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REGULAR ARTICLE

Azospirillum amazonense inoculation: effects on growth, yield and N_2 fixation of rice (*Oryza sativa* L.)

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Abstract Bacteria of the genus *Azospirillum* are considered to be plant-growth promoting bacteria (PGPR) and stimulate plant growth directly either by synthesising phyto-hormones or by promoting nutrition by the process of biological nitrogen fixation (BNF). Although this genus extensively studied, the effects of inoculation and the possible BNF contribution of the *Azospirillum amazonense* specie are very scarce. The aim of this study was to isolate, characterise and evaluate auxin production and nitrogenase activity of this species and to select, by inoculation of rice plants, promising isolates based on their ability to improve plant growth, yield and the BNF contribution. One hundred and ten isolates obtained from rice were characterised and grouped according to colony features. Forty-two isolates, confirmed as *A. amazonense* by the fluorescent in situ hybridization (FISH) technique, were tested for auxin production and nitrogenase activity in vitro. Subsequently plant growth promotion related to plant nutrition effect was evaluated, in vitro and in greenhouse experiments. The BNF contribution to plant growth was evaluated using the ^{15}N isotope dilution technique. All *A. amazonense* strains tested produced indoles, but only 10% of them showed high production, above 1.33 μM mg protein^{-1}. The nitrogenase activity also was variable and only 9% of isolates showed high nitrogenase activity and the majority (54%) exhibited a low potential. The inoculation of selected strains in rice under gnotobiotic conditions reduced the growth of root and aerial part when compared to the control, showing the negative effects of excess of phytohormone accumulation in the medium. However, in the greenhouse experiment, inoculation of strains of *A. amazonense* increased grain dry matter accumulation (7 to 11.6%), the number of panicles (3 to 18.6%) and nitrogen accumulation at grain maturation (3.5 to 18.5%). BNF contributions up to 27% were observed for *A. amazonense* Y2 (wild type strain). The data presented here is the first report that the PGPR effect of *A. amazonense* for rice plants grown under greenhouse conditions is mainly due the BNF contribution as measured by ^{15}N isotope dilution technique, in contrast to the hormonal effect observed with other *Azospirillum* species studied.

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Introduction

Rice is the world's most important food crop and a primary source of food for more than half the world's population. More than 90% of rice is grown and consumed in Asia where 60% of the people on earth live. According to various estimates, we must produce 40% more rice by 2025 to satisfy the growing demand without adversely affecting the resource base. This increased demand will have to be supported without expansion of cropping lands, or increased water use, fewer chemical additions and less labour. In accordance to Ladha and Reddy (2003), nitrogen is the major limiting nutrient for rice production. In this context, biological N_2 fixation (BNF) has been considered an alternative, which may prove to be a better solution to supply nitrogen to the cropping systems of the future (Jensen and Nielsen 2003). It is widely recognised that BNF can benefit the rice crop. Using the ^{15}N-isotope dilution technique as a measure for BNF, it was observed that certain rice varieties grown in N-deficient soil could obtain up to 30% of this element from BNF. Moreover, significant differences for BNF contribution between rice varieties have been observed (Boddey et al. 1995b). The commercial cultivars IR 42 and IR4432-28-5, for instance, showed the highest BNF contribution, while IAC 4440 the lowest contribution, suggesting that it could be considered a low N_2-fixing cultivar and a potential negative control for BNF experiments in rice crops (Boddey et al. 1995b). Further studies conducted at the International Rice Research Institute (IRRI) in the Philippines using the same methodology also found similar differences in BNF contributions and once again, the IR42 variety was found to be amongst those with the highest BNF contribution (Shrestha and Ladha 1996; Malarvizhi and Ladha 1999).

The plant tissue and rhizosphere of wetland rice are largely colonised by aerobic heterotrophic nitrogen-fixing bacteria including species of *Azospirillum*, *Herbaspirillum*, *Burkholderia*, *Azoarcus*, *Pseudomonas* (Baldani et al. 1997; Ladha and Reddy 2003). Most species of the genus *Azospirillum* are known to act as plant growth-promoting rhizobacteria (PGPR) and stimulate plant growth directly either by synthesising phytohormones or by promoting improved N nutrition through BNF. Extensive studies on *Azospirillum* genetics, biochemistry and physiology have been carried out making this genus one of the best-characterised genera among associative PGPR (Steendhoudt and Vanderleyden 2000). The *A. amazonense* species, which was initially isolated from forage grasses and plants belonging to the Palmaceae family in Brazil by Magalhães et al. (1983) has not been included in this accumulated knowledge. This species has been identified in association with cereals such as maize, sorghum, rice, sugarcane and *Brachiaria*, mainly in tropical countries (Baldani et al. 1997; Baldani 1984; dos Reis et al. 2000, 2004). In vitro studies suggest that *A. amazonense* is better adapted to soil acidity than *A. brasilense* or *A. lipoferum* (Magalhães et al. 1983), and this characteristic is an advantage for its adaptation to the majority of Brazilian soils. In addition, it was shown that *A. amazonense* species may be able to fix nitrogen in the presence of nitrogen (Hartmann et al. 1986). In this study, the BNF inhibition of *A. amazonense* was observed in presence of 10 mM NH_4Cl, while in *A. lipoferum* and *A. brasilense* species the BNF was completely inhibited by the addition of 1 mM of NH_4Cl (Hartmann et al. 1986). Unlike *A. brasilense* and *A. lipoferum* species, physiological and genetic studies point out that *A. amazonense* does not denitrify (Kloos et al. 2001). In soils with little O_2 concentration, as in a rice-flooded soil system, the denitrification processes could mean substantial loss of nitrogen. These physiological characteristics make *A. amazonense* a promising species for inoculation studies in wetland rice. To date, only a few inoculation studies evaluated the potential of *A. amazonense* as a plant growth-promoting organism. Thus, in this paper we investigated the occurrence, auxin production, nitrogenase activity and inoculation effects of *A. amazonense* isolates on growth, yield and BNF contribution to rice plants.

