	12,35(c)	R(a)	8,48(b)	R(a)	4
UFLA 04-243	24,59(g)	19,38(f)	13,58(d)	R(a)	R(a)	17,14(e)	25,16(g)	30,83(h)	11,94(c)	R(a)	9,23(b)	R(a)	4
UFLA 04-0314	28,78(g)	19,92(e)	14,77(d)	6,99(b)	R(a)	20,85(e)	25,98(f)	33,17(h)	20,01(e)	R(a)	10,48(c)	R(a)	3

Table 4 continued

Strains	AZI	STR	ERY	AMP	CHL	RFM	KAN	NAL	CLA	AMO	GEN	VAN	Σ(R)
UFLA 04-546	24,25(f)	21,10(e)	13,69(c)	R(a)	R(a)	18,82(d)	24,83(f)	33,35(g)	14,50(c)	R(a)	10,84(b)	R(a)	4
UFLA 04-559	24,32(f)	19,48(e)	13,96(c)	R(a)	R(a)	16,70(d)	24,38(f)	23,81(f)	15,02(c)	R(a)	11,01(b)	R(a)	4
∑(R)	22	9	21	27	28	2	6	10	22	25	11	28	

Antibiotic resistant bacteria (halo = 0.00 = R), azithromycin (AZI), streptomycin (STR), erythromycin (ERY), ampicillin (AMP), chloramphenicol (CHL), rifamycin (RFM), kanamycin (KAN), nalidixic acid (NAL), clarithromycin (CLA), amoxicillin (AMO), gentamicin (GEN) and vancomycin (VAN). In each line, values followed by the same letter are not significantly different according to the Scott-Knott test at 5 % probability

state are quite recent (Oliveira-Longatti et al. 2013; Silva et al. 2012). This strain was isolated from Amazon region by using siratro as trap species. In our work we show that *B. fungorum* able to establish symbiosis with cowpea is also able to fix N_2 in the free living state. Our strain was isolated from bauxite mining areas. The positive controls BR 5401^T and ORS 571^T, which did not form a pellicle when sodium lactate was replaced by mannitol, confirm the results found by Dreyfus et al. (1998) and Moreira et al. (2006) that the strains of *Azorhizobium* do not use mannitol as a carbon source. The pellicle is only formed in medium with lactate.

The solubilization of P–Ca by the strains from *Burkholderia* and *Enterobacter* has also been previously reported (Collavino et al. 2010; Peix et al. 2001b). When inoculated into common bean plants, the strain SAOCV2 from the *Burkholderia cepacia* species caused a significant (44 %) increase in the P content of plants that were cultivated in the soil relative to plants that were cultivated in non-inoculated soil (Peix et al. 2001b). Collavino et al. (2010) showed that the strain *Enterobacter aerogenes* R4M-A significantly increased the phosphorus content of the aerial part of the beans grown in insoluble phosphate conditions compared to beans inoculated with the strain *Burkholderia sp.* R4M-F or non-inoculated plants.

The three strains currently approved for the inoculation of the cowpea bean were tested by Marra et al. (2011) in GELP culture medium (yeast extract, glucose, peptone, soil extract, and mineral salts) and UFLA 03-84 and BR 3267 showed a solubilization index (SI) of 2.12 and 1.78 for P–Ca and 1.22 and 1.20 for P–Al, respectively. INPA 03-11B did not solubilize P–Al and P–Ca medium. In our study, UFLA 03-84 did not grow in GES culture medium containing P–Ca. However, it showed a SI for P–Al similar to that reported by Marra et al. (2011). The SI for P–Ca and P–Al in the BR 3267 strain were similar between the studies. In our study, the strain INPA 03-11B grew, but did not solubilize the culture medium with P–Al, and had a SI of 1.56 for P–Ca. The main difference between these two culture media is that GELP medium contains peptone and yeast extract, which seems to affect the solublization ability of INPA 3-11B and UFLA 3-84 depending on the type of phosphate.

The presence of P–Ca-solubilizing bacteria with a high solubilization index (SI > 4) has previously been observed in bacteria from Amazonian soils. This high solubilization potential was observed by Hara and Oliveira (2004) in a non-identified nodule-forming bacterium, indicating that bacteria with high potential to solubilize insoluble phosphate might be found in Amazonian soils.

The strains of the genera *Burkholderia* and *Bradyrhizobium* that did not produce IAA in the absence of L-tryptophan probably did not have an active indole-3-pyruvate

pathway (IpyA) under these culture conditions, because the ability of nodule-forming bacteria to synthesize IAA involves three synthesis pathways: indole-3-acetamide (IAM), indole-3-pyruvate (IpyA) and tryptamine (TAM) (Theunis et al. 2004). However, the IpyA pathway is independent from L-tryptophan, which is a precursor amino acid in the other two pathways. In soil conditions, the exposure of the roots to exogenous bacterial IAA could affect the plant growth in different ways including pathogenesis, growth inhibition, or phytostimulation (Spaepen et al. 2007). The IAA, as well as other hormones, stimulates plant growth only within a narrow concentration range; the lower concentrations of IAA are not effective, and the higher concentrations are toxic (Biswas et al. 2000). Studies have verified that the *Rhizobium leguminosarum* strain TV-13, which produces 171.17 μg mL^{-1} of IAA, negatively affected the growth of lettuce seedlings. Conversely, strains of *Bradyrhizobium* sp. (T6-4, T6-12, C-3, V-10), which produce from 1.2 to 3.3 μg mL^{-1} of IAA, were shown to increase the vigor of seedlings relative to treatment without inoculation (Schlindwein et al. 2008). In our study, values as high as those mentioned by other authors were not observed for the nodule-forming and endophytic bacteria, indicating that these strains might act as phytostimulants; this hypothesis needs to be verified in studies testing the effect of inoculation in plants.

Our study corroborates the study of Florentino et al. (2012) showing that strain, UFLA 03-84 (*Bradyrhizobium* sp.), has resistance to all of the studied antibiotics; this strain is an approved inoculant for the cowpea bean (Lacerda et al. 2004; Soares et al. 2006). This demonstrates a selective advantage over the remainder of the microorganisms, making this strain more competitive in the soil and thus indicating an indispensable trait for the establishment of symbiosis; this may explain its success as an inoculant. All of the *Bradyrhizobium* strains in this study, including the other two strains approved for the cowpea bean (BR 3267 and INPA 03-11B), had considerable resistance to at least eight among the 12 antibiotics tested. These results indicate that the cowpea symbiotic nitrogen-fixing strains are well adapted to overcome amensalistic relationships among the biological populations in the soil that can produce antibiotics, as eight out of the 12 antibiotics tested are produced by microorganisms.

The performance of other biotechnological processes that both contribute to plant growth and have the ability to adapt to several types of stress adds considerable value to the diazotrophic free-living and/or symbiotic bacteria. To evaluate the joint action of these processes on plant growth under the complex, heterogeneous, and dynamic conditions of the field, edaphic systems is being investigated in the next phase of this study.

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