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Bradyrhizobium japonicum, *B. elkanii* and *B. diazoefficiens* Interact with Rice (*Oryza sativa*), Promote Growth and Increase Yield

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Abstract

Bradyrhizobium is a genus of plant growth-promoting rhizobacteria (PGPR) that have been studied for several decades mainly for the ability to fix diazotrophic nitrogen after having been established endosymbiotically inside root nodules of the legumes of Fabaceae. The aim of this work was to evaluate the capability of *Bradyrhizobium* to promote the growth of crops belonging to other families, in this case, rice (*Oryza sativa*), both in laboratory and in field trials. For laboratory test, surface-sterilized rice seeds were soaked with cultures of each strain and planted in pots. Plant length and dry weight were measured after 35 days. For the field test, rice seeds of varieties Yeruá La Plata and Gurí INTA were inoculated with the three best strains observed in the laboratory test and planted in plots. After 60 days of growth, plant length and dry weight were measured. At harvest time, we measured the dry weight of the aerial part, yield and thousand-grain weight. Inoculation with any of the three species described provoked significant increments compared to the uninoculated control at least in one of the parameters measured, both in the laboratory and in the field tests. *Bradyrhizobium japonicum* E109 was the strain that promoted rice growth the most in the lab while *Bradyrhizobium elkanii* SEMIA 587 was the strain that promoted rice growth the most in the field, with increments in yield of approximately 1000 kg/ha. Data obtained suggest that the *Bradyrhizobium* species promoted all rice growth and yield.

Introduction

It has been predicted that the world population will reach 11 billion by the year 2100 [1], 87% of it will be living in developing countries. Within many of these countries, food demand is on rise and rice is a staple food [2] for more than 3 billion. Since there is not much more surface to be cultivated the only way to increase production is by increasing yields of crops and this might be achieved by improving crop management as well as soil nutrients.

Many agricultural systems managements led to the loss of soil fertility (mainly macronutrients) due to subsequent harvests in monoculture strategies and often soils are amended with mineral fertilizers. Among them, nitrogen, the most limiting factor of growth and yield, is applied in 2–3 splits while 15 to 20 kg of N is needed for every ton of rice yield [3]. However, fertilizers pose a threat to environment since water sources might be contaminated with nitrates as well as the atmosphere that might be contaminated with N oxides as well as CH_4. In addition, their cost cannot be afforded by farmers.

The availability of environmentally friendly and cheaper fertilizers might lead to increase production within developing countries, protecting soils and water sources. Because of this, scientists are concentrating their efforts in developing bio-fertilizers such as microbes with the ability to mineralize nutrients within industrial wastes and unavailable soil nutrients, produce plant hormones and/or compete with plant pathogens [4–7].

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The main source for the isolation and further development of biofertilizer is the soil as it contains plant growth-promoting (PGP) bacteria, microorganisms that colonize plant roots enhancing plant growth [8]. Plant growth-promoting rhizobacteria (PGPR) predominantly live in the rhizosphere but also can grow within, or around tissues and stimulate plant growth by different mechanisms such as phosphate solubilization, siderophore production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormones production, antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance (ISR), promoting beneficial plant–microbe symbioses and interference with pathogen toxin production, among others [9–11]. These mechanisms were grouped in direct and indirect ones, while the former ones occur within the plant affecting directly plant metabolism, the latter ones occur outside of the host. While direct mechanisms include a balance of plant growth regulators, biological nitrogen fixation, phosphate solubilization, production of phytohormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins, indirect mechanisms include synthesis of siderophores, chitinases, glucanases and/or antibiotic molecules that trigger ISR. PGPR modulates plant stress markers under abiotic stress and production of ACC deaminase.

Biological nitrogen (N) fixation, mainly through the legume-rhizobium symbiosis, has been a way humanity increased productivity in sustainable agricultural systems [12]. Because of this, inoculants of nitrogen-fixing bacteria are produced on large scale in many countries.