Materials and methods

Origin and identification of the A. amazonense isolates Samples of rice cultivars IR 42 (IRRI, Philippines) and IAC 4440 (Instituto Agronômico da

Campinas, Brazil) were collected from previous greenhouse experiment carried out with two hydromorphic soils from rice fields of Rio de Janeiro and Goiás State–Brazil (Rodrigues et al. 2006). Subsequently, these rice samples were used for isolating and counting of *A. amazonense* populations. The most probable number of this diazotroph species was estimated in semi-solid LGI medium using the McCrady probability table (Döbereiner et al. 1995). For screening and isolation of *A. amazonense*-like colonies, pellicles from semi-solid cultures were streaked onto LGI and potato agar plates. After morphological characterisation, a cluster of *A. amazonense*-like isolates obtained from two rice cultivars (Table 2) were analysed by the fluorescent in situ hybridization (FISH) technique. Strains of species of *Burkholderia* spp. (M130), *Herbaspirillum seropedicae* (Z67 ^{T}), *Azospirillum brasilense* (Cd) and *A. amazonense* (Y6 ^{T}, Y1 ^{T}, Y2 ^{T} and CBamC) were used as controls. Cells obtained by centrifugation of cultures at the exponential growth phase were treated for 1 h in paraformaldehyde 4%, washed twice with PBS for 5 min and suspended in ethanol/PBS (1:1). For the FISH reaction, these fixed cells (10 μl) were deposited on gelatin-coated slides (0.075% gelatin–0.01% CrK $(SO_4)_2$) and dried at room temperature. Prior to hybridisation, the cells were dehydrated by dipping the slide into a sequence of ethanol solutions (50, 80 and 98%). The cells were overlaid with 9 μl of the hybridisation buffer containing 50 ng ml^{-1} of the oligonucleotide probe, 50% of formamide, 0.9 M of NaCl, 20 mM of Tris–HCl pH 8.0 and 0.01% of SDS. The 16S rRNA based oligonucleotide probes used for identification (Interactive, Ulm, Germany) were labelled at the 5′ end with fluorescent protein Cy3, which emits red fluorescence when excited. The oligonucleotide probes used are listed in Table 1. The slides were incubated in a humid chamber for 2 h at 46°C and then the excess probe was removed with a wash solution buffer (20 mM of Tris–HCl pH 8.0; 0.1% SDS; 5 mM of EDTA pH 8.0 and 18 mM NaCl). After drying at room temperature, the slides were analysed by epifluorescence microscope (AxioPlan Model, Zeiss). The identity of *A. amazonense*-like isolates was confirmed by emission of red fluorescence (positive signal). The isolates confirmed as *A. amazonense* were deposited in the Embrapa Agrobiologia Culture Collection, Seropédica, Brazil (Table 2) and then evaluated for their potential as PGPRs.

Physiologic characterisation: auxin production and nitrogenase activity Production of indolic compounds by *A. amazonense* isolates was estimated by the colorimetric microplate method using the Salkowsky reagent (Sarwar and Kremer 1995). The experiment was performed in triplicate with 42 randomly selected isolates. Sterile water was used as blank and type strains of diazotrophic species were used as controls. Cultures grown overnight and adjusted at $DO_{436\ nm}$ 0.5 were inoculated in modified liquid LGI medium (without yeast extract, vitamins, bromothymol blue) containing KNO_3 1 g l^{-1} as nitrogen source and supplemented with 100 μg ml^{-1} of L-tryptophan (VETEC, Rio de Janeiro, Brazil) as precursor for the phytohormone metabolic pathway. Cultures were grown at 30°C for 72 h on a rotary shaker at 125 rpm in the dark. When the cultures reached the stationary phase, the cells were centrifuged (7,000 g, 5 min) and 150 μl of supernatant were mixed on polystyrene microplates with 100 μl of Salkowsky reagent (1 ml of $FeCl_3.6H_2O$ 0.5 M in 50 ml of $HClO_4$ 35%) and left to stand in the dark for 30 min at room temperature. The colour intensity was then measured at 492 nm in a spectrophotometer (Lab

Table 1 Probes used in the fluorescent in situ hybridization (FISH) analysis

Probes*	Target	rRNA position	References
EUB338 (50% FA)	Domain Eubacteria	16S (502–516)	Amann et al. (1990)
BET42a (50% FA)	β-Subclass of *Proteobacteria*	23S (1027–1043)	
GAM42a (50% FA)	γ-Subclass of *Proteobacteria*	23S (1027–1043)	
ALF1b (50% FA)	α-Subclass of *Proteobacteria*	16S (19–35)	Manz et al. (1992)
Aam1250c (50% FA)	*A. amazonense*		Stoffels et al. (2001)

All probes were labelled with photoactive protein Cy3 that emit red fluorescence. In parenthesis is % of formamide (FA) used in the hybridisation procedure.

systems iEMS Reader MF) and the standard curve of synthetic indole-3- acetic acid (VETEC) was used for auxin quantification.

