Vol. 13(42), pp. 2351-2361, 18 October, 2018
DOI: 10.5897/AJAR2018.13256
Article Number: 4B5FBF158995
ISSN: 1991-637X
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African Journal of Agricultural Research

Full Length Research Paper

Evaluation of diazotrophic bacteria from rice varieties (*Oryza sativa* L.) grown in Rio de Janeiro State, Brazil

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Received 19 May, 2018; Accepted 5 September, 2018

The indiscriminate application of nitrogen fertilizer in order to increase rice crop productivity contributes to environmental degradation. One of the possible alternatives to ease up this problem is the use of sustainable and cost-effective technologies such as biofertilizers to promote plant growth. This work aimed to isolate, characterize and select diazotrophic bacteria associated with rice cultivars EPAGRI-109 and BRS TROPICAL. About 39 bacteria belonging to the genera *Azospirillum*, *Herbaspirillum*, *Paenibacillus*, *Ideonella* and *Pseudomonas* were isolated. Based on the best results of acetylene reduction assay (ARA), indolic compounds production and phosphate solubilization, the isolates were selected as potential plant growth promoters which were tested on rice cultivars IR 42, EPAGRI 109 and BRS TROPICAL under axenic condition and the most efficient isolates promoted up to 31% of dry weight of the evaluated rice cultivars. Two promising isolates were selected in this work (*Paenibacillus* species strain EP3-16N and *Nitrospirillum* species strain EP4-1L), however, more studies are necessary to confirm this capacity in field conditions.

Key words: Plant growth promoters, biological nitrogen fixation, rice cultivars.

INTRODUCTION

Rice (*Oryza sativa* L.) has been playing an important nutritional role for more than half of the world population, and about 486.67 million ton of rice was consumed worldwide in 2016/2017 (United States Department of Agriculture [USDA], 2018). Brazil is the world's 10th largest rice producer and it is produced mostly in the wetlands ecosystem of the Southern region under flooded condition (USDA, 2018).

Rice cropping requires considerable amounts of nutrients for development and growth and, nitrogen is particularly important for rice cultivation. Association of this crop with several nitrogen-fixing bacteria might contribute a part of nitrogen required by the plant (Choudhury and Kennedy, 2005).

Nitrogen contribution derived from biological nitrogen fixation (BNF) is a promising alternative to circumvent N

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losses associated to rice cropping. The BNF occurs in some prokaryotic organisms, such as, bacteria and cyanobacteria which are capable of converting N_2 into NH_3 (Baca et al., 2000; Baldani and Baldani, 2005; Kraiser et al., 2011).

The enzyme nitrogenase which is present in free-living or symbiotic diazotrophic bacteria is extremely versatile as it transforms N_2 into ammonia and catalyzes the reduction of several other substrates such as acetylene. As a consequence, the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) became the basis of indirect estimation of nitrogenase activity (Boddey et al., 1990; Stoltzfus et al., 1997; Moreira and Siqueira, 2006; Mus et al., 2016).

Many genera of diazotrophs such as *Azospirillum, Herbaspirillum, Burkhoderia* and *Sphingomonas* have been isolated from rice and have shown positive effects on many rice varieties (Baldani et al., 2000; Tran Van et al., 2000; Baldani and Baldani, 2005; Rodrigues et al., 2006; Rodrigues et al., 2008; Pedraza et al., 2009; Araújo et al., 2013).

These microorganisms colonize the interior and exterior of plant organs exerting beneficial functions (in addition to BNF) to diseases either directly or indirectly by antagonizing the pathogenic fungi through siderophores, chitinase and antibiotics production as well as phosphate solubilization, phytohormones production, etc (Downing et al., 2000; Baldani et al., 2002; Lee et al., 2004; Videira et al., 2009; Figueredo et al., 2010).

Indole acetic acid (IAA) is the most abundant phytohormone that regulates various aspects of plant developmental functions (Teale et al., 2006) reflected by the complexity of their biosynthesis, transport and signaling pathways (Santner et al., 2009). Studies have revealed great variability of auxin production among these strains; also, their inoculation promotes plant growth (Asghar et al., 2002; Khalid et al., 2004; Kuss et al., 2007; Richardson et al., 2009).

Solubilization of phosphates is presented by many bacteria and fungi in soil and in association with different plant species altogether. The metabolic processes involved in P solubilization and mineralization include excretion of hydrogen ions, release of organic anions, siderophores and phosphatases (Richardson, 2001). Solubilization of phosphate compounds is very common *in vitro,* but it has not been reported frequently because of technical difficulties in uncontrolled environment (Khan et al., 2007; Estrada et al., 2013).

Rice cultivars EPAGRI-109 and BRS TROPICAL are recommended for planting in Rio de Janeiro State and their associations with plant growth promoting bacteria have been previously reported (Kuss et al., 2007; Viana et al., 2015); however, no data is available to diazotrophic bacteria associated with these cultivars in Rio de Janeiro State.

According to some authors, native isolates of diazotrophic bacteria may contribute to greater nitrogen gains in Poaceae since it is locally adapted to environmental conditions (Santos et al., 2015; Viana et al., 2015).

The work aimed to isolate, characterize and select diazotrophic bacteria associated with rice cultivars IR-42, EPAGRI-109 and BRS TROPICAL grown in Rio de Janeiro State.

MATERIALS AND METHODS

Sampling

Two rice cultivars (*O. sativa* L.), EPAGRI-109 and BRS TROPICAL, and their rhizospheric soils were used for enumeration and isolation of diazotrophic bacteria. Samples from cultivar EPAGRI-109 were obtained at field condition while those of cultivar BRS TROPICAL were derived from seeds cultivated at greenhouse condition because during the isolation none was planted in the field.