Bradyrhizobium japonicum [13], *Bradyrhizobium elkanii* [14] and *Bradyrhizobium diazoefficiens* [15, 16], commonly known as rhizobia, even though they are not the only symbionts of soybean, they have been used the most to formulate commercial inoculants in the main producing area of soybean around the world that includes Brazil, Argentina, Paraguay and Uruguay. *Bradyrhizobium* include slow-growing [17, 18], gram-negative soil bacteria that develop nodules on soybean. There is an ample diversity of these organisms that is shown by the broad array of serological groups (serogroups) [18]. Genotypic and phenotypic variations of *B. japonicum* strains have been reported in terms of DNA fingerprints, internal transcribed spacer (ITS) sequences between 16 and 23SrDNA, denitrification, symbiotic associations and nitrogen fixation [19].

It has already been demonstrated that *Bradyrhizobium* not only benefits legumes in terms of biological nitrogen fixation but it also promotes the growth of many crops [20]. Chebotar et al. [21] found that co-inoculation of *B. japonicum* and rhizobacteria increased nodulation, nitrogenase activity, plant growth and yield of soybean. Antoun et al. [20] inoculated radishes with 266 collection strains of rhizobia and bradyrhizobia and found that while some bacteria reduced plant growth others promoted it, among the latter ones a *B. japonicum* was identified and it was found that it might enhance growth of maize.

More recently, *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* strains have been identified as endophytes of different rice cultivars and species growing naturally or under cultivation in different geographical regions around the world [22]. It has been recognized that these legume symbionts might promote the growth of non-legumes, like cereals such as rice (*Oryza breviligulata* and *Oryza sativa*), wheat and maize. Chaintreuil et al. [23] found photosynthetic *Bradyrhizobium* strains living endophytically within stems of the legume Aeschynomene, as well as rice *O. breviligulata*.

Regarding rice plants, Mano and Morisaki [24] found *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* living associated with the roots of rice. Furthermore, Sreevidya et al. [25] showed that rhizobia colonized root epidermal cells, including root hairs of transgenic rice (*Oryza sativa* L. cv. Murasaki) whose growth was significantly promoted by *Rhizobium leguminosarum* var. *viciae* or *Bradyrhizobium japonicum* USDA110, respectively.

Therefore, the aim of this study was to evaluate the effect of inoculating commercial strains of *Bradyrhizobium japonicum, Bradyrhizobium elkanii* and *Bradyrhizobium diazoefficiens* on rice in laboratory as well as in field experiments and determine the plant growth-promoting effect of *Bradyrhizobium* strains on rice plants.

Materials and Methods

Strains, Growth and Rice Inoculation Conditions

Five commercial *Bradyrhizobium* strains, two *Bradyrhizobium japonicum* (SEMIA 5079 and E109), two *Bradyrhizobium elkanii* (SEMIA 587 and SEMIA 5019) and one *Bradyrhizobium diazoefficiens* (SEMIA 5080) from the collection of the Universidad Nacional de La Plata, Argentina, were used in this study.

Bradyrhizobium strains stock cultures were kept in the freezer at − 80 °C with 10% polyethylene glycol (PEG). Slants of the isolate were done on Yeast Extract Mannitol medium (YEM) [26] that were inoculated and grown for a week in the darkness at 28 °C. 5 mL cultures of each isolate were grown by inoculating tubes with 5 mL of YEM with a loop from the slants kept in the refrigerator. These were incubated in an orbital shaker at 150 rpm for 7 days. Then an aliquot of the culture was inoculated and grown in YEM. Five ml cultures were transferred to 50 mL of liquid YEM medium and incubated at 30 °C for 1 week. Bacteria were grown to mid log phase at OD_{600} of 0.6–1.

Seeds of Italian cultivar Baldo were surface sterilized by immersing them for 10 min in 95% ethanol, rinsed twice with sterilized distilled water and then immersed 10 min in 10% calcium hypochlorite (CaOCl) and finally rinsed six times with sterile distilled water. Then, they were placed in sterile petri dishes with moistened filter papers and were incubated at room temperature in the darkness. Seedlings with 1 to 2 cm long roots were inoculated with a bacterial suspension obtained by resuspending pelleted cells of a Mid log phase bacterial cultures in sterile PBS to a concentration of 1×10^8 cells/mL. Inoculation was performed by soaking germinated seeds in the inoculant suspension or in sterile PBS (negative control) for 20 min. 2-weeks old seedlings were transferred to plastic pots containing local unsterilized soil. Complete randomized design (CRD) with three replicates per each treatment was followed. Pots were watered with tap water once in every two days and maintained at a constant water level above the soil surface.