The cell sediments of these cultures were used for protein quantification by the Lowry method (Lowry et al. 1951). The cultures (10 ml) were centrifuged (5,000×*g*, 15 min), re-suspended an equal volume of sterilised water and diluted (100 µl cell suspension: 400 µl water). This cell suspension (500 µl) was mixed with NaOH 1 M (500 µl) and boiled (100°C, 5 min) to disrupt the membranes and release the total cellular contents. To the cellular extracts (500 µl) were added 2.5 ml of fresh solution (100:1:1) of Na_2CO_3 (5%):$CuSO_4$ (1%): $KNaC_4H_4O_6.4H_2O$ (2%). After 10 min of reaction 500 µl Folin–Ciocalteu reagent (1 M) was added. After 30 min of incubation in the dark the protein content of each sample was measured at 750 nm using spectrophotometer (PE Lambda Bio, Model 11). A standard curve of serum albumin was used for calibration of the protein method and protein quantification.

The nitrogenase activity was estimated using the acetylene reduction assay (ARA; Boddey 1987) on vials (10 ml) containing 5 ml of modified N-free semisolid LGI medium (without yeast extract, vitamins and bromothymol blue) inoculated with 50 µl of adjusted cell suspension. After growth at 30°C for 48 h, the vials were sealed with rubber stopper (SubaSeal®) and 1 ml of acetylene gas was injected to a final concentration of 10% (*v*/*v*) of the flask volume. Samples were removed after a 1 h incubation period and assayed for ethylene using a gas chromatograph, equipped with a flame ionisation detector (Perkin Elmer–Autosystem II). Ethylene concentration was determined using a standard calibration curve and a digital integrator (PE Nelson model 1022). For the quantification of protein in cultures grown on semi-solid medium, a 1 M NaOH solution was used for cell lyses. Total cell extracts were diluted twice and was used for Lowry reaction as described above.

Inoculation of A. amazonense isolates in rice plants Two inoculation experiments were carried out using the rice variety IR 42, considered highly efficient in BNF (Boddey et al. 1995b; Shrestha and Ladha 1996). For the gnotobiotic experiment, rice seeds were surface disinfected and pre-germinated on agar plates according to Döbereiner et al. (1995). The inoculum consisted of bacterial suspension adjusted to optical density 0.8 at 436 nm. The experiment was conducted using test tubes (120 ml) containing 50 ml of sterilized nitrogen-free semi-solid Hoagland medium (Hoagland and Arnon 1950) modified by the addition of Bacto-agar (0.6%). Before the agar solidification, 1.0 ml of inoculum was added into each test tube and after that, one pre-germinated seed was transferred to each test tube. This experiment consisted of 24 treatments, including 18 *A. amazonense* isolates (including strains $Y1^T$ and $Y2^T$), 2 strains of *A. brasilense* (Sp 245 and Cd), 1 strain of *A. lipoferum* ($Sp59b^T$) and a non-inoculated control. The experimental design was completely randomised with ten replicates. Plants were harvested after growth at 28°C for 30 days and a 12 h photoperiod. The root system was excised, washed, dried with blotting paper and stained by immersion in 0.1% toluidine solution for 30 s. To measure surface area and length, the roots were carefully spread over a transparent plastic sheet using humidified cotton and placed on a computer scanner. The image was analysed using the SIARCS® 3.0 software (Crestana et al. 1994; Jorge and Crestana 1996). Their aerial parts were weighed after drying at 65°C for 3 days.

Greenhouse experiment Ten promising isolates were chosen by auxin production, ARA activity and inoculation effects for a greenhouse experiment at Embrapa Agrobiologia using an Argisol serie Itaguaí (Ramos et al. 1973). Based on soil analysis (Embrapa-SNLCS 1979), the soil acidity was corrected using dolomitic lime (750 mg kg^{-1}) and fertilised with phosphorus (100 mg kg^{-1}), potassium (100 mg kg^{-1}) and micronutrients (75 mg kg^{-1} of FTE BR 12). The total soil N content of the Argisoil was measured and obtained an amount of 766 mg N kg^{-1}. This amount was not enough for a normal rice growth for the period. For this reason and due to this soil has low ^{15}N natural abundance $\delta^{15}N=5$ ‰ (data not shown), the soil of all treatments were fertilised with 80 mg kg^{-1} N (ammonium sulphate) labelled with 1% at ^{15}N exc. in order to have a minimum soil N availability for plant growth and to apply ^{15}N isotope dilution technique (Boddey et al. 1995a). An additional control treatment with 80 mg kg^{-1} was also evaluated. After liming and fertiliser application, the soil was incubated for 40 days with

at approximately 50% water holding capacity. Each pot containing 10 kg of fertilised soil was sown with four rice seeds, following the inoculation of a bacterial suspension containing 10^8 cells mL^{-1} (1 ml per seed). After 10 days, only two healthy plants were maintained. The submersion of the rice plants was conducted after the plant reached 15 cm high and the 3 cm of water was maintained until the end of the experiment. Fifteen treatments were evaluated, including ten *A. amazonense* isolates selected from the gnotobiotic experiment, plus strains $Y2^T$ and Cd, and a non-inoculated control. The experimental design was randomised blocks with 3 replicates. At grain maturation, harvested straw and grain were dried at 65°C. The grains obtained were weighed and their mass divided by the whole plant biomass (straws and grains) to calculate the harvest index. The yield was evaluated by number of panicles, dry mass of grains and the growth from mass of straw. The nitrogen content of straw and grain was measured using the semi-micro Kjeldahl method (Bremmer and Mulvaney 1982). The nitrogen content of grain was divided by nitrogen content of whole plant (grain and straw) to calculate the nitrogen harvest index. To evaluate the ^{15}N enrichment, the samples of grain were finely ground and analysed on the Finnigan DeltaPlus continuous-flow isotope ratio mass spectrometer at Embrapa Agrobiologia as described by Ramos et al. (2001). The BNF contribution was estimated applying the ^{15}N isotope dilution technique (Boddey et al. 1995a) using the rice variety IAC 4440 as non-N_2-fixing control. By this technique, the input of unlabelled nitrogen derived from BNF was calculated from the proportional dilution of the plant ^{15}N enrichment derived from the labelled soil. If the inoculation increases the BNF and the nitrogen is assimilated, the rice plant ^{15}N enrichment will be lower than the non-inoculated treatment or control plant (IAC 4440).

