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Rhizobial Inoculation, Alone or Coinoculated with *Azospirillum brasilense*, Promotes Growth of Wetland Rice

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ABSTRACT: Rhizobia and associative bacteria promote growth in rice plants (*Oryza sativa* L.) through a series of mechanisms, but most studies on inoculation have been performed based on inoculation with these bacteria in a separate or singular manner. The objective of this study was to assess the efficiency of single/isolated inoculation and inoculation combined with symbiotic rhizobia from forage legume and with *Azospirillum brasilense* on promoting growth and the root colonization process in wetland rice. Two rhizobia among four isolates from a greenhouse and a laboratory experiment were selected that efficiently promoted seed germination and rice plant growth in a sterilized substrate and in soil. The two most efficient isolates (UFRGS Vp16 and UFRGS Lc348) were inoculated alone or in combination with a commercial product containing *A. brasilense* in two field experiments using two wetland rice cultivars over two growing seasons. In the field experiments, these isolates coinoculated with *A. brasilense* promoted larger increases in the agronomic variables of wetland rice compared to the control without inoculation. Confocal laser microscopy confirmed the presence of inoculated bacteria tagged with *gfp* (UFRGS Vp16, UFRGS Lc348, and *A. brasilense*) colonizing the root surface of the rice seedlings, mainly in the root hairs and lateral roots.

Keywords: Plant growth-promoting rhizobacteria, *Oryza sativa*, *gfp* tagging.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal in the world, feeding two-thirds of the world's population. Brazil is one of the ten main rice-producing countries, and the southern region, where wetland rice cropping is concentrated, accounts for 70 % of Brazilian production (Conab, 2012). Rice cropping areas are characterized by flat topography and hydromorphic soils with deficient natural drainage characteristics. These areas present constraints to agriculture and are mainly used for wetland rice cropping and livestock rearing.

Using N-fixing forage in winter is a way to increase yield in these areas, especially considering that nitrogen (N) is the main factor limiting rice production (Ladha and Reddy, 2003). The *Lotus* and *Trifolium* genera are preferred because they tolerate flood conditions. Inoculation of forage with rhizobial strains efficient in biological N fixation (BNF) can assist successive rice crops, either through plant residue decomposition or through an increase in N soil content. Furthermore, several studies have demonstrated that rhizobia and other symbiotic bacterial genera establish endophytic associations and promote rice growth (Yanni et al., 2001; Singh et al., 2006; Yanni and Dazzo, 2010).

These bacteria live inside roots and stems and stimulate rice plant growth due to phytohormones, such as indole acetic acid (IAA) (Chen et al., 2005; Bhattacharjee et al., 2012), phosphate solubilization (Bakhshandeh et al., 2014), and production of ACC-deaminase, which reduces ethylene levels in roots (Chinnadurai et al., 2009). Indirectly, these bacteria protect plants against pathogens by releasing substances, such as antibiotics and cell-wall lytic enzymes, or by inducing a systemic defense response (Dutta et al., 2007).

Studies on the use of legume symbiotic bacteria for non-leguminous plant growth are relatively recent and rare compared with ecological and physiological studies on the *Azospirillum* genus (Pereira et al., 1988; James et al., 2000; Khorshidi et al., 2011). The mechanisms underlying plant growth induced by inoculation with *Azospirillum* include auxin production by these bacteria (Steenhoudt and Vandereyden, 2000) and contributions by BNF, which ranged from 9.2 to 27.7 % and produced increases of up to 11.6 % in grain yield, 18.6 % in number of panicles, and 18.5 % in total N in rice grains in a greenhouse experiment (Rodrigues et al., 2008).

However, neutral results have also been reported for inoculation of rice cultivars with *Azospirillum* strains in field experiments (Boddey et al., 1995; Sasaki et al., 2010; Souza et al., 2012). In Brazil, a combined inoculation containing more than one *Azospirillum* strain has been commercially used for rice, maize, and wheat (Hungria et al., 2010; Brasil, 2011). In another study (Oliveira et al., 2006) showed that inoculation with five associative bacteria (*Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Herbaspirillum rubrisubalbicans, Azospirillum amazonense*, and *Burkholderia tropica*) increased sugarcane yield.

Our hypothesis was that the combination of rhizobia and associative bacteria in wetland rice can increase yield and reduce chemical fertilizer use for this cereal. The objective of this study was to assess the efficiency of separate inoculation or coinoculation with rhizobia and *A. brasilense* on the growth of wetland rice.

MATERIALS AND METHODS

The experiments were conducted in three stages. First, experiments were conducted in the laboratory and greenhouse at the School of Agronomy of the Federal University of Rio Grande do Sul (Universidade Federal do Rio Grande do Sul - UFRGS), Porto Alegre, RS, Brazil. The aim was to characterize the rhizobia UFRGS Vp16 isolated from nodules of white clover (*Trifolium repens*) and the rhizobia UFRGS Lg111, UFRGS Lc336, and UFRGS Lc348 isolated from nodules of bird´s foot trefoil (*Lotus corniculatus*) as growth

promoters of the IRGA 422CL rice cultivar. The isolates belong to the UFRGS rhizobia collection and proved to be efficient in fixing N with their respective hosts, along with high production of indoleacetic acid (IAA), in previous studies (Frizzo, 2007; Machado, 2011; Alves et al., 2012). The isolates that were most efficient in the lab and greenhouse experiments were used in the rice field experiments. Finally, two rhizobia isolates, UFRGS Lc348 and UFRGS Vp16, and two *A. brasilense* isolates were *gfp* tagged and used to inoculate rice seedlings, which were monitored for colonization.