EPAGRI–109 plants were collected during January 2013 100 days of growth (DAG) and 120 DAG during the vegetative growth stage in Campo dos Goytacazes, RJ, Brazil field.

Seeds of BRS TROPICAL were inoculated with Sp 245 to evaluate if the inoculation would alter bacteria groups associated with rice plants. The seeds of BRS TROPICAL were sown in a randomized experimental design in the greenhouse using 12 pots each containing 16 seeds and 20 kg un-sterile soil of an Ultisol soil collected from Embrapa Agrobiologia experimental field at Seropédica, RJ. The treatments were inoculated and un-inoculated with *Azospirillum brasilense* strain Sp 245 with 4 replicates.

Isolation of diazotrophic bacteria

EPAGRI-109 and BRS TROPICAL plants were individually wrapped in plastic bags and brought to the Laboratory of Grasses at Embrapa Agrobiologia, RJ for the extraction of soil adhered to roots (rhizospheric soil), shoots and roots. Diazotrophic bacteria were isolated using the serial dilution technique. 10 g rhizospheric soil was transferred to flasks containing 90 ml saline solution of K_2HPO_4 (100 mg L^{-1}), $MgSO_4$ (50 mg L^{-1}), NaCl, (20 mg L^{-1}), $CaCl_2.2H_2O$ (50 mg L^{-1}), and FeEDTA (16.4 mg L^{-1}). The roots and shoots were washed in tap water and then surface disinfested in chloramine T (1%), chopped into 10-cm pieces and 10 g were transferred to flasks containing 90 ml saline solution. After homogeneization by blending, each sample was diluted up to 10^{-6} level with saline solution. Thereafter, 0.1 ml of each dilution extract was used to inoculate vials containing 5 ml JNFb (Döbereiner et al., 1995), Nfb (Döbereiner et al., 1995) and LGI (Magalhães et al., 1983) semi-solid media. The diazotrophs population per gram of fresh tissue was enumerated by the Most Probable Number (MPN) technique according to the McCrady's table (Döbereiner et al., 1995).

Acetylene reduction activity (ARA)

Isolates from EPAGRI-109 were obtained using JNFb, NFb and LGI nitrogen free semi-solid media and those from BRS TROPICAL were obtained using JNFb and NFb semi-solid media. The positive growth was evaluated after the appearance of a typical diazotrophic bacterial pellicle in the subsurface of the medium after incubation for 7 to 10 days at 30°C. The bacteria were purified on potato agar medium (Döbereiner et al., 1995).

ARA was performed to estimate nitrogenase activity following Boddey et al. (1990). Isolates were cultured in NFb, JNFB or LGI semi-solid (without bromothymol blue) and incubated at 30°C, then evaluated after 24, 48, 72 and 96 h in different flasks. Flasks were

sealed with rubber caps, injected with 10% acetylene (v/v of gas phase) and incubated for 1 h at 30°C. Aliquots of 0.5 ml of gas samples were analysed through a Perkin Elmer F11 model gas chromatograph with Porapak N column (50 cm, 40°C). Diazotrophic bacteria *A. brasilense* Sp245, *Nitrospirillum amazonense* CBAmC and *Herbaspirillum seropedicae* ZAE94 were used as positive control. Total proteins were determined according to Bradford method (Bradford et al., 1976). Absorbance was recorded through a Labsystems iEMS Microplate Reader MF at 595 nm and protein contents were estimated using bovine serum albumin (BSA) calibration curve (7.5 to 150.0 µg ml^{-1}) as external standard.

Indolic compounds production

Total indolic compounds produced by isolates were quantified in microplates as described by Sarwar and Kremer (1995). For this test, 1 ml of bacterial culture previously cultured for 24 h in DYGS medium (Rodrigues Neto et al., 1986) was inoculated into 5 ml of DYGS medium supplemented with L-tryptophan at the final concentration of 100 µg ml^{-1}. The tubes remained in the dark under agitation of 150 rpm at a temperature of 30°C for 48 h. 1 ml aliquots were removed and centrifuged at 5000 ° g for 15 min.

In microplate 96-well, 150 µl aliquot of the 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) previously prepared. The samples remained in the dark for 30 min at room temperature and the absorbance reading was done on a microplate reader (Labsystem IEMS Reader MF, Labsystem) to a wavelength of 540 nm. The quantification of indole compounds was evaluated using a calibration curve prepared with serial dilutions of synthetic IAA standards (5 to 100 µg tryptophan ml^{-1}). Cultures of *Gluconacetobacter diazotroficus* (PAL5), *A. brasilense* (Sp245), *H. seropedicae* (ZAE94) and *N. amazonense* (CBAmc) were used as positive controls. Concentration was estimated with a standard calibration curve of indole acetic acid (10 to 80 mg ml^{-1}). Values are presented as mg of indolic compounds per mg of proteins, determined by the Bradford method (Bradford et al., 1976). After the determination of ARA, 20 µl of pellicle and culture medium mix was added into 20 µl 1 M NaOH and 60 µl of sterile distilled water and placed in a water bath at 90°C for 5 min to promote cell disruption. Subsequently, 900 µl of the Bradford reagent was added, the tubes were mixed by vortex and incubated for 3 min at room temperature.

Phosphate solubilization

Phosphate solubilization was evaluated using NBRIP agar medium containing $Ca_3(PO_4)_2$ (Nautiyal, 1999). Cells were grown in DYGS liquid medium at 30°C for 24 h at 150 rpm; thereafter, culture optical density was adjusted to $O.D_{0.9\text{-}1.0}$ at 600 nm. Aliquots (20 µL) of each culture were spotted onto NBRIP solid media, in triplicate. Growth and halo formation (mm) were measured at 48 h intervals during 15 d incubation at 30°C.