Statistical analysis was performed using the System for Statistical Analysis (SAEG; Arthur Bernardes Foundation, UFV, Viçosa, MG), the Lilliefors test for the normal distribution of data, the Cochran and Bartlet test for variance homogeneity, and the Scott Knott and LSD tests at 5% probability for the analysis of variance. The Pearson test (Snedecor and Cochran 1980) was used in the correlation analysis.

Results

Counts of diazotrophic bacteria cultivated in semi-solid LGI medium using the MPN technique and the total number isolates are showed in Table 3. In all plant parts at several stages of developments, including seeds, diazotrophic bacteria were detected and isolated. The counts were not influenced by soil type but plant parts were decisive. Counts in washed roots showed the highest numbers, followed by surface sterilised roots and aerial plant tissue. The number of isolates was highest in rice roots, representing 74% of the total (110 isolates; Table 3). All isolates of this group showed white colonies of dry aspect, with a wrinkled surface and lens-like elevation on plates containing potato medium, as described by Döbereiner et al. (1995). However, a survey of colonies of these bacteria grown on LGI plates showed that colonies were circular, 1 to 5 mm diameter in size, defined borders, and dry consistency, but differed in colour (white or white-yellow), colony morphology (smooth, rough or wrinkled) and elevated (plane, concave or convex) colonies. After initial grouping by colony morphology characteristics on LGI plates, 42 isolates (Table 2) were selected to confirm their identity by FISH using 16S rRNA based oligonucleotide probes. The labeled Cy3 probes used enabled all isolates assayed to be identified as *A. amazonense*, including the type strains $Y2^T$, $Y6^T$, $Y1^T$ and CbamC, as observed by fluorescent red colour under the epifluorescence microscope (Table 2). The cells of other species used as negative controls (*Burkholderia* spp. strain M130, *Herbaspirillum seropedicae* type strain $Z67^T$ and *Azospirillum brasilense* type strain Cd) did not show fluorescence, thus confirming the specificity of the probe.

Further analysis of these isolates for auxin production and nitrogenase activity in in vitro assays showed differences in potential among isolates tested (Fig. 1). The potential for nitrogen fixation was compared as specific activity among isolates and varied from 4.5 to 188.9 nmol of C_2H_4 evolved mg $protein^{-1}$ h^{-1}. According to the values observed, the isolates could be grouped as strains of low (up to 48.4 nmol C_2H_4 mg $protein^{-1}$ h^{-1}), medium (from 57.0 to 123.9 nmol C_2H_4 mg $protein^{-1}$ h^{-1}) and high (higher than 155.7 nmol C_2H_4 mg $protein^{-1}$ h^{-1}) potential. The highest and lowest specific nitrogenase

Table 2 Identification of *A. amazonense* isolates by Fluorescent Hybridisation (FISH)

Isolates	EUB338	BET42a	GAM42a	ALF1b	AZO440	Aam1250c	References
BR 11742	+	−	−	+	+	+	This work
BR 11754	+	−	−	+	+	+	This work
BR 11749	+	−	−	+	+	+	This work
BR 11745	+	−	−	+	+	+	This work
BR 11751	+	−	−	+	+	+	This work
BR 11750	+	−	−	+	+	+	This work
BR 11753	+	−	−	+	+	+	This work
BR 11737	+	−	−	+	+	+	This work
BR 11746	+	−	−	+	+	+	This work
BR 11828	+	−	−	+	+	+	This work
BR 11736	+	−	−	+	+	+	This work
BR 11871	+	−	−	+	+	+	This work
BR 11829	+	−	−	+	+	+	This work
BR 11856	+	−	−	+	+	+	This work
BR 11857	+	−	−	+	+	+	This work
BR 11741	+	−	−	+	+	+	This work
BR 11739	+	−	−	+	+	+	This work
BR 11855	+	−	−	+	+	+	This work
BR 11874	+	−	−	+	+	+	This work
BR 11875	+	−	−	+	+	+	This work
BR 11876	+	−	−	+	+	+	This work
BR 11877	+	−	−	+	+	+	This work
BR 11830	+	−	−	+	+	+	This work
BR 11831	+	−	−	+	+	+	This work
BR 11835	+	−	−	+	+	+	This work
BR 11832	+	−	−	+	+	+	This work
BR 11755	+	−	−	+	+	+	This work
BR 11748	+	−	−	+	+	+	This work
BR 11743	+	−	−	+	+	+	This work
BR 11744	+	−	−	+	+	+	This work
BR 11752	+	−	−	+	+	+	This work
BR 11869	+	−	−	+	+	+	This work
BR 11870	+	−	−	+	+	+	This work
BR 11850	+	−	−	+	+	+	This work
BR 11851	+	−	−	+	+	+	This work
BR 11872	+	−	−	+	+	+	This work
BR 11852	+	−	−	+	+	+	This work
BR 11853	+	−	−	+	+	+	This work
BR 11854	+	−	−	+	+	+	This work
BR 11873	+	−	−	+	+	+	This work
BR 11834	+	−	−	+	+	+	This work
BR 11833	+	−	−	+	+	+	This work
$Y2^T$ (BR11140)[a]	+	−	−	+	+	+	Magalhães et al. (1983)
$Y6^T$ (BR11141)[a]	+	−	−	+	+	+	Magalhães et al. (1983)
$Y1^T$ (BR11142)[a]	+	−	−	+	+	+	Magalhães et al. (1983)
CbamC (BR11145)[a]	+	−	−	+	+	+	Magalhães et al. (1983)
Cd (ATCC 29710)[b]	+	−	−	+	+	−	Eskew et al. (1977)
$Z\ 67^T$ (BR11175)[b]	+	+	−	−	−	−	Baldani et al. (1986)
M130 (BR11340)[b]	+	+	−	−	−	−	Baldani (1996)

[a] Positive control: $Y2^T$, $Y6^T$, $Y1^T$ and CbamC (*A.amazonense*).