Taxonomic identification of the isolates

For taxonomic identification of the UFRGS Vp16, UFRGS Lg111, UFRGS Lc336, and UFRGS Lc348 isolates, total bacterial DNA was extracted using the Wizard® Kit (Promega Corp., Madison, WI, USA) in accordance with manufacturer instructions. The DNA region of the gene that codifies the 16S ribosomal portion was amplified with the 8F and 1492R primers (Edwards et al., 1989) following the protocol described by Stroschein et al. (2011). The fragments were sequenced using the MegaBace 500 (Amersham Biosciences) capillary electrophoresis system. Partial sequences of the 16S ribosomal region for the homologous strains were obtained from GenBank using the BLAST 2.0 program.

Laboratory and greenhouse experiments

The first experiment was to assess the effect of inoculating the four rhizobia on rice seed germination. The seeds were surface-disinfected with 70 % ethanol and 0.3 % sodium hypochlorite (0.3 %) and then washed with sterile distilled water. Fifty rice seeds were placed in Petri dishes with a paper towel and 3.3×10^7 rhizobia in 5 mL of yeast mannitol (YM) broth was applied per seed. This quantity of bacterial cells is above the application rate recommended by RELARE (2014) (1.2×10^6 cells seed^{-1}) to test inoculant efficiency.

Bacterial cells in the broth were counted using a Neubauer chamber. The bacteria were grown at 28 °C in an incubator with 120 rpm orbital shaking for seven days in YM broth. The control seeds received 5 mL YM sterilized broth. The Petri dishes were placed in a chamber at 28 °C, and the germinated seeds were counted and removed every 24 h and evaluated at the end of the sixth day. The initial germination was determined based on the number of germinated seeds divided by the total number of germinated seeds at the end of the sixth day. A completely randomized design with four replications was used.

In the second experiment, we assessed the effect of inoculating the four rhizobia on rice plants cultivated in a sterilized substrate containing a mixture of vermiculite and sand (2:1). The rice seeds were surface disinfected as previously described and pre-germinated on a paper towel moistened with sterile distilled water in a germination chamber at 28 °C for 24 h, and six seeds were placed in a 500 mL plastic pot containing a sterilized substrate. The seedlings were thinned two days after transplanting, leaving only two seedlings per pot. Seven days after transplanting, the plants were inoculated with 3.3×10^7 rhizobia in 5 mL of YM broth per seedling. Bacterial cells in the broth were counted using a Neubauer chamber. The control plants received 5 mL sterilized YM broth. The plants were maintained in a greenhouse and watered daily with a nutritive solution (Sarruge, 1975). Thirty-seven days after transplanting, the shoot was cut, separated from the root system, placed in a chamber, and oven dried at 65 °C until constant weight. A completely randomized design with four replications was used.

In the third experiment, we assessed the growth-promoting ability of rhizobial isolates on rice plants. The soil, classified as *Chernossolo Ebânico Carbonático vertissólico* (Typic Haplustoll) (Embrapa, 2006), was collected at a 0.00-0.20 m depth in a marsh area with native pasture in the municipality of Caçapava do Sul, RS (Table 1).

Plastic pots with a 2.5 L capacity were used with 1.7 kg of soil, which was previously mixed with simple super phosphate and potassium chloride, equivalent to 90 kg ha^{-1} of P_2O_5 and

Table 1. Chemical properties of the soils used in the experiment

Experiment	Soil[(1)]	Clay	OM	pH(H_2O)	P	K	Ca^{2+}	Mg^{2+}	Al^{3+}
		g kg^{-1}			mg dm^{-3}		$cmol_c$ dm^{-3}		
Pots with soil	*Chernossolo*	200	380	5.2	8.0	65.0	7.0	3.0	0.6
Cachoeirinha, RS	*Gleissolo*	340	120	5.3	16.3	42.0	2.1	0.9	0.4
Cachoeira do Sul, RS	*Planossolo*	140	160	5.2	9.8	101.0	3.0	1.1	0.6

[(1)] Soil classification according to Embrapa (2006). Clay: pipette method; OM: organic matter, Walkley-Black method; pH in water, v/v; P, K: extractor Mehlich-1; Ca, Mg, Al: extractor 1 mol L^{-1} KCl.

50 kg ha^{-1} of K_2O, based on the liming manual and fertilization recommendations for the states of Rio Grande do Sul and Santa Catarina. The soil was flooded for 16 days before sowing, maintaining a 5 cm layer of water above the soil. The seeds of each cultivar were treated as described above, and six seeds were sown per pot. Before sowing, the seeds were inoculated with 3.3×10^7 rhizobia in 5 mL of YM broth per seedling. The control plants received 5 mL sterilized YM broth. Bacterial cells in the broth were counted using a Neubauer chamber. After 10 days, the seedlings were thinned, leaving three plants per pot, and N was applied as a urea solution (760 mg L^{-1}), equivalent to 40 kg ha^{-1} of N. Two non-inoculated control treatments were used, in which N equivalent to 40 kg ha^{-1} or 80 kg ha^{-1} was added. After fertilization, flooding conditions were re-established. Thirty days after sowing, a second N application was made, which was exactly the same as the initial treatment. Sixty days after sowing, the number of tillers per plant was counted, and the plants were cut at ground level. The plant shoot was oven dried at 65 °C to constant weight to determine shoot dry matter (SDM). A completely randomized design with four replications was used.