Molecular characterization

Total DNA extraction was performed using cetyl trimethylammonium bromide (CTAB) protocol as described in Sambrook et al. (1989). The isolates were inoculated in liquid DYGS medium at 30°C for 24 h under constant agitation and the bacterial culture was transfered to 2 ml microtube centrifuged at 4,000 x g for 8 min. The pellet was suspended in 567 µl $T_{10}E_1$ (10 mM Tris pH 8.0 and 1 mM NA_2EDTA) and homogenized vigorously; further, 30 µl of 10% SDS was added and homogenized by inversion with 3 µl of Proteinase K (20 mg ml^{-1}) and then incubated at 65°C for 20 min.

After 100 µl of 5 M sodium chloride was added and homogenized by inversion, an additional 80 µl of CTAB / NaCl (10% CTAB in 0.7 M NaCl) was added and the incubation was carried out at 65°C for 20 min. 700 µl phenol-chloroform-alcohol isoamyl alcohol (25:24:1) was added and left overnight. The material was centrifuged for 10 min at 4°C at 13,000 × g. The supernatant was transferred to a new microtube and 700 µl chloroform-isoamyl alcohol (24:1) was added and centrifuged for 10 min at 4°C at 13,000 × g. The supernatant was thereafter recovered and transferred to a new 1.5 ml microtube to which 0.6 volume of ice-cold isopropanol was added. The material was incubated at 20°C for 30 min. Afterwards, the material was again centrifuged for 10 min at 4°C at 13,000 × g and the supernatant was discarded. The material was further subjected to centrifugation for 10 min at 4°C to 13,000 x g, with 70% ice-cold ethanol. The material was dried at room temperature and then suspended in 100 µl $T_{10}E_1$ (10 mM Tris pH 8.0 and 1 mM NA_2EDTA).

The DNA quantification and qualification was assessed by electrophoresis on 0.7% agarose gel. 5 µl of sample was used together with 2 µl of sample buffer (0.25% bromophenol blue and 40% sucrose), along with 3 µl of 1 kb Plus DNA ladder standard (invitrogen® Cat. No. 10787-018). The samples were migrated in 80 v for 90 min in 1X TAE buffer (0.04 M Tris acetate and 1 mM EDTA). The gel was stained with an ethidium bromide solution (0.5 µg ml^{-1}) and visualized with ultraviolet light on KODAK® Gel Logic 100 Photodocumentator (KODAK Scientific Imaging Systems, Cat. No. 172.8468) and one computer. Gels were analyzed using the KODAK® 1D Image Analysis (KODAK Molecular Imaging Systems, Cat. Nr. 811.2344).

The 16S rRNA gene was amplified using the universal pair of primers: Amp-1: (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).

Amplification was carried out in 50 µl final reaction volume which contained 10% (final volume) 10X PCR buffer; 3 µl $MgCl_2$ (50 mM); 1 µl dNTP (200 mM); 1 µl of Taq DNA polymerase (INVITROGEN®) (1.25U µl $^{-1}$); 1 µl of native DNA (50 ng µl) and the volume made up to 50 µl with PCR grade water. The PCR schedule was: one denaturation cycle (95ºC for 5 min), followed by 34 intermittent cycles (94°C for 15 s, 60°C for 45 s and 72°C for 2 min), and one final extension cycle (72°C for 30 min), followed by cooling (4°C for 24 h).

For identification and phylogenetic analysis, PCR products of each isolate were purified using Wizard® genomic DNA purification kit (cat. A1120, PROMEGA Corporation, USA) and then sequenced at the Genomic Laboratory of Embrapa Agrobiologia. Sequences were deposited in the GenBank at accession numbers KT619165 - KT619177.

Inoculation experiment

The 12 most promising isolates (TR-I1, TR-N1, EP3-13J, EP4-2J, EP4-1L, EP3-2L, EP3-16N, EP4-8N, EP3-1L, EP3-7L and EP4-5J) based on plant growth promoting tests (Table 3) were selected to be tested in an inoculation experiment for growth promotion of rice in the Laboratory of Grasses, Embrapa Agrobiologia, Seropédica, RJ in a completely randomized factorial design (3×20) with four replicates using three cultivars of irrigated rice (IR 42, EPAGRI-109 and BRS TROPICAL) and were inoculated with 12 diazotrofic bacteria isolates (TR -I1, TR-N1, EP3-1L, EP3-2L, EP3-7L, EP3-13J, EP3-14J, EP3-16N, EP4-1L, EP4-2J, EP4-5J, EP4-7J), two bacterial mixtures comprising of MIX 1 (EP4-2J, EP3-13J and EP3-14J) and MIX 2 (TR-I1, TR-N1, EP4-1L, EP4-5J and EP3-14J), positive controls *H. seropedicae* Z94 (BR11417), *A. brasilense* Sp245 (BR11005), *N. amazonense* CBAMC (BR11145) provided by Diabotrophic Bacteria Collection of Embrapa Agrobiologa - CRB

Table 1. MPN of bacteria from shoots, roots and rhizospheric soil of rice cultivar BRS TROPICAL grown at greenhouse condition and MPN of bacteria from shoots, roots and rhizospheric soil of rice cultivar EPAGRI-109 from production area at Campo dos Goytacazes, RJ. Mean values of 3 replicates.

Cultivar	BRS TROPICAL					
	Shoots		Roots		Rizospheric soil	
	NFB	JNFB	NFB	JNFB	NFB	JNFB
Inoculated	5.65	5.04	6.04	6.04	5.15	5.15
Non inoculated	3.40	4.14	6.15	6.15	4.65	4.04

	EPAGRI 109								
	LGI	NFB	JNFB	LGI	NFB	JNFB	LGI	NFB	JNFB
Plot 103	4.15	3.65	4.04	6.04	5.15	5.15	6.04	6.04	4.15
Plot 104	5.15	4.65	5.15	6.04	6.15	6.04	4.65	6.04	5.15

Johanna Döbereiner and a commercial inoculant (AZOTOTAL), an uninoculated treatment and a nitrogen treatment with 2.60 mM of nitrogen as ammonium nitrate.