[b] Negative control: Cd (*A. brasilense*), $Z67^T$ (*Herbaspirillum seropedicae*) and M130 (*Burkholderia* spp.).

[c] Signal positive (+) or negative (−) to refer, respectively, at presence or absence of fluorescence with probes tested labeled with Cy3.

Table 3 Counting of diazotrophic bacteria using LGI semi-solid medium without nitrogen addition and number of *A. amazonense* isolates obtained from plant samples of two rice varieties planted in pots containing two different rice soils

[a]Plant Stage	Treatments	[b]Log cells g^{-1} fresh weight				Number of isolates
		Washed roots	Surface sterilised roots	Stems	Seed	
Vegetative	IR 42/GO	7.1±0.0	6.0±0.5	5.6±1.1	–	9
	IR 42/RJ	7.1±0.1	5.3±1.0	5.6±0.4	–	9
	IAC/GO	7.1±0.1	6.2±0.9	5.6±1.1	–	8
	IAC/RJ	7.1±0.0	5.8±1.4	6.1±1.6	–	5
Flowering	IR 42/GO	7.1 ±0.1	5.9±1.0	5.8±1.5	–	1
	IR 42/RJ	6.9±0.5	5.3±1.5	5.9±1.5	–	2
	IAC/GO	6.4±0.4	5.1±0.9	5.5±0.5	–	0
	IAC/RJ	6.4±0.8	5.9±1.1	5.8±1.6	–	3
Grain filling	IR 42/GO	6.6±1.3	5.4±2.1	5.1±2.0	–	11
	IR 42/RJ	5.8±2.3	5.4±2.0	5.5±2.0	–	8
	IAC/GO	6.5±0.4	5.4±2.1	4.6±1.9	–	10
	IAC/RJ	6.6±1.1	5.2±1.9	5.0±1.8	–	10
Maturation	IR 42/GO	6.7±1.4	6.0±1.2	7.1±0.1	6.4±0.9	10
	IR 42/RJ	7.1±0.1	5.8±2.4	7.0±0.4	5.3±0.7	9
	IAC/GO	7.1±0.1	5.4±1.7	6.7±1.2	5.6 ±1.3	10
	IAC/RJ	6.9±0.5	5.6±0.6	7.0±0.6	5.3±0.3	5
Number of isolates		36	45	25	4	110

[a] Rice plant samples collected at different plant stages and obtained in an earlier experiment using two rice varieties: IR 42 and IAC4440 (Rodrigues et al. 2006).

[b] Most Probable Number of cells expressed in Log and standard deviation at $p=0.05$. Mean of four replicate samples.

activity was observed in cultures of BR 11833 and BR 11877, respectively. Only 9% of isolates showed high nitrogenase activity and the majority (54%) exhibited low potential.

In this study, when the culture media was supplemented with tryptophan the *A. amazonense* isolates showed production of indoles at rates varying from 0.06 to 2.02 μM mg protein^{-1} (Fig. 1). According to the different abilities to produce auxins, isolates could be classified in relation to efficiency as low (up to 0.42 μM mg protein^{-1}), medium (from 0.46 to 0.85 μM mg protein^{-1}) and high (higher than 1.33 μM mg protein^{-1}) indole producers. Most isolates assayed (90%) were considered to produce and accumulate auxin in low or medium concentration (up to 0.6 μM mg protein^{-1}), and only 10% showed high production.

Based on these analyses, ten isolates were chosen according to their efficiency for indole production and nitrogen fixation (low, medium and high) for evaluation of their potential as rice growth promoters. Under gnotobiotic conditions, the majority of *A. amazonense* isolates showed inhibitory effects on plant growth (Table 4). Stem height was reduced by 38% and stem dry matter by 55%, while root length reduced by up to 55% and root area to 54%. The strongest inhibitory effects were observed by inoculation of isolate BR11746 that reduced the dry matter and height of stem and the area and length of roots. Although the effects were not significant, some promoting effects were observed. Increases of 53 and 32% in area and length of root, respectively, were observed by inoculation of BR11755 isolate. The BR11831 isolate increased in 12% the dry matter accumulation of rice stem. There was no significant correlation between auxin production and nitrogenase activity with the growth parameters evaluated (Table 5). When correlation analysis (r Pearson) between auxin production or nitrogenase activity was associated with the evaluated growth parameters negative and positive values were observed, respectively, but neither was significant at $p=0.05$ (Table 5).