Field experiments

The most responsive rhizobial isolates in the laboratory and greenhouse experiments were used in two field experiments conducted in the municipalities of Cachoeirinha, RS (29° 55' 30" S and 50° 58' 21" W) during the 2011/2012 growing season and Cachoeira do Sul, RS (30° 02' 20" S and 52° 53' 38" W) during the 2012/2013 growing season. In Cachoeirinha, RS, the soil was classified as a *Gleissolo Háplico Ta Distrófico típico* (Typic Haplaquents) and in Cachoeira do Sul, RS as a *Planossolo Háplico Eutrófico arênico* (Typic Albaqualf) (Embrapa, 2006). Soil chemical properties in the two experiments (0.00-0.20 m) are in table 1. Both areas are located in traditional rice-producing municipalities in the state of Rio Grande do Sul (southern Brazil) and belong to the Rice Experimental Station (Estação Experimental do Arroz - EEA) of the Rio Grande Rice Institute (Instituto Riograndense do Arroz - IRGA).

In the first year, the Puitá INTA rice cultivar was used. A bifactorial arrangement was used, with two N application rates (60 and 120 kg ha^{-1} N) and five treatments as single and combined inoculants of the UFRGS Lc348 and UFRGS Vp16 isolates and the commercial inoculant Azototal® based on *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) from Total Biotecnologia Indústria e Comércio Ltda. The product used was a liquid formula, and 100 mL of the commercial product per hectare is recommended according to company recommendations. The manufacturer lists an inoculant concentration of 2.0×10^8 cells mL^{-1}. A non-inoculated control treatment was also used, for a total of 12 treatments.

During the second year in the region of Cachoeira do Sul, the IRGA 422CL rice cultivar was used, which has greater grain yield potential. A bifactorial arrangement was used, with three N application rates (0, 70, and 140 kg ha^{-1}) and the inoculant treatments as previously described. A non-inoculated control treatment was also used, for a total of 18 treatments.

Split application of N in the form of urea was made: ⅔ at the V3 and V4 stages before flooding (10 days after emergence) and ⅓ as topdressing upon tillering. Upon sowing, 90 kg ha^{-1} K_2O as potassium chloride and 60 kg ha^{-1} P_2O_5 as triple super phosphate were applied.

In both experiments, the area was previously treated with glyphosate, and the soil was conventionally prepared using a disc plow. Phytosanitary management was conducted in accordance with the recommendations of the South Brazilian Wetland Rice Society (Sociedade Sul-Brasileira de Arroz Irrigado - Sosbai, 2007).

For the commercial inoculating product based on *A. brasilense,* 3.5 mL of the commercial product was mixed; we estimated that 5.3×10^7 cells were applied per m^2 per plot. The bacterial cells in the broth were counted using a Neubauer chamber. In the plots that received the rhizobia- and *A. brasilense*-based commercial product mixture, 3.5 mL rhizobia broth was mixed; we estimate that 2.5×10^8 cells were applied per m^2 per plot. The broth was mixed before inoculating the plots. To produce the bacterial inoculant, the UFRGS Vp16 and UFRGS Lc348 isolates were grown in 250 mL flasks with YM culture medium and incubated at 28 °C in an orbital incubator with 120 rpm shaking for six days. Thereafter, 3.5 mL broth with 7.5×10^8 cells mL^{-1} was mixed in 8 L sterilized distilled water, which generated a 3.3×10^5 cell mL^{-1} suspension. The total volume of the suspension (8 L) was manually applied on the whole plot over the crop row using a watering can shortly after sowing the plot; we estimated that 1.7×10^8 rhizobia cells were applied per m^2 in the plot.

A randomized block design in split plots was used, with four replicates. The main plot was the inoculant, and the split plot contained the N application rates. Each plot consisted of thirteen 6-m-long rows, which were spaced at 0.17 m, for a total area of 13.26 m^2. Screens were constructed between the plots with the inoculated treatments to prevent between-plot contamination.

The number of tillers per plant, yield components (grains per tiller and number of tillers per m^2), and plant SDM were assessed for one linear meter in two rows of each split plot over a 0.34 m^2 area. After oven drying at 65 °C, the samples were weighed and ground to determine the N content in accordance with Tedesco et al. (1995), as well as SDM total N. To determine plant height, the length from the base of the plant (soil surface) to the tip of the longest tiller was measured for 10 plants per experimental unit, which were randomly selected. Grain yield was obtained by manually harvesting the central rows of each split plot and corrected to 130 g kg^{-1} moisture.

Analysis of variance was applied to the results, and the means were compared using the Tukey test ($p \leq 0.05$). Statistical analyses were performed using R, version 3.0.3 (Team RDC, 2014).

Rice seedling colonization by *Azospirillum* and rhizobia tagged with *gfp*

A plasmid containing the *gfp* gene was constructed using a pBBR1MCS-2 vector with genes resistant to the antibiotic kanamycin. The method described by Rouws et al. (2006) was used to generate electrocompetent cells for the *A. brasilense* strains Ab-V5 and Ab-V6, and the methodology described by Garg et al. (1999) was used for the UFRGS Vp16 and UFRGS Lc348 rhizobia bacterial cultures. The two *A. brasilense* pure strains belong to the UFPR bacteria collection.

Seeds of the rice cultivar Puitá INTA were dehulled and surface disinfected through successive immersion in alcohol (70 %) for 1-min, followed by sodium hypochlorite (2.5 %) for 1-min, and seven consecutive washings in sterilized distilled water. The seeds were pre-germinated for eight days at 28 °C. After this period, each seedling was transplanted to a 25 cm-long, 24 mm-diameter test tube with a previously sterilized nutrient agar solution (6 %) (Sarruge, 1975).