The bacteria were reactivated in 125-ml Erlenmeyer flask containing 50 ml DYGS medium, at 30°C agitation at 150 rpm for 24 h.The experiment was conducted in 120-ml test tubes containing 50 ml Hoagland's agar solution (6 g L^{-1}), without nitrogen. The tubes were covered with autoclaved cottons. After sterilization and prior to the solidification of the agar, each tube was inoculated with 2 ml pre-grown bacterial culture. The uninoculated treatment was inoculated with sterile culture medium. The seeds were disinfested according to Hurek et al. (1994) and pre-germinated on agar plates (1%) for 48 h. A pre-germinated seed was planted in each test tube. The experiment was terminated at 30 DAP, thereafter shoot and root dry weight were analyzed.

Statistical analysis

The normality (Lilliefors test) and homogeneity of variance (Bartlet's test) of the data were processed with SAEG 8.0 program (Euclydes, 1983). Analysis of variance was performed using the SISVAR 5.0 program (Ferreira, 2003) and a comparison of means by the Scott-Knott test at a 5% probability (Scott and Knott, 1974).

RESULTS AND DISCUSSION

Isolation of diazotrophic bacteria

The MPN counts of cultivar EPAGRI-109 varied from 10^4 cells g root frw $^{-1}$ and 10^6 cells g frw^{-1} in rhizospheric soil while the shoot values were 10^3 to 10^4 cells g root frw^{-1}. Interestingly, root associated bacteria of un-inoculated plants of BRS TROPICAL of the greenhouse were similar to EPAGRI-109 grown under field condition. In addition, number of bacteria in shoots and rhizospheric soil were higher in plants inoculated with *A. brasilense* Sp245 (Table 1).

The microbial population intimately associated with the rice plants was of 10^6 cells g frw^{-1} in cultivar EPAGRI 109 and in un-inoculated BRS TROPICAL plants. When BRS TROPICAL plants were inoculated with *A. brasilense* Sp245 the MPN values for shoots grossly doubled over in un-inoculated plants. Pedraza et al. (2009) also observed bacterial MPN increased on phyllosphere of rice cultivars in response to inoculation of *Azospirillum* strains. The inoculation treatment affected the bacterial counts in shoots and rhizosphere because *A. brasilense* Sp245 effectively colonized and established at these sites and altered the population of diazotrophs.

We obtained isolates in shoots (10), roots (8) and rhizospheric soil (9) from BRS TROPICAL and only isolates in shoots (9) and roots (3) from cultivar EPAGRI-109 (Table 2).

Hardoim et al. (2011) observed that some rice cultivars have a strong influence on the population composition of bacterial communities in their rhizosphere, selecting similar bacterial communities while other genotypes select divergent bacterial communities. Both bacterial adaptation and plant genotype contribute to the formation of bacterial communities associated with rice roots. This behavior could also be explained by the development stage which influences different exsudates production in roots (Chaparro et al., 2014).

Reduction of acetylene activity

The isolates were screened for *in vitro* eficiency of nitrogen fixation in semi-solid media (NFb, JNFb and LGI) (Figure 1A and B). Isolates obtained from cultivar EPAGRI-109, fixed 0.36 to 1462.0 nmol C_2H_4 mg protein^{-1} (Figure 1A). The isolates EP4-2J and EP4-3J presented ARA of 1021 and 1462 nmol C_2H_4 mg protein^{-1}, respectively. The ARA values were higher than those of positive control, that is, *A. brasilense* Sp245 (702 nmol C_2H_4 mg protein^{-1}), *H. seropedicae* Z94 (305 nmol C_2H_4 mg protein^{-1}) and *N. amazonense* CBAmC (53 nmol C_2H_4 mg protein^{-1}). The EP3-8J, EP4-4J, EP4-6J, EP4-7J, EP4-9N and EP4-10N isolates could not grow in semi-solid medium and were not evaluated by ARA. Isolates obtained from BRS TROPICAL cultivar showed ARA range of 3.16 to 416 nmol C_2H_4 mg protein^{-1} (Figure 1B).

Table 2. Isolates from rice cultivars EPAGRI-109 and BRS TROPICAL according to isolation source and semisolid medium.

Cultivar	Isolation source	Semi-solid medium	Isolates
EPAGRI-109	Shoots	LGI	EP3-1L, EP3-2L, EP3-3L, EP3-4L, EP3-7L
		JNFb	EP3-8J, EP3-9J, EP3-10J, EP4-7J
		NFb	EP4-8N
	Roots	LGI	EP4-1L
		JNFb	EP3-11J, EP3-12J, EP3-13J, EP4-2J, EP4-3J
		NFb	EP4-9N, EP4-10N
	Rhizospheric soil	LGI	EP3-5L, EP3-6L
		JNFb	EP3-14J, EP4-4J, EP4-5J, EP4-6J
		NFb	EP3-15N, EP3-16N, EP3-17N
BRS TROPICAL	Shoots	JNFb	TR-N3, TR-N4, TR-N5, TR-N6, TR-N7, TR-N8, TR-N9, TR-N10, TR-N11
		NFb	n.d.
	Roots	JNFb	TR-N1, TR-N2
		NFb	TR-I1
	Rhizospheric soil	JNFb	n.d.*
		NFb	n.d.*

*n.d.: not detected.

Table 3. Selected isolates and their main characteristics for potential use as plant growth promoter.