Finally, these isolates were evaluated in a greenhouse experiment in order to measure the BNF contribution to rice plants. *A. amazonense* inoculants changed the growth and productivity of rice plants, by promoting positive or negative effects that were expressed in comparison to a control that received

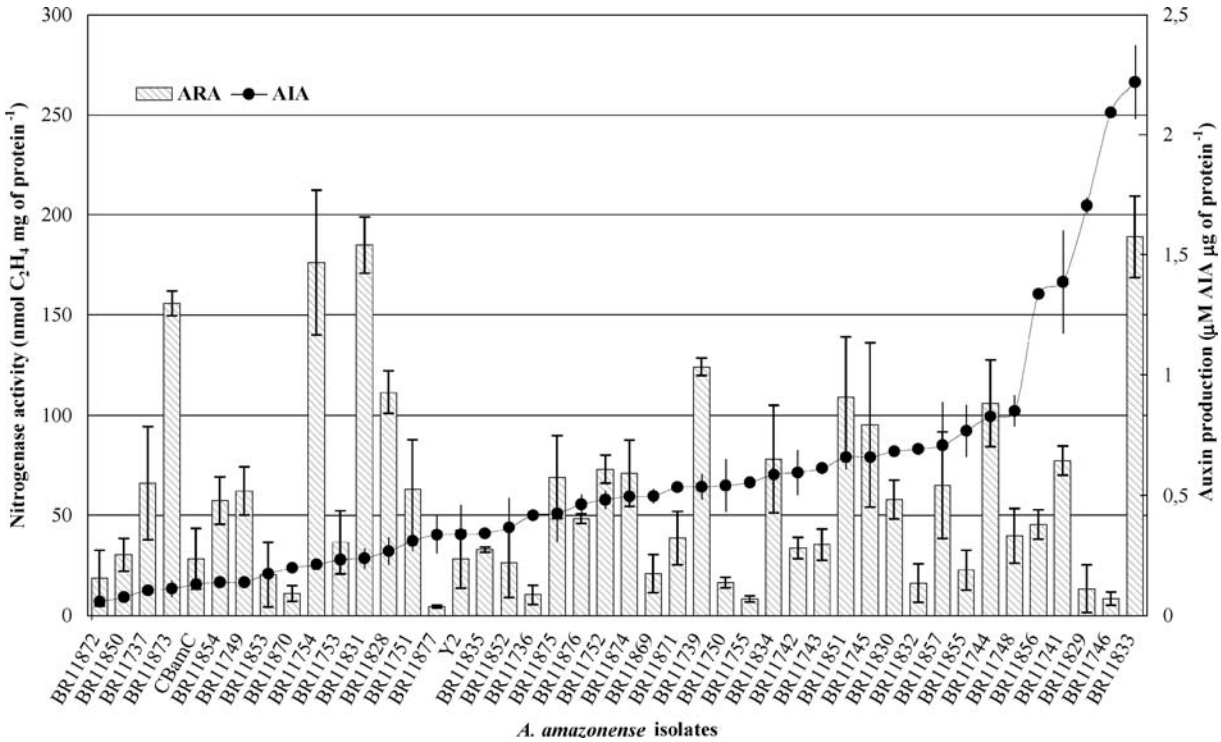

Fig. 1 Analysis of isolates for auxin production and nitrogenase activity in in vitro assays

80 mg N kg^{-1} soil (Table 6). Increases of 16% were observed in dry matter of the stem by inoculation of strain Cd (*A. brasilense*). Grain dry matter showed a positive effect by the inoculation with *A. amazonense* isolates BR 11833 and BR11752 (up to 10%). Reduction in this parameter was also observed by the inoculation of isolates BR11741 and strain Cd that reduced, respectively, in 10 and 11.5% the dry matter of grains. The panicle number was increased by inoculation of all *A. amazonense* isolates. The influence of inoculation on the productivity was observed by changes in the harvest index of grain. This parameter was used to quantify the dry matter that was transported from stem to the grain. Although the results were not significant, the treatments BR11755, BR11746, BR 11833 e BR11752 increased the harvest index compared to the control.

Likewise, the inoculation caused positive and negative alterations in the nitrogen accumulation in rice, however these differences were not significant in the conditions assayed (Table 7). With stems, increases of 10% and reductions up 22% were observed by inoculation of Cd and BR11746, respectively. In grains, increases in N accumulated of 18% (BR11755), 14% ($Y2^T$) and 11% (BR11746) were observed. Using the ^{15}N isotope dilution technique it was possible to estimate the percent of N derived from the air (%Ndfa) in the rice crop IR 42. In this study, BNF contributions of 6 to 27% were found (Table 8). The inoculation of $Y2^T$ strain showed the highest BNF contribution equivalent at 120 mg of N per pot, followed by isolates BR11752 and BR11834.

Discussion

Counting of diazotrophic bacteria using the most probable number (MPN) showed that the highest populations were present in the washed roots rather than the surface-sterilised roots. Since the root samples were crushed to obtain the extract, this plant part reflects endophytic and epiphytic population while surface disinfested roots showed that part of this population inhabits the inner root parts. Similar results were found by Magalhães et al. (1983), who observed the preferential colonisation of roots and rhizosphere of grasses by *A. amazonense*, with occasional colonisation of endophytic tissues. The results showed that this species colonises internal tissues of rice and also shows their preference for

Table 4 Influence of *Azospirillum* inoculation on growth parameters evaluated at 30 days after planting of the rice seedlings variety IR 42

Treatments	Stem height (cm plant^{-1})	Root length[a] (cm plant^{-1})	Root area[b] (cm^2 plant^{-1})	Stem dry matter (mg plant^{-1})
Control	17.3[a]	188.1[a]	5.8	8.7[a]
BR11833	15.2[b]	135.8[a]	5.2	6.2[b]
BR11831	18.3[a]	157.4[a]	6.6	9.8[a]
BR11739	18.2[a]	178.5[a]	6.0	7.7[a]
BR11834	17.1[a]	130.9[b]	4.7	6.8[b]
BR11741	15.7[b]	143.7[a]	6.1	8.2[a]
BR11752	14.8[b]	135.7[b]	4.1	6.6[b]
BR11872	13.7[b]	83.9[b]	3.3	4.0[b]
BR11870	17.7[a]	149.3[a]	5.5	8.0[a]
BR11746	10.8[b]	83.1[b]	2.7	3.9[b]
BR11755	16.7[a]	248.5[a]	8.9	7.7[a]
BR11142	15.2[b]	71.8[b]	3.0	5.3[b]
BR11140	18.1[a]	172.7[a]	6.6	8.8[a]
Sp 59b^T	17.5[a]	149.5[a]	7.1	9.2[a]
Sp 245	15.3[b]	95.2[b]	4.4	7.2[a]
Cd	14.6[b]	118.0[b]	4.1	5.8[b]

Values represent mean of seven replicate samples. Values followed by the same letter did not differ significantly from *F* test at 5% of significance.