The transformed *Azospirillum* (Ab-V5gfp and Ab-V6gfp) cultures were grown in Dygs culture medium containing 50 µg mL^{-1} kanamycin for four days at 28 °C. At the end of this period, a 1 mL aliquot of the bacterial culture with 1.5 × 10^9 cells mL^{-1} was inoculated per test tube. The transformed rhizobium cultures (Vp16gfp and Lc348gfp) were grown in LM culture medium with 50 µg mL^{-1} kanamycin for seven days at 28 °C. After that, 1 mL of the bacterial culture at 8.5 × 10^8 cells mL^{-1} was used to inoculate each test tube. The number of bacterial cells in the broth were counted under an optical microscope using a Neubauer chamber. The tubes containing the inoculated rice seedlings were kept in a lighted room with a daily 12 h photoperiod. In addition to the seedling treatments with inoculation, seedlings were grown in test tubes without inoculation. Three replicates were generated for each treatment.

At 10 and 20 days after inoculation, the rice seedlings were removed from the tubes and washed in sterile water. Longitudinal cuts were made by hand in the root hair zone, the lateral root emergence zone, and in the root division and elongation zone, as well as at the base of the stem and plant leaves. The fragments obtained were mounted on glass slides and blocked in 10 % sterile glycerol. The cuts were visualized using a confocal laser scanning microscope (Olympus FV1000) equipped with a 488 nm filter to detect the green fluorescence from the bacteria with the GFP protein. The images were collected in a confocal series and analyzed using the Olympus Fluoview 3.1 program. On each day of assessment, cuttings from three seedlings per treatment were evaluated.

RESULTS

Taxonomic characterization of the isolates

Partial sequencing of the 16S rDNA of the isolates used in the present study identified the UFRGS Vp16 isolate as belonging to the *Burkholderia* genus (100 % similarity) and the UFRGS Lg111, UFRGS Lc336, and UFRGS Lc348 isolates as belonging to the *Mesorhizobium* genus, with more than 99 % similarity.

Laboratory and greenhouse experiments

Inoculation with UFRGS Lc348 accelerated initial germination of the IRGA 422CL cultivar seeds by more than 38 % ($p \leq 0.05$) compared with the control (Table 2)., The UFRGS Vp16 and UFRGS Lg111 rhizobia also stimulated initial germination compared with the control, but at lower values (21 and 20 %, respectively). Inoculation with UFRGS Vp16 and UFRGS Lc336 increased the SDM of the RGA 422CL rice cultivar plants grown in sterilized substrate by 11 and 8 %, respectively. The RDM increased by 156 % when inoculated with UFRGS Vp16.

When rice was cultivated in pots with an established soil microbial population, plants inoculated with UFRGS Lc336 and that received 40 kg ha^{-1} N exhibited the same number of tillers as the control plants without inoculation that received 80 kg ha^{-1} N. However, SDM production was similar among the treatments with rhizobia inoculation.

Field experiments

For the Puitá INTA cultivar grown in the Cachoeirinha region, coinoculation with UFRGS Vp16 and a commercial inoculating product containing *A. brasilense* was compared with the control without inoculation and yielded average increases of 36.5 % in SDM, 50.3 % in SDM total N, 4.7 % in plant height, and 11.6 % in panicles m^{-2} at the two N application rates (Table 3). When using 60 kg ha^{-1} N, inoculation with UFRGS Vp16 in association with *A. brasilense* increased the tillers per plant by 18.9 %. The increase in SDM in this treatment corresponded to 1.9 Mg ha^{-1} compared with the control treatment without inoculation. Combined inoculation with UFRGS Lc348 and *A. brasilense* led to increases in SDM total N (25.7 %), in plant height (3.1 %), and in panicles m^{-2} (13.1 %) at the two N application rates,

Table 2. Initial germination, shoot (SDM) and root dry matter (RDM), and tillering of IRGA 422CL rice cultivar plants inoculated with isolates of rhizobia native to southern Brazil grown in pots with sterilized substrate and soil

Treatment	Sterilized substrate			Soil	
	Initial germination[(1)]	SDM	RDM	Tiller	SDM
	%	mg per plant		tiller per plant	mg per plant
UFRGS Vp16	53.9 b	331.0 a	813.0 a	5.56 ab	2.36 b
UFRGS Lc348	61.6 a	283.0 b	353.0 c	4.78 b	2.33 b
UFRGS Lg111	53.6 b	245.0 b	554.0 b	5.56 ab	2.14 b
UFRGS Lc336	45.4 c	323.0 ab	382.0 c	6.22 ab	2.03 b
Control 0 (kg ha^{-1} N)	44.6 c	297.0 b	318.0 c	-	-
Control 40 (kg ha^{-1} N)	-	-	-	5.22 ab	2.20 b
Control 80 (kg ha^{-1} N)	-	-	-	6.78 a	2.73 a
CV (%)	8.6	8.5	19.8	8.1	7.5

[(1)] Initial germination: percentage of seeds germinated on the third day compared with the number of seeds germinated on the sixth day. Means followed by the same letters do not differ statistically (Tukey, $p \leq 0.05$).

on average, and, when using 60 kg ha^{-1} N, an increase in tillers per plant (25 %), compared with the control treatment without inoculation (Table 3). Among the treatments with single inoculation, inoculation with the commercial product containing *A. brasilense* yielded a 4 % increase in plant height with 60 kg ha^{-1} N; the treatment with the Puitá INTA cultivar and single inoculation was the only treatment that promoted plant growth in rice plants.