Several parameters were measured within plants of the pot assay. The determinations made on the rice plants were shoot and root length (cm) and dry weight (g), shoot and root weight (g), chlorophyll content of leaves at two stages of growth; days to fifth leaf and plant height (cm) at fifth leaf stage; days to flowering, plant height (cm), culm number, leaf number and chlorophyll content (µg/mL) were measured in infected Baldo rice grown in pots.

The Chlorophyll content was measured (i) 2 weeks after inoculation, (ii) at the V5 vegetative stage corresponding to the upper part of the last leaf developed that acquired at least 80% of its size, which was the flag leave at the V5, and (iii) at flowering stage. An 0.2 g of fresh plant leaf was frozen and kept at − 20 °C for 5 days. Chlorophyll was measured as described by Arnon [27]. Briefly, samples were homogenized in 5 mL of 80% acetone at 4 °C until complete leaf decolouration, then they were centrifuged at 2500 rpm for 10 min then absorption of the extracts was measured using a spectrophotometer at 663 nm (A_{663}), 647 nm (A_{647}) and 470 nm (A_{470}) in a Sp-2000UV spectrophotometer. Total chlorophyll content and carotenoid content were calculated using the formula used by Sumanta, et al. [28].

$$\text{Chlorophyll a}\left(C_a\right) = 12.25A_{663} - 2.79A_{647}$$

$$\text{Chlorophyll b}\left(C_b\right) = 21.5A_{647} - 5.1A_{663}$$

$$\text{Carotenoid}\ \left(C_{x+c}\right) = \left(1000A_{470} - 1.82C_a - 85.02C_b\right)/198$$

Re-Isolation of *Bradyrhizobium* from Inoculated Plants

Samples of 0.2 g of leaf, shoot and root were collected from inoculated and non-inoculated plants 15 days after inoculation. Each sample was thoroughly washed with tap water, rinsed with sterile distilled water and dried before proceeding to surface sterilize them by immersion in 95% ethanol for 2 min followed by 6 washes with sterile distilled water followed by an immersion in 70% calcium hypochlorite for 10 min. After rinsing the samples, 100 µL of the final wash was plated on Tryptone-Yeast extract (TY, Casein enzymic hydrolysate 6.0 g/L, Yeast extract powder 3.0 g/L, Agar 12.0 g/L, pH 7.2) agar plates. TY plates with no growth confirmed sterility of the samples that were used to isolate endophytic bacteria. Then, samples were crushed in sterile mortars supplemented with 20 µL of sterile water in the laminar hood under sterile conditions. Then 5 µL was transferred to an Eppendorf tube containing 495 µL of sterile water and, after vortexing, 0.1 mL aliquots were spread on YEM agar and incubated overnight before monitoring. We analysed the bacterial content on three replicates of each treatment.