Isolate	ARA	Indolic compounds	Phosphate solubilization	16S rDna Blast analysis
TR-I1	++	+	+	*Azospirillum* spp.
TR-N1	+	+	+	*Herbaspirillum* spp.
TR-N7	+	+	+	*Herbaspirillum* spp.
EP3-13J	+++	++	++	*Azospirillum* spp.
EP3-14J	+++	++	+	*Azospirillum* spp.
EP3-15N	+	++	+	*Azospirillum* spp.
EP3-16N	+	+++	+	*Paenibacillus* spp.
EP4-1L	+	+	+	*Nitrospirillum* spp.
EP4-2J	+++	+++	++	*Azospirillum* spp.
EP4-3J	+++	++	+	n.d.
EP4-5J	++	++	++	n.d.
EP4-8N	+	+	n.d.	*Pseudomonas* spp.

Nitrogenase activity evaluated as nmol C_2H_4 mg protein^{-1}: (+) < 100; (++) ≥100; (+++) ≥ 500. Indolic compounds production mg protein^{-1}: (+) from 0.3 to 10; (++) from 10 to 20; (+++) > 20 µg. Solubilization halo in mm: (-) not observed; (+) < 2.0; (++) from 2.0 to 5.0; (+++) >5.0 n.d. = not determined.

None of them presented ARA comparable to *A. brasilense* Sp245 (601 nmol C_2H_4 mg protein^{-1}) but TR-I1 presented ARA more than double to that of *H. seropedicae* Z94 (198 nmol C_2H_4 mg protein^{-1}). We selected 3 strains obtained from BRS TROPICAL (TR-I1, TR-N1 and TR-N7) and 15 from EPAGRI-109 (EP3-1L, EP3-2L, EP3-3L, EP3-4L, EP3-6L, EP3-7L, EP3-13J, EP3-14J, EP3-15N, EP3-16N, EP4-1L, EP4-2J, EP4-3J, EP4-5J and EP4-8N) based on the best ARA results. These isolates were further evaluated for indolic componds production and phosphate solubilization.

Although, N-free semi solid medium was used for diazotyroph isolation, all isolates from EPAGRI-109 were not able to grow and/or reduce acetylene to ethylene

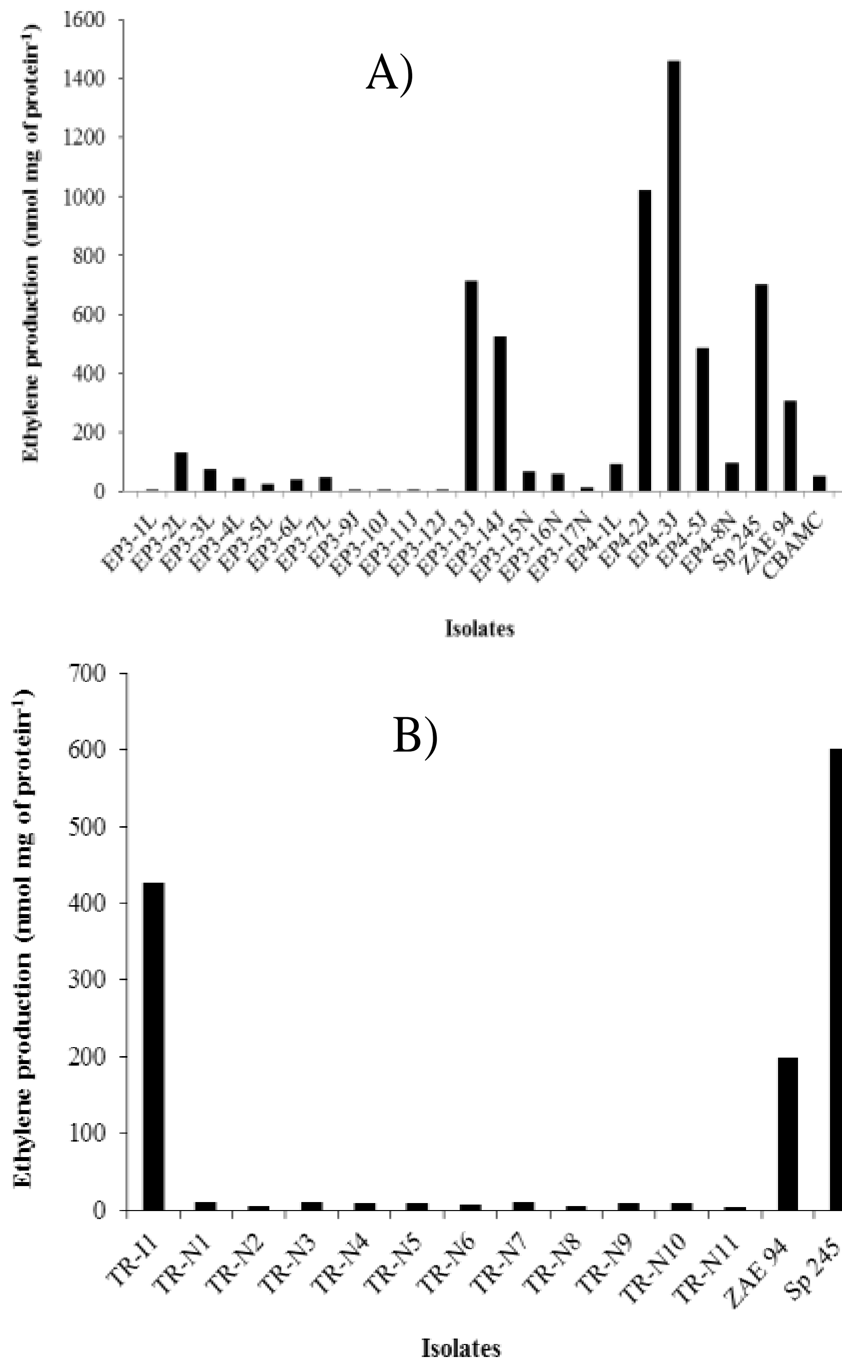

Figure 1. Estimative of nitrogenase activity measured by Acetylen Reduction Activity (ARA) of diazotrophic bacteria isolates from rice cultivar (A) EPAGRI-109 and (B) BRS-TROPICAL.