For the IRGA 422CL cultivar (Table 4), as with the Puitá INTA cultivar, combined inoculation with UFRGS Vp16 and the inoculating product containing *A. brasilense* also exhibited outstanding results for many variables (SDM, SDM total N, grain yield, grains per panicle, and panicles m^{-2}) compared with the control treatment without inoculation. Coinoculation with UFRGS Vp16 and *A. brasilense* increased the number of panicles by 19.1 % when 70 kg ha^{-1} N was applied, and SDM increased 21.8 and 18.1 % when 70 and 140 kg ha^{-1} N was applied, respectively. When fertilized with 140 kg ha^{-1} N, the number of grains per panicle in the plants inoculated with these bacteria increased by 13.8 %, and SDM total N increased by 38.4 %. Furthermore, for this treatment, over 70 kg ha^{-1} N accumulated in SDM in the average of three N application rates. Additionally, in the plants that received application rates of 0 and 70 kg ha^{-1} N, inoculation with UFRGS Vp16 increased SDM (by 29.4 and 19.5 %, respectively).

An increase in grain yield was observed when the IRGA 422CL cultivar was coinoculated with UFRGS Vp16 and the commercial product containing *A. brasilense,* an 18.4 % increase compared with the control without inoculation using 70 kg ha^{-1} N. This grain yield increase corresponded to 1.424 Mg ha^{-1} and was greater than the control without inoculation at twice the N rate (140 kg ha^{-1} N). Grain yields were unaffected by the inoculations among the treatments with double the N application rate (140 kg ha^{-1}).

Nitrogen only affected the IRGA 422CL cultivar in regard to total N in the shoot and tillers per plant (Table 4). In the IRGA 422CL cultivar, at higher N application rates, only the number of tillers per plant did not show differences in response to the N factor.

Colonization of rice seedlings inoculated with *gfp*-tagged *Azospirillum* and rhizobia

Neither the *A. brasilense* Ab-V5gfp and Ab-V6gfp nor the rhizobia were observed in the inoculated rice seedling fragments from the base of the stem and the leaves at either 10 or 20 days after inoculation. However, these bacteria heavily colonized the surface of the root hair zone (Figures 1a and 1b). These bacteria also colonized the intercellular spaces of the root epidermis tissue (Figure 1c). Cells of the four marked bacteria were either alone or formed dense cell aggregations at the root hair zone of the rice seedlings and the lateral root emission zone (Figure 1d).

Table 3. Production of shoot dry matter (SDM), SDM total N, grain yield, plant height, grains per panicle, panicles m^{-2}, and tillers per plant of the wetland rice cultivar Puitá INTA inoculated with diazotrophic bacteria in the presence of 60 and 120 kg ha^{-1} of nitrogen

N	Control	UFRGS Vp16	UFRGS Lc348	*Azospirillum*	UFGRS Vp16 + *Azospirillum*	UFRGS Lc348 + *Azospirillum*	Mean	CV
kg ha^{-1}								%
				SDM (Mg ha^{-1})				
60	6.19	6.85	6.76	7.37	8.55	7.58	7.22 A	
120	7.11	7.31	7.55	7.11	8.49	8.05	7.60 A	16.7
Mean	6.65 b	7.08 ab	7.16 ab	7.24 ab	8.52 a	7.82 ab		
				SDM total N (kg ha^{-1})				
60	182.0	197.4	206.9	212.2	273.6	245.9	219.7 A	
120	212.6	214.0	212.2	197.0	252.1	250.3	223.0 A	17.7
Mean	197.3 b	205.7 b	209.6 b	204.6 b	262.9 a	248.1 a		
				Grain yield (Mg ha^{-1})				
60	5.82	5.80	6.12	6.51	6.29	6.43	6.16 ns	
120	6.12	6.16	6.35	6.48	6.22	6.62	6.33	8.1
Mean	5.97 ns	5.98	6.24	6.50	6.26	6.53		
				Plant height (cm)				
60	85.5	87.7	87.0	88.9	89.3	88.5	87.8 A	
120	86.8	88.1	88.0	88.1	88.9	89.2	88.2 A	8.4
Mean	86.2 b	87.9 ab	87.5 ab	88.5 a	89.1 a	88.9 a		
				Grains per panicle				
60	51.5	56.9	54.8	50.8	60.4	54.3	54.8 ns	
120	52.2	49.6	50.8	51.9	51.7	48.1	50.7	11.7
Mean	51.9 ns	53.3	52.8	51.4	56.1	51.2		
				Panicles m^{-2}				
60	427.9	441.2	415.4	494.9	480.9	461.0	453.6 ns	
120	448.5	448.5	426.5	463.5	497.8	530.1	469.2	15.0
Mean	438.2 b	444.9 ab	421.0 b	479.2 ab	489.4 a	495.6 a		
				Tillers per plant				
60	1.6 Ab	1.5 Ab	1.5 Ab	1.6 Ab	1.9 Aa	2.0 Aa	1.7	
120	1.3 Aa	1.4 Aa	1.4 Aa	1.5 Aa	1.3 Ba	1.4 Ba	1.4	32.1
Mean	1.5	1.5	1.5	1.6	1.6	1.7		

Means followed by the same lowercase letters in the line and uppercase letters in the column do not differ (Tukey, $p \leq 0.05$). ns: not significant.

DISCUSSION

The stimuli to initial germination of the IRGA 422CL cultivar seeds shown by inoculation with isolates UFRGS Vp16 and UFRGS Lc348 is an important result of this study. An increase in speed of germination is sought in the fields because it increases crop uniformity, especially in the panicle maturation period, which reduces loses in the harvest period. Additionally, faster germination reduces the period of heterotrophism and reduces the chances of attack by soil pathogens (Araújo et al., 2010). Stroschein et al. (2011) observed that rhizobia isolated from alfalfa increased the speed of germination of rice seeds and stimulated the growth of rice seedlings.