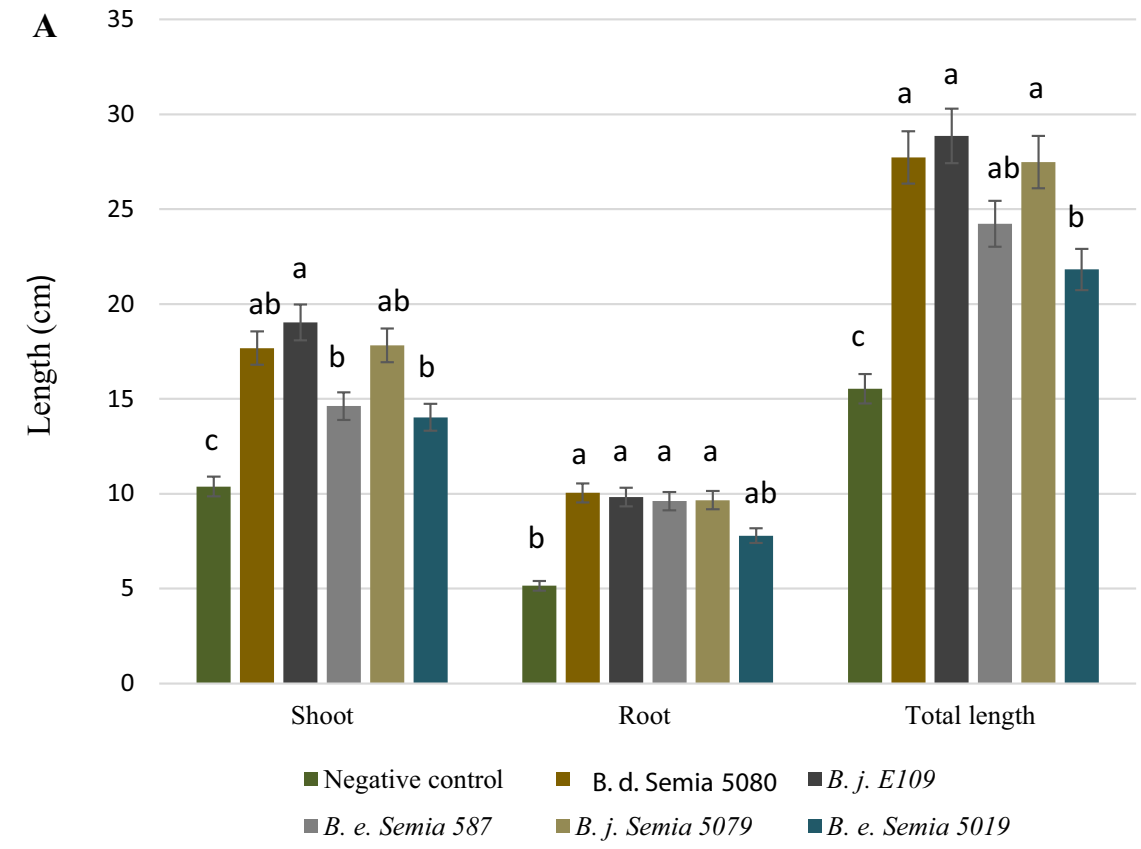
Genetic Characterization of Re-Isolated Strains

Bacterial colonies re-isolated from inoculated plants were cultured in nutrient broth (NB; Beef Extract 1.0 g/L, Yeast Extract 2.0 g/L, pH 6.8) liquid medium that was agitated in an orbital shaker at 180 rpm at 30 °C for 4 days. One ml culture of each organism was transferred into 1.5 mL microcentrifuge tubes and DNA was extracted by using a Genomic DNA Purification Kit (Promega) following the instructions of the supplier. The *nifH* gene was amplified by using universal primers: *NifH F2*, 5′-TGYGAYCCIAAIGCIGA-3′ and *NifH R6*, 5′-TCIGGIGARATGATGGC-3′ [29].

Field Test

B. diazoefficiens SEMIA 5080 (83), *B. japonicum* E109 (84), *B. elkanii* SEMIA 587 (85) were used to inoculate rice seeds that were sown in the field to evaluate bacterial growth promotion effect on two rice varieties, Gurí INTA ssp. Indica (G) and Yeruá La Plata ssp. Japonica (Y) (Argentina).