[a] Data modified by square root for variance analysis.

[b] Data do not have variance homogeneity and normal distribution.

roots, since 74% of isolates were derived from this plant tissue.

The variability of the nitrogenase activity of *Azospirillum* has been observed previously *in vitro* by Han and New (1998), with ARA varying from 0 to 155 nmol of C_2H_4 mg protein^{-1} h^{-1}, in pure cultures of *A. lipoferum* and *A. brasilense* obtained from soils

Table 5 Regression between auxin production, nitrogenase activity and plant growth parameters evaluated in the gnotobiotic experiment using rice variety IR 42

Correlation	R *pearson*
Auxin production × stem height	−0.21
Auxin production × stem dry matter	−0.19
Auxin production × root length	−0.09
Auxin production × root area	−0.02
Nitrogenase activity × stem height	+0.07
Nitrogenase activity × stem dry matter	+0.07
Nitrogenase activity × root length	+0.06
Nitrogenase activity × root area	+0.16

Table 6 Evaluation of dry matter, number of panicles and harvest index of rice cultivar IR42 inoculated with several strains of *A. amazonense* and compared with a strain of *A. brasilense* (Cd) under greenhouse conditions

Treatments	Dry matter		Panicles		Harvest index (%)[c]
	Stem (g pot^{-1})	Grain (g pot^{-1})	Total[b]	(Number pot^{-1})	
Control I[a]	50.7	41.6	92.2	23.3	45.9
Control II-[a]	37.3	27.8	65.1	18.3	42.4
BR11833	50.8	45.7	96.5	24.0	47.3
BR11831	54.0	41.3	95.2	25.0	42.9
BR11739	51.9	40.0	91.9	24.7	43.4
BR11834	45.9	41.5	87.4	24.3	47.6
BR11741	51.9	37.5	89.3	26.7	41.8
BR11752	45.3	46.4	91.6	27.7	50.7
BR11872	44.0	38.0	82.0	25.0	46.4
BR11870	51.7	40.4	92.1	25.3	43.9
BR11746	49.6	44.5	94.1	25.0	47.3
BR11755	48.6	45.0	93.5	25.7	48.1
Y2^T (BR11140)	49.8	41.7	91.1	24.7	45.5
Cd (ATCC29710)	59.1	36.8	95.8	23.0	38.1

Values represent mean of three replicate samples. Values followed by the same letter did not differ significantly from *F* test at 5% of significance.

[a] Control not-inoculated and fertilized with 80 (control I) and 50 (control II) mg N kg^{-1} soil, respectively.

[b] Sum of stem and grains dry matter.

[c] Relation grain/total dry matter ×100.

of different regions. In another study, the nitrogenase activity of *Azospirillum* isolates ranged from 17.6 to 49.6 nmol C_2H_4 ml^{-1} h^{-1}, being higher in cultures of *A. brasilense* than for *A. lipoferum* isolates (Mascarua-Esparza et al. 1988). Our results showed large differences in the nitrogen fixing ability of *A. amazonense* isolates. The capacity to synthesise auxin also differed among the *Azospirillum* species. Radwan et al. (2002) obtained similar results for several *Azospirillum* reference strains, reporting a high capacity of the *A. brasilense* strain (Cd) to produce IAA (379 μM ml^{-1} of culture), contrasting with the low production of the *A. amazonense* type strain Y6^T (19 μM ml^{-1}), the lowest of the *Azospirillum* genus. dos Reis et al. (2004) also evaluated indole acetic acid production from several strains of *A. amazonense* and data ranged from 35 to 110 μM ml^{-1}. This specie has been considered less efficient in indole production but it is important to point out that most of the studies were made with reference strains. In our work, we

Table 7 Evaluation of nitrogen accumulated and nitrogen harvest index of rice cultivar IR42 inoculated with several strains of *A. amazonense* and compared with a strain of *A. brasilense* (Cd) under greenhouse conditions

Treatments	Nitrogen accumulated[c]			Nitrogen harvest index (%)[d]
	Stem (mg N pot^{-1})	Grain (mg N pot^{-1})	Total[b]	
Control I[a]	223.2	419.4	642.52	67.1
Control II[a]	143.0	256.8	400.68	63.4
BR11833	201.8	433.8	634.48	68.2
BR11831	219.8	434.7	656.44	65.6
BR11739	216.5	437.4	653.92	66.8
BR11834	220.0	423.3	636.97	65.7
BR11741	245.5	441.1	688.51	64.2
BR11752	173.7	454.4	622.18	73.1
BR11872	179.1	395.4	573.86	68.8
BR11870	237.4	436.8	673.67	64.2
BR11746	173.1	466.5	631.24	74.2
BR11755	191.6	496.9	688.47	72.2
$Y2^T$ (BR11140)	204.1	481.1	685.87	69.4
Cd (ATCC29710)	246.3	388.2	633.79	60.9

Values represent mean of three replicate samples. Values followed by the same letter did not differ significantly from *F* test at 5% of significance.

[a] Control not-inoculated fertilized with 80 (control I) and 50 (control II) mg N kg^{-1} soil, respectively.