The isolate UFRGS Vp16 exhibited outstanding promotion of growth in rice plants cultivated in a sterilized substrate (Table 2) and in field experiments with the IRGA 422CL cultivar (Table 4). In the IRGA 422CL cultivar, this isolate exhibited high relative efficiency indexes among the treatments in which diazotrophic bacteria were inoculated

separately (Table 4). This cultivar also exhibited increased SDM production, as well as an increased number of grains per panicle. The isolate UFRGS Vp16 was obtained from clover nodules (*Trifolium repens*) and exhibited a highly efficient nitrogen-fixing capability in this legume (Alves et al., 2012) and high production of indole acetic acid (IAA) (Machado, 2011). Bacteria of the *Burkholderia* genus, which includes UFRGS Vp16, have great growth-promoting capacity in rice plants, as shown by Baldani et al. (2000), Van et al. (2000), Chen et al. (2005), and Govindarajan et al. (2008).

Table 4. Production of shoot dry matter (SDM), shoot dry matter total N (SDM total N), grain yield, plant height, grains per panicle, panicles m^{-2}, and tillers per plant of wetland rice cultivar IRGA 422CL inoculated with diazotrophic bacteria at 0, 70, and 140 kg ha^{-1} of nitrogen

N	Control	UFRGS Vp16	UFRGS Lc348	*Azospirillum*	UFGRS Vp16 + *Azospirillum*	UFRGS Lc348 + *Azospirillum*	Mean	CV
kg ha^{-1}								%
				SDM (Mg ha^{-1})				
0	5.21 Bb	6.75 Ba	5.48 Bab	6.40 Bab	5.44 Bab	6.20 Bab	5.91	
70	8.95 Ab	10.70 Aa	9.81 Aab	10.25 Aab	10.90 Aa	9.94 Aab	10.09	10.9
140	10.08 Ab	11.42 Aab	11.16 Aab	11.14 Aab	11.90 Aa	11.18 Aab	11.15	
Mean	8.08	9.62	8.82	9.26	9.41	9.11		
				SDM total N (kg ha^{-1})				
0	68.2	89.7	65.4	76.3	59.3	80.0	73.2 C	
70	211.3	267.4	222.7	248.0	264.8	266.4	246.8 B	18.3
140	270.2	314.1	310.3	348.7	436.8	364.1	340.7 A	
Mean	183.2 b	223.7 ab	199.5 b	224.3 ab	253.6 a	236.8 ab		
				Grain yield (Mg ha^{-1})				
0	5.43 Ba	5.49 Ba	5.28 Ba	6.11 Ba	5.52 Ca	5.34 Ba	5,53	
70	7.74 Ab	8.44 Aab	7.77 Ab	8.79 Aab	9.16 Aa	8.30 Aab	8,37	8.1
140	8.16 Aa	8.90 Aa	8.05 Aa	8.28 Aa	8.14 Ba	9.06 Aa	8.43	
Mean	7.11	7.61	7.03	7.73	7.61	7.57		
				Plant height (cm)				
0	76.2	77.0	75.4	76.1	76.3	76.5	76.3 C	
70	89.0	91.0	89.9	90.5	90.3	91.8	90.4 B	9.3
140	94.8	94.6	97.8	94.9	96.4	95.2	95.6 A	
Mean	86.7 a	87.5 a	87.7 a	87.2 a	87.7 a	87.8 a		
				Grains per panicle				
0	51.2	53.7	49.9	58.2	53.4	59.0	54.2 C	
70	57.8	61.7	60.3	64.4	60.3	59.7	60.7 B	10.8
140	67.4	79.3	72.1	75.3	86.9	73.3	75.7 A	
Mean	58.8 b	64.9 ab	60.8 ab	66.0 ab	66.9 a	64.0 ab		
				Panicles m^{-2}				
0	610.4 Aa	620.3 Ba	533.0 Ba	550.0 Ba	553.7 Ca	581.6 Ba	574.8	
70	789.0 Ab	817.6 Ab	723.5 Ab	807.3 Ab	939.7 Aa	776.5 Ab	808.9	15.4
140	752.9 Aa	861.0 Aa	814.0 Aa	844.9 Aa	763.2 Ba	820.6 Aa	809.4	
Mean	717.4	766.3	690.2	734.1	752.2	726.2		
				Tillers per plant				
0	2.5	2.3	2.2	2.2	2.5	2.3	2.3b	
70	2.7	3.0	2.5	2.7	2.6	2.9	2.7a	19.7
140	2.4	2.8	2.8	3.0	3.0	2.7	2.8a	
Mean	2.5 ns	2.7	2.5	2.6	2.7	2.6		

Means followed by the same lowercase letters in the line and uppercase letters in the column do not differ (Tukey, $p \leq 0.05$). ns: not significant.