A liquid YEM culture of 5 mL of each *Bradyrhizobium* strain was prepared at room temperature for inoculation. After 24 h of culture, it was transferred to 100 mL of YEM liquid medium and incubated at 30 °C for 1 week. A bacterial suspension of each *Bradyrhizobium* strain was prepared as described above. At the moment of inoculation, they were supplemented with 5% sucrose as adhesive and then seeds were soaked in a plastic container containing 50 mL of a bacterial suspension of 1×10^9 cfu/mL. Seeds were sown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Shoot
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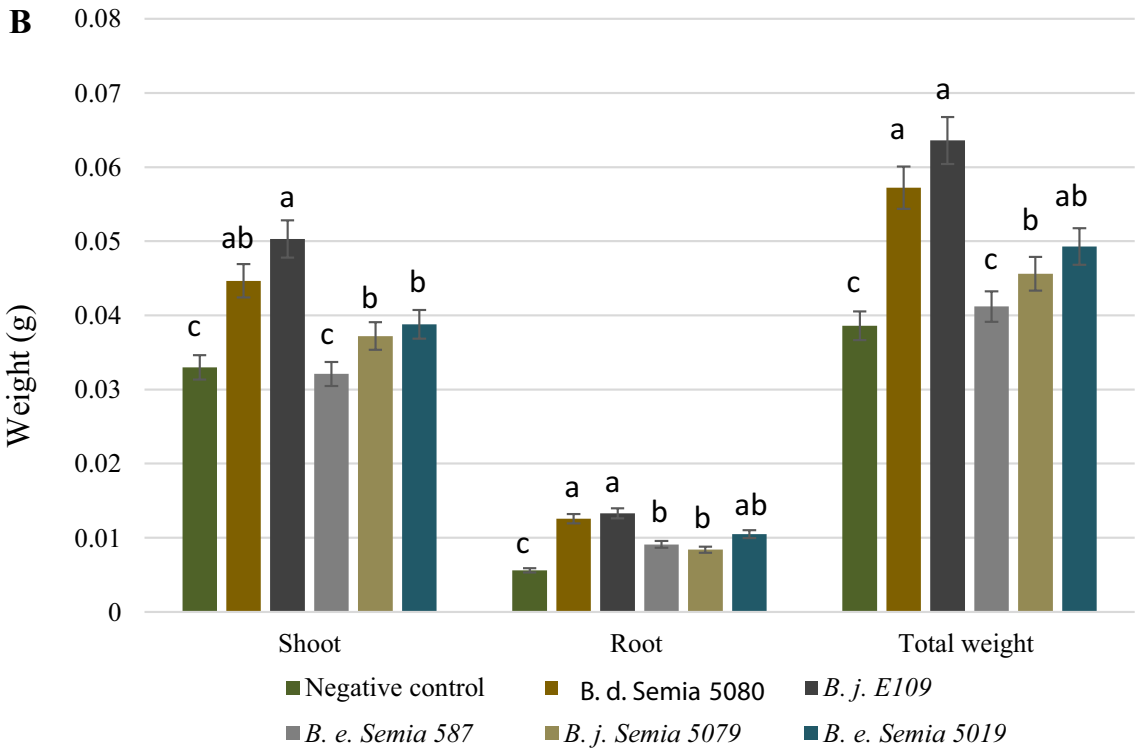
Negative control
B. d. Semia 5080
B. j. E109
B. e. Semia 587
B. j. Semia 5079
B. e. Semia 5019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c
b
ab
Shoot
Root
Total weight
Negative control
B. d. Semia 5080
B. j. E109
B. e. Semia 587
B. j. Semia 5079
B. e. Semia 5019

◂**Fig. 1** Length (**A**) and dry weight (**B**) of rice plants cv Baldo non-inoculated (Negative control) and inoculated with *B. diazoefficiens* SEMIA 5080 (*B. d. Semia 5080*), *B. japonicum* E109 (*B. j. E109*), *B. elkanii* SEMIA 587 (*B. e. Semia 587*), *B. japonicum* SEMIA 5079 (*B. j. Semia 5079*) and *B. elkanii* SEMIA 5019 (*B. e. Semia 5019*), after 2-weeks. Similar letters indicate that differences were not statistically significant

in lots of 5 × 2 m (5 rows, 20 cm between each) and plant density was of 400 seeds per square meter. The seeds of each inoculated plot were mixed with 15 mL of culture. Rice plots were managed under irrigation without flooding and were not amended with chemical fertilizers. Chyalofop was applied for preventative barnyard grass control.

The parameters measured were determined at two stages of growth development, 2 months after sowing and at harvest. At the first sampling date, three plants were collected at random from each plot and the following parameters were determined: shoot height (cm) and weight (g), root length (cm) and weight (g), total plant length (cm) and total plant weight (g). At harvest, 3 linear meters per plot were harvested and yield (g/m^2) and thousand grains weight (PMG, g) were calculated and 1 linear meter of each plot was used to calculate aerial dry matter (g/m^2).