[b] Sum of nitrogen accumulated in stem and grains.

[c] Nitrogen accumulated calculated with the data of % N.

[d] Ratio of nitrogen grain/nitrogen total ×100.

Table 8 BNF contribution of rice cultivar IR42 inoculated with several strains of *A. amazonense* and compared with a strain of *A. brasilense* (Cd) under greenhouse conditions

Treatments	Atom $^{15}N_{exc}$ in grain	%Ndfa[a]	mg N pot^{-1}
Control	0.63 ab	6.3	29.9 e
IAC 4440	0.67 a	–	–
BR11833	0.58 cd	13.5 bcd	62.3 bcd
BR11831	0.59 bcd	12.1 bcd	54.3 cd
BR11739	0.61 bc	9.7 cd	43.1 cd
BR11834	0.55 d	18.2 b	70.7 bc
BR11741	0.61 bc	9.2 cd	40.8 d
BR11752	0.55 d	18.3 b	83.1 b
BR11872	0.57 cd	15.2 bc	59.9 bcd
BR11870	0,58 bcd	13,3 bcd	57,6 bcd
BR11746	0.59 bcd	11.9 bcd	54.7 cd
BR11755	0.59 bcd	12.1 bcd	60.0 bcd
$Y2^T$ (BR11140)	0.49 e	27.7 a	120.1 a
Cd (ATCC 29710)	0.57 cd	14.7 bc	59.4 bcd

Values represent mean of three replicate samples. Values followed by the same letter did not differ significantly from *F* test at 5% of significance.

[a] BNF contribution obtained by the ^{15}N isotope dilution technique calculated using the data of % at ^{15}N excess by the plant pot planted with rice variety IAC4440 used as a non-N_2-fixing control.

found high variability in IAA production among the isolates, tested in replicates, and showing up to 15 times higher efficiency in IAA production compared with the reference strains BR11145 and $Y2^T$ under the same experimental conditions. Since the majority of isolates showed a low potential for auxin production and nitrogenase activity, the search for inoculants requires a large screening program.

The high efficiency in PGP-bacteria to produce IAA must be evaluated carefully. Since roots are very sensitive to IAA, relatively low concentrations of exogenous IAA can inhibit root growth (Scott 1972; Lambrecht et al. 2000). Patten et al. (1999) suggested that the level of auxin synthesised by the plant itself might be an important factor in determining whether bacterial IAA will stimulate or inhibit growth in a plant. Plant roots may have endogenous levels of IAA which are suboptimal or optimal for growth, the additional input into the IAA pool by bacteria could modify endogenous auxin to either optimal or supra-optimal levels, resulting in the induction or inhibition of plant growth, respectively.

In the present study, the effects of inoculation in the experiments in vitro and under greenhouse conditions differed between the isolates assayed. In the gnotobiotic experiment, increases and reductions in plant growth were observed, with predominance of the inhibition of stem and root growth. This inhibition may be related to the bacterial auxin released on seedling roots during the assay. Similar studies with wheat and rice seedlings showed reduced length and root surface area for *Azospirillum* and *Herbaspirillum* inoculation. These effects were attributed to production of indoles and the use of strains of *Azospirillum* that were more efficient in indole production (Radwan et al. 2004). The results obtained by the inoculation with *A. amazonense* provide evidence that this species produces auxin in pure culture and in association with roots. The absence of a correlation between the auxin

production in culture medium and the changes on plant growth (Table 4) suggest that others factors may influence the plant response such as BNF itself and/or other phytohormones. The production of cytocynin and giberelins by *Azospirillum* spp. has been related to plant dry matter accumulation and stimulatory effect (Tien et al. 1979; Costacurta and Vanderleyden 1995; Steendhoudt and Vanderleyden 2000).

Because of their potential as plant promoting bacteria, *Azospirillum* spp. have been used as inoculants to improve the growth and yield of cereal crops (Bashan and Holguin 1997; Bashan 1998; Reis et al. 2000; Baldani et al. 2000; Bashan et al. 2004; Baldani and Baldani 2005). In accordance with Okon and Labandera-Gonzalez (1994), 60 to 70% of inoculation studies with *A. lipoferum* and *A. brasilense* species have had success, although in only 5–30% of these studies the responses to inoculation were statistically significant.

Under greenhouse conditions, the inoculation of *A. amazonense* changed the rice growth. Between the isolates tested some showed beneficial effects on the parameter evaluated: productivity, nitrogen accumulation and contributions of N_2 fixation to rice; while others inhibited growth. This confirms the necessity of screening of bacterial inoculants, principally in different conditions of assay. The isolates BR11755, BR11746, BR 11833 and BR11752, which increased the dry matter of grain, the panicle number and N accumulation and showed high BNF contributions (11 to 18% Ndfa) are promising bacterial inoculants for furthers studies. Similar results were obtained by Baldani et al. (2000) who reported a BNF contribution of 18% in rice inoculated with different strains of *Herbaspirillum* and 20% with *Burkholderia* spp. Similarly, a recent study performed by Ferreira et al. (2003) showed increments of 20% in the nitrogen accumulated in the grain of rice cultivar IR42 inoculation of *H. seropedicae.*

Inoculation experiments with *A. amazonense* are rare even in Brazil where it is a typical tropical bacterial species that has been mainly isolated from cultivated plants and pasture. This work shows that this bacterium can act as a plant growth promoter and can contribute to nitrogen accumulation by rice, via the biological N_2 fixation too. More studies must be realised to elucidate the mechanisms of growth promotion. Also, it is essential that isolation and screening experiments be performed to reveal the potential of *A. amazonense* and wetland rice interaction in field conditions, and to identify the conditions and factors necessary to obtain better benefits of inoculation to this crop.

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