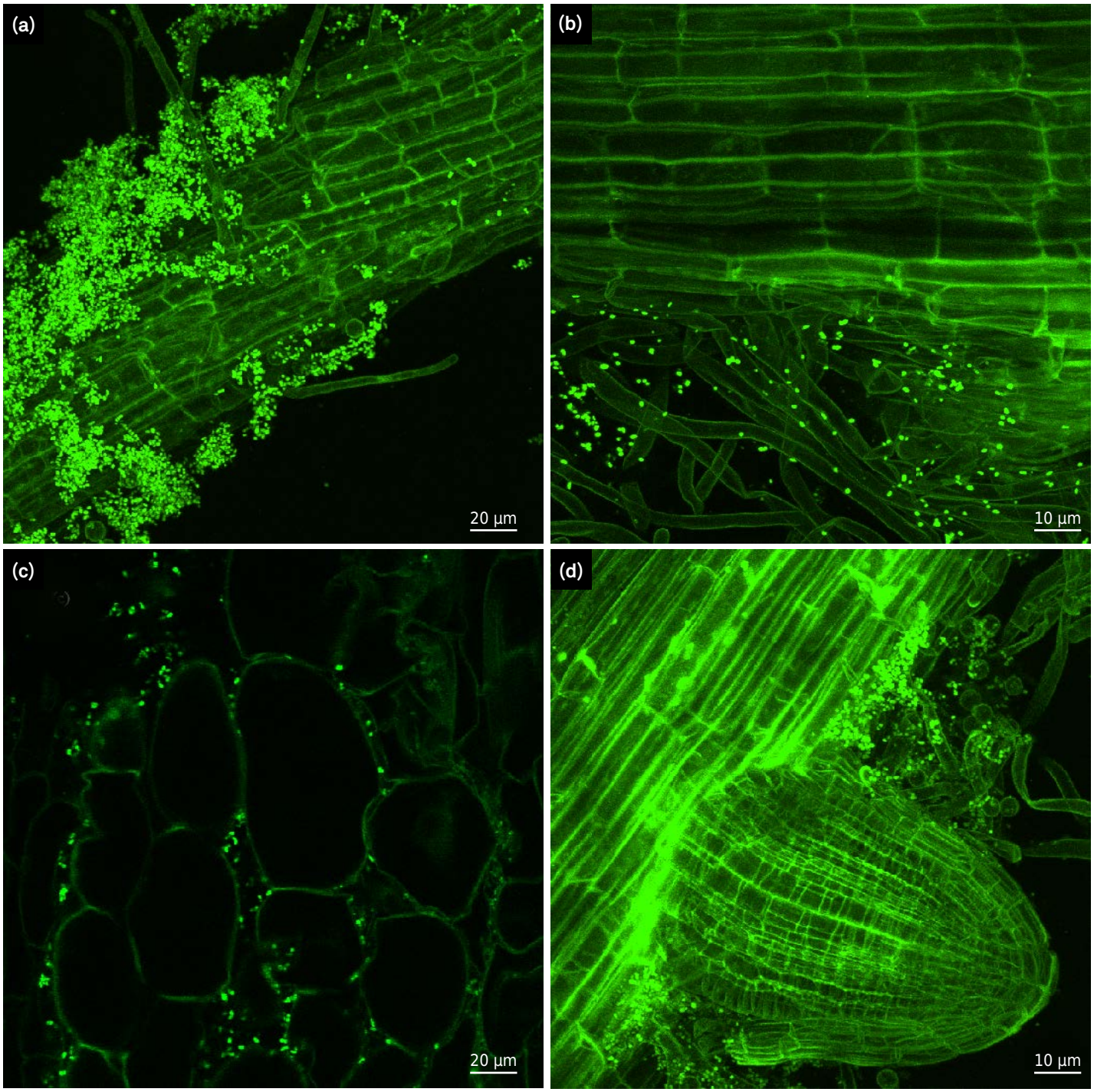

Figure 1. Confocal laser scanning microscopy micrographs of longitudinal cuts in rice seedling fragments. (a) Rhizobia *Burkholderia* sp. Vp16gfp colonizing the rhizoplane in the rice seedling root hair zone after 10 days of growth. (b) Rhizobia *Mesorhizobium* sp. Lc348gfp colonizing the rhizoplane in the rice seedling root hair zone after 20 days of growth. (c) *A. brasilense* Ab-V5gfp in the intercellular colonization of the epidermis in the branching zone of the rice seedling root after 20 days of growth. (d) Colonization by the rhizobia *Burkholderia* sp. Vp16gfp in the rice seedling lateral root emission region after 20 days of growth.

The commercial inoculant product containing *A. brasilense* and UFRGS Vp16 were inoculated separately, which promoted the growth of the rice cultivar used in the field experiments. Inoculation with the same bacteria in the two rice cultivars produced different results, perhaps due to differences in the plant/microorganism growth-promoting interactions, suggesting that cultivar specificity may exist. This is suggested by findings of Hardoim et al. (2011) in a survey of microbial communities present in 10 rice cultivars, in which both bacterial adaptation and plant genotype contribute to shaping the dynamic bacterial communities associated with roots of rice plants. Differences in the responses

of IRGA 422CL and Puitá INTA cultivars to inoculated bacteria observed in this study might be attributable to genetic differences between cultivars. One likely basis for the highly variable results observed with single inoculation with bacteria in plants is variable root colonization, due to problems with inoculum survival or unfavorable conditions for the bacteria (Haas and Defago, 2005; Raaijmakers et al., 2009); if the bacterium cannot colonize the rhizosphere, it cannot interact with the plant (Yanni et al., 2001; Sasaki et al., 2010). Despite these findings, our results with gfp-tagged *Azospirillum* and rhizobia showed these bacteria heavily colonizing the surface of the root hair zone and intercellular spaces of the root epidermis tissue. These potential concerns demonstrate the complexity of interactions involved in the rhizosphere among the plant, the introduced bacteria, and the remaining rhizosphere population, neutral or deleterious, which significantly interferes with the capacity of the isolate to promote plant growth (Antoun et al., 1998). The secondary metabolism compounds production in rice cultivars, which may signal that an effective bacteria/plant interaction may be influenced in different ways by bacteria in the rhizosphere environment and may be used to determine differences in growth-promoting effects in cultivars, which has been demonstrated in maize varieties and through interactions with the *Azospirillum* genus (Walker et al., 2011).