Data Collection and Analysis

In pot experiments, a two-way variance (ANOVA), means (ANOM), and simple regression analysis was conducted using Minitab® (version 15.1.0.0.) statistical software; statistically significant differences were established at the $P < 0.05$ level. Significant difference between means of each treatment was compared using the Duncan Multiple Range Test (DMRT) using SAS software (9.1 version, USA).

In the case of field trials, the design was a randomized block design with 3 replicates per treatment. All the data were analysed by the analysis of variance ANOVA (one-way analysis of variance) at the 0.05 level ($P < 0.05$). The level of significance was calculated by using Fisher's LSD test (Least significant difference) ($P < 0.05$) for multiple comparisons. The statistical program InfoStat (version 2011) was used.

Results and Discussion

Bradyrhizobium Effect on Rice Growth

Growth of rice plants was assessed by measuring root, shoot and whole plant length and dry weight along plant development at the seedling stage, 2 weeks after inoculation with *Bradyrhizobia*. The other two developmental stages corresponded with the moment rice plants developed the 5th leaf and the development of the first primary tiller, when the second node's tiller emerges and roots start growing. In Fig. 1, we present plant growth at the first step of plant development. Inoculation promoted plant growth, whether measured by shoot or plant height and dry weight, independently of the *Bradyrhizobium* isolate inoculated. However, it should be highlighted that two strains, *B. diazoefficiens* SEMIA 5080 and *B. japonicum* E109, promoted plant growth the most, in particular in term of dry weight (Fig. 1A). Furthermore, the analysis of plant height and plant dry weight showed that they looked similar suggesting that any of them can be a reliable index of rice growth. Therefore, we used this information to make non-destructive measurements at the 5th leaf stage.

Plant development is important and is an indirect index of plant growth so we determined the number of days it took plants of each treatment to reach the 5^{th} leaf stage as well as the flowering stage (Fig. 2). It can be seen that inoculation with *B. japonicum* and *B. diazoefficiens* strains substantially reduced the number of days required to get to the fifth leaf stage, while it was not the same when *B. elkanii* was the inoculated bacteria (Fig. 2A). Observation of inoculation effects on days to flowering showed that the *B. japonicum* E109 was the strain that anticipated flowering the most, seventeen days earlier that the non-inoculated control. However, also *B. japonicum* SEMIA 5079 and *B. elkanii* SEMIA 587 significantly anticipated flowering (Fig. 2B and Table 1). This phenomenon of plant growth anticipation might be related with either a particular molecule synthesized and released within the plant rhizosphere either by one or the other strains used or to the different ability of these two species of rhizobia to colonize plant tissue and live as an endophyte and promote growth.

At the flowering stage, plant growth was assessed also by measuring plant height showing that *B. diazoefficiens* SEMIA 5080, *B. japonicum* E109 and *B. japonicum* SEMIA 5079 were the ones that promoted growth the most (Table 1).

To assess the influence of rice inoculation on nitrogen nutrition and photosynthesis, we analysed Chlorophyll a (*Ch a*), Chlorophyll b (*Ch b*) and Carotenoids (Table 2). We found some significant differences that have been further analysed by Principal Component Analysis (PCA) and Hierarchal Cluster Analysis (HCA).

Attempts to recover *Bradyrhizobium* strains from inoculated rice seedlings resulted in the isolation of slow-growing (at least 6–7 days) rhizobia. They did not form spores and developed circular, opaque, rarely translucent, white and convex colonies, phenotypically similar to those developed by *Bradyrhizobium* strains used for the inoculation. The result of this re-isolation and the observed average number of Colony Forming Units (CFU/g) are 26 ± 1, 378 ± 12 and 2 ± 1 for leaves, shoots and roots, respectively. These results suggest that *Bradyrhizobium* locates predominantly in shoots, confirming previous reports of its endophytism in rice [23, 24, 30]. When genomic DNA from re-isolated