under the experimental conditions. Some isolates from EPAGRI-109 showed higher ARA than *A. brasilense* Sp245 (Figure 1A). In contrast, isolates from BRS TROPICAL showed lower ARA than *A. brasilense* Sp245, although 16S rDNA homology showed that TR-I1 is closely related to this species (Figure 1A, Table 4). Variations of ARA values among the isolates of the same species have been reported previously by several authors (Staal et al., 2001; Videira et al., 2012; Estrada et al., 2013). Although an indirect technique, ARA was used for indirect nitrogen fixation ability measurement because it is inexpensive and practical but since several factors (such as pH, carborn source, temperature and dissolved oxygen) can interfere with the performance of the isolates

Table 4. Dry weight of shoots and roots (g) of irrigated Rice cultivars at 30 days after inoculation with diazotrophic bacteria in Hoagland's solution.

Treatment	Shoot dry weight (g)			Root dry weight(g)		
	IR42	Tropical	Epagri-109	IR42	Tropical	Epapri-109
Uninoculated	0.0098^{Ab}	0.0128^{Aa}	0.0133^{Ba}	0.0054^{Ba}	0.0056^{Aa}	0.0065^{Aa}
Sp 245	0.0103^{Ab}	0.0166^{Aa}	0.0141^{Ba}	0.0054^{Ba}	0.0066^{Aa}	0.0050^{Ba}
ZAE 94	0.0077^{Ab}	0.0121^{Aa}	0.0131^{Ba}	0.0055^{Ba}	0.0050^{Ba}	0.0061^{Aa}
CBAMC	0.0088^{Aa}	0.0116^{Aa}	0.0115^{Ba}	0.0051^{Ba}	0.0050^{Ba}	0.0045^{Ba}
AZO TOTAL	0.0084^{Ab}	0.0114^{Aa}	0.0130^{Ba}	0.0069^{Aa}	0.0066^{Aa}	0.0061^{Aa}
TR-I	0.0102^{Aa}	0.0139^{Aa}	0.0126^{Ba}	0.0055^{Ba}	0.0053^{Ba}	0.0041^{Ba}
TR-N1	0.0079^{Ab}	0.0118^{Aa}	0.0138^{Ba}	0.0049^{Ba}	0.0063^{Aa}	0.0057^{Aa}
EP3-1L	0.0072^{Ab}	0.0130^{Aa}	0.0136^{Ba}	0.0041^{Ba}	0.0053^{Ba}	0.0056^{Aa}
EP3-2L	0.0093^{Aa}	0.0109^{Aa}	0.0130^{Ba}	0.0051^{Ba}	0.0043^{Ba}	0.0048^{Ba}
EP3-7L	0.0076^{Ab}	0.0138^{Aa}	0.0125^{Ba}	0.0048^{Ba}	0.0052^{Ba}	0.0045^{Ba}
EP3-13J	0.0057^{Ab}	0.0127^{Aa}	0.0132^{Ba}	0.0040^{Bb}	0.0048^{Bb}	0.0063^{Aa}
EP3-14J	0.0086^{Aa}	0.0108^{Aa}	0.0117^{Ba}	0.0053^{Ba}	0.0051^{Ba}	0.0058^{Aa}
EP3-16N	0.0083^{Ab}	0.0095^{Ab}	0.0173^{Aa}	0.0052^{Ba}	0.0050^{Ba}	0.0054^{Aa}
EP4-1L	0.0097^{Ab}	0.0139^{Aa}	0.0129^{Ba}	0.0071^{Aa}	0.0061^{Ab}	0.0052^{Bb}
EP4-2J	0.0091^{Ab}	0.0119^{Aa}	0.0136^{Ba}	0.0054^{Bb}	0.0056^{Ab}	0.0068^{Aa}
EP4-5J	0.0085^{Ab}	0.0132^{Aa}	0.0150^{Ba}	0.0051^{Ba}	0.0041^{Ba}	0.0055^{Aa}
EP4-8N	0.0079^{Ab}	0.0140^{Aa}	0.0132^{Ba}	0.0050^{Ba}	0.0055^{Aa}	0.0053^{Ba}
MIX 1	0.0088^{Ab}	0.0100^{Ab}	0.0129^{Ba}	0.0057^{Ba}	0.0056^{Aa}	0.0055^{Aa}
MIX 2	0.0055^{Ab}	0.0128^{Aa}	0.0099^{Ba}	0.0045^{Bb}	0.0066^{Aa}	0.0055^{Ab}
Nitrogen	0.0108^{Ab}	0.0134^{Ab}	0.0185^{Aa}	0.0037^{Ba}	0.0036^{Ba}	0.0044^{Ba}
Mean	0.0085	0.0125	0.0135	0.0052	0.0054	0.0055
CV%	-	19.91	-	-	17.59	-

Mean values of 4 triplicates. Means followed by distinct letters, upper case in column and lower case in row, differ by Scott Knott's test at 5% probability.

it have been used mostly to qualify than quantify their BNF potential.

Production of indolic compounds

Production of indolic compounds varied among the isolates (EP3-13J, EP3-14J EP3-16N and EP4-8N) and positive control strain (*H. seropedicae* ZAE94). The *H. seropedicae* ZAE94 and the isolates EP3-13J, EP3-14J EP3-16N as well as EP4-8N produced indole compounds during the first 24 h, with most of them attaining optimum production at 72 h; however, isolate EP4-8N accumulated indole compounds only after 48 h (Figure 2).