In our study, UFRGS Lc348 and UFRGS Vp16 isolates promoted growth in rice plants when they were coinoculated with the commercial inoculating product containing *A. brasilense*. In both field experiments, combined inoculation with UFRGS Vp16 - *A. brasilense* commercial inoculating product resulted in more variables with higher values compared with the other treatments. Plant hormone production (Chen et al., 2005; Bhattacharjee et al., 2012), phosphate solubilization (Alikhani et al., 2006; Bakhshandeh et al., 2014), and BNF (Rodrigues et al., 2008) or indirect mechanisms, such as plant protection from pathogens (Dutta et al., 2007), were indicated as important bacteria growth-promoting mechanisms when used to inoculate plants. Ueda et al. (1995) stated that for combined inoculation with growth-promoting bacteria, each isolate can occupy a different niche in the plant, substituting expected competition with a cooperative effect. In the present study, combined inoculation with bacteria showed better results than when the bacteria were inoculated alone in the plants, which has also been demonstrated in other studies. In field experiments by Govindarajan et al. (2008), the authors observed increases from 9.5 to 23.6 % in rice growth from combined inoculation with *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Azospirillum lipoferum*, and two *Burkholderia vietnamiensis* isolates; inoculation with each bacterium alone promoted rice plant growth by only 3.1 to 12.8 %. In Brazil, Oliveira et al. (2006) observed yield increases in sugarcane when it was inoculated with a combination of five diazotrophic bacteria. Notably, in the studies indicated above, combined inoculation with diazotrophic bacteria was studied; however, here we studied combined inoculation with associative bacteria of the *Azospirillum* genus as a commercial product along with rhizobia isolated from legumes (clover and bird's foot trefoil).

Diazotrophic bacteria of the *Azospirillum* genus have been defined as facultative endophytes (Döbereiner et al., 1995) and have been detected colonizing both the root surface and intercellular space of root epidermis cells without infecting the cortex cells and xylem tissues (Ramos et al., 2002; Rothballer et al., 2003). These results are in agreement with the present study because cells from the strains *A. brasilense* Ab-V5gfp and Ab-V6gfp were not detected colonizing tissues in the cortex, xylem, and shoots (stem and leaves) of rice seedlings. However, Chi et al. (2004) detected *A. brasilense* Yu62, which was used to inoculate rice and tobacco seeds, densely colonizing the plant stem and leaves.

In addition to *Azospirillum*, other diazotrophic bacteria genera have shown controversial results regarding their capacity to colonize shoot tissue after inoculation of seeds. Elbeltagy et al. (2001) inoculated wild rice seeds (*Oryza officinalis*) with diazotrophic

bacteria from the *Herbaspirillum* genus with *gfp* and showed that these bacteria colonized the rice seedling sprouts. However, Zakria et al. (2007) did not detect *gfp*-labelled bacteria from this genus in rice shoot tissues. Confocal microscopy was used to analyze plant fragments inoculated with the rhizobia Vp16gfp and Lc348gfp; their cells were not detected colonizing the more internal rice seedling root and shoot tissues. However, the rhizobia and *Azospirillum* isolates densely colonized the rhizoplane of inoculated rice seedlings, mainly in the root hair zone. Chi et al. (2004) showed that rhizobia can systemically ascend to rice plant shoots when inoculated on seeds. The authors identified populations with more than 9×10^{10} rhizobia (from seed inoculation) per cm^3 of shoot tissue and suggested that bacterial cells disseminated through the aerenchyma and conducting vessels ascend to the stem and leaves. Furthermore, the authors observed that certain rhizobium species and strains can persist in the interior of rice tissue until the plant initiates its reproductive phases.

Using rhizobia with a *gus* gene inserted through plasmid conjunction, Osório Filho (2009) studied rhizobium infection in plantlets from bird's foot trefoil (*Lotus corniculatus*), clover straw (*Trifolium vesiculosum*), and rice (*Oryza sativa*) and observed that the rhizobia penetrated the legume roots, concentrating only on the nodules and nodule primordia. In rice seedlings, the author observed bacteria colonizing the root and canopy tissue, especially in the secondary roots, root cortex, and leaf vascular tissue. Rhizobium penetration has also been observed in rice roots with the formation of large bacterial aggregates and between cells of the lateral root emergence region and root cortex (Webster et al., 1997).

To obtain higher rice grain yields with more efficient use of applied N, symbiotic bacteria can be combined with other plant-growth-promoting bacteria, such as *Azospirillum*, as an efficacious alternative. As demonstrated by the results of field experiments, the treatment with half the N application rate in combination with rhizobium and a commercial product containing two *A. brasilense* strains (UFRGS Vp16 + inoculating product) showed that the same grain yield can be obtained as with full N application rates without inoculation.

The results of the present study indicate high potential for increasing wetland rice production through coinoculation with plant-growth-promoting microorganisms such as rhizobia and *Azospirillum*, which may reduce production costs and potential environmental impacts from nitrogen fertilizers.

CONCLUSIONS

Initial germination, and shoot and root dry matter of rice cultivated in a greenhouse increase with UFRGS Vp16 inoculation.

Combined inoculation with UFRGS Vp16 and the commercial inoculating product containing *Azospirillum brasilense* promotes the growth of IRGA 422CL and Puitá INTA rice cultivars grown in the field.

Confocal laser microscopy confirms the presence of inoculated bacteria colonizing the rice seedling root surface, mainly in the root hairs and lateral root emergence sites.

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