Indolic compound production by isolates TR-N1, TR-N7, EP3-1L, EP3-7L, EP3-15N, EP4-1L, EP4-2J, EP4-3J and EP4-5J could be detected after 24 h, in which their maximum production was observed at 48 h and then decreased up to 72 h. Interestingly, TR-I1 and EP3-7L behaved similarly to *A. brasilense* Sp245 and accumulated small amounts of indolic compounds after 72 h, that is, EP3-2L after 72 h had minimal production after 72 h but *A. amazonense* CBAmC and isolates EP3-3L and EP3-4L did not produce indolic compounds (data not shown).

Indolic compounds production by isolates was similar to those previously reported for rice plants isolates, an average production of 1.8 and 53 µg IAA ml^{-1} (Kuss et al., 2007; Rodrigues et al., 2008; Araújo et al., 2013). After 48 h, amount of indole accumulation was lesser than after 72 h probably due to degradation of indole compounds as reported by Leveau and Gerards (2008). Based on positive control, the organisms could be clustered into: Group 1 - Isolates that produced indole compounds comparable to *H. seropedicae* (EP3-13J, EP3-16N, EP4-2J and EP4-3J); Group 2 - Isolates that produced lower indoles than *H. seropedicae* ZAE94 but higher than that of *A. brasilense* Sp245 (TR-N1, TR-N7, EP3-14J, EP3-15N and EP4-5J); and Group 3 - Isolates that produced indoles comparable to *A. brasilense* Sp245 (TR-I1, EP3-1L EP3-2L, EP3-6L, EP3-7L, EP4-1L and EP4-8N).

Phosphate solubilization

41% of isolates was able to solubilize inorganic phosphates *in vitro;* the solubilization halo of isolates

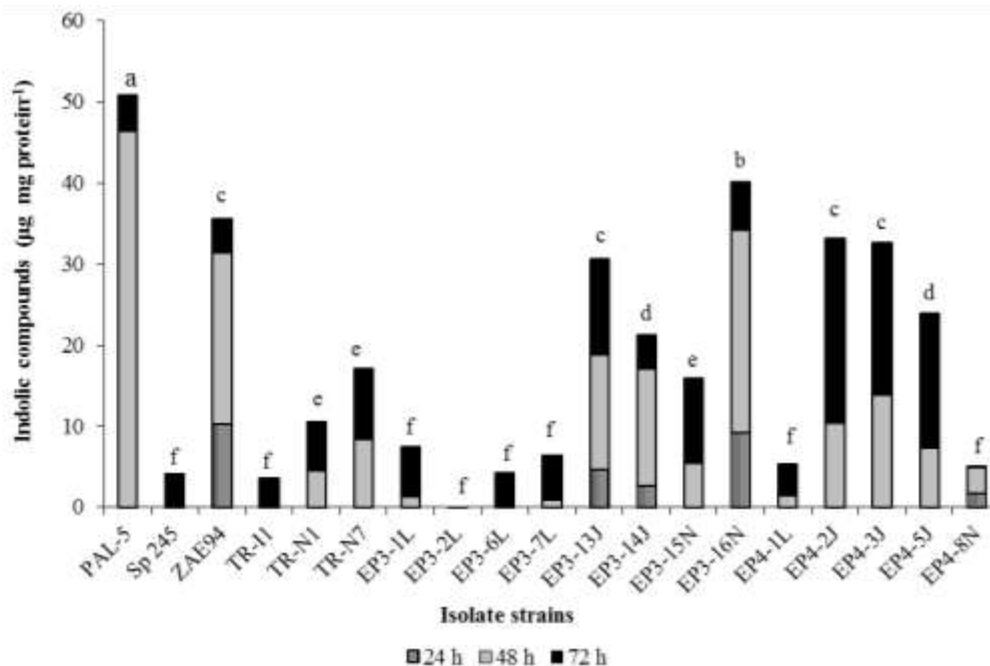

Figure 2. Indolic compounds production of isolates from irrigated rice cultivars at 24, 48 and 72 h of growth in Dygs medium suplemented with L-tryptofhan.

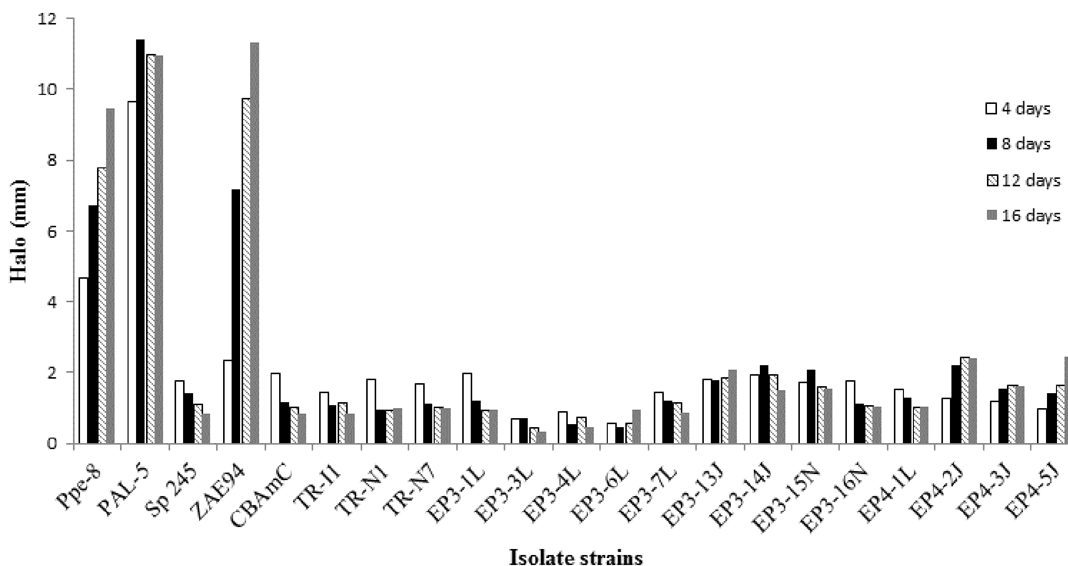

Figure 3. Solubilization activity of isolates of irrigated Rice cultivars at 4, 8, 12 and 16 days of growth in NBRIP agar medium.

varied from 0.33 to 2.45 mm during the four evaluations (Figure 3). These values are in the same range of those observed for *A. brasilense* Sp245 and *N. amazonense* CBAmC used as positive control. Phosphates solubilization was higher for *G. diazotrophicus* PAL5, *Burkhoderia tropica* Ppe8 and *H. seropedicae* ZAE94. However, no trend of P solubilization by the isolates could be established until 16th day of evaluation. Some isolates produced greater solubilization halo on first day and others produced bigger halos of solubilization on the last day of analysis.

Phosphorus (P) is important in several physiological

processes of plants, especially in photosynthesis, carbon metabolism and membrane formation, being a structural component of many coenzymes, phospho-proteins, phospholipids, DNA of all living organisms and responsible for the transfer and storage of energy which is used for growth and reproduction (Anand et al., 2016).

Despite this, about 95 to 99% of the phosphorus present in the soils is unavailable to the plants due to the fixation of P that is adsorbed on the mineral particles of the soil or precipitated by the ions Al^{3+} and Fe^{3+} in solution (Wu, 2005; Sharma et al*.,* 2013; Anand et al*.,* 2016).

Soil microorganisms play a key role in the soil P dynamics and subsequent soil P availability. Phosphates solubilizers, especially bacteria, increase the solubilization of insoluble phosphorus compounds through the release of phosphatic and phytase enzymes and enzymes that are present in various soil micro-organisms (Vassileva et al., 2000; Anand et al*.,* 2016).

Molecular characterization

Amplification of 16S rDNA from all isolates generated fragments in the range of 1500 base pairs. The sequences varied in the range of 1208 to 1479 bp and Blast analysis revealed their phylogenetic relationship, based on similarity to sequences deposited at GenBank (GenBank accession numbers KT619165 - KT619177). The diazotrophic isolates belong to Alfaproteobacteria (*Azospirillum* and *Nitrospirillum* species), Betaproteobacteria (*Herbaspirillum* and *Ideonella* species) and Gammaproteobacteria (*Pseudomonas* species) within Proteobacteria. In addition, Firmicutes (*Paenibacillus* species) within Bacilli was also identified (Table 3).

Some authors found these same genera associated with rice plants and closely related to diazothrofic bacteria (Baldani and Baldani, 2005; Breidenbach et al*.,* 2016).

The most promising isolates (TR-I1, TR-N1, EP3-13J, EP4-2J, EP4-1L, EP3-2L, EP3-16N, EP4-8N, EP3-1L, EP3-7L and EP4-5J) based on plant growth promoting tests (Table 3) were selected to be tested in an inoculation experiment.

Inoculation experiment

The results of diazotrophic bacteria inoculation under axenic conditions showed significant improvement in dry weight of the inoculated rice cultivars. The shoots of EPAGRI-109 were more responsive to inoculation than BRS TROPICAL and IR42 which did not significantly respond to any treatment (Table 4). The EP3-16N significantly improved shoot wt., that is, about 30% higher than the uninoculated plants and was similar to that of nitrogen supplemented cultivar EPAGRI-109.

Root dry weight of the cultivar IR42 increased more with inoculation of the isolate EP4-1L and commercial inoculant AZOTOTAL recording 27 and 31% improvement, respectively. However, growth of bacterized BRS TROPICAL and EPAGRI-109 were not statistically different from the uninoculated plants, rather cultivars were negatively affected for inoculation of some bacteria (Table 4). The results support that in addition to the intrinsic characteristics of the bacteria, interactions between genotype and genotype-environment would directly interfere in the growth promotion efficiency of host plants also (Oliveira, 1994; Reis et al., 2000; Kennedy, 2004; Hungria et al*.,* 2016).

However, the results conform to several studies which have shown that in axenic conditions diazotrophic bacteria was promising for field conditions. The *in vitro* evaluation allows selection of a large number of strains with potential plant growth promoting functions (Baldani et al*.,* 2000; Araújo et al*.,* 2013).

Evaluation under axenic conditions enabled Araújo et al. (2013) to select plant growth promoting isolates and host cultivars for testing in greenhouse, and found dstinct increases in biomass, that is, by 48 and 21% of Cana Forte and Cana Roxa varieties due to inoculation of *N. amazonense* strains AR3122, respectively. In addition, some varieties respond more to inoculation than others. The bacteria that promote yield with lower nitrogen fertilizer are greatly relevant for sustainable agriculture.

Application of inoculant Azototal containing *A. brasilense* strains Abv5 and Abv6 has been approved by the Ministry of Agriculture Livestock and Food Supply (MAPA) in Brazil for maize, wheat and rice to reduce nitrogen fertilizers by up to 50% which implies in savings of R$ 1.5 billion per year to farmers.

Conclusion

Thirty-nine isolates of diazotrophic bacteria were isolated from rice cultivars EPAGRI-109 and BRS TROPICAL, about 49% of which occurred from shoots, 28% from roots and 23% in rhizospheric soil. Among them, eight isolates belonged to the genus *Azospirillum* spp. and two isolates were identified as *Herbaspirillum* spp.

Paenibacillus spp. strain EP3-16N increased in biomass of plants mainly the cultivar EPAGRI-109 and *Nitrospirillum* spp. strain EP4-1L in roots of cultivar IR-42. However, more studies are necessary to confirm this capacity in field conditions.

The use of molecular tools must be considered in future studies to elucidate the microorganism diversity and interaction in rice plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the fellowships granted and the Brazilian Agricultural Research Corporation (Embrapa) for the financial support received.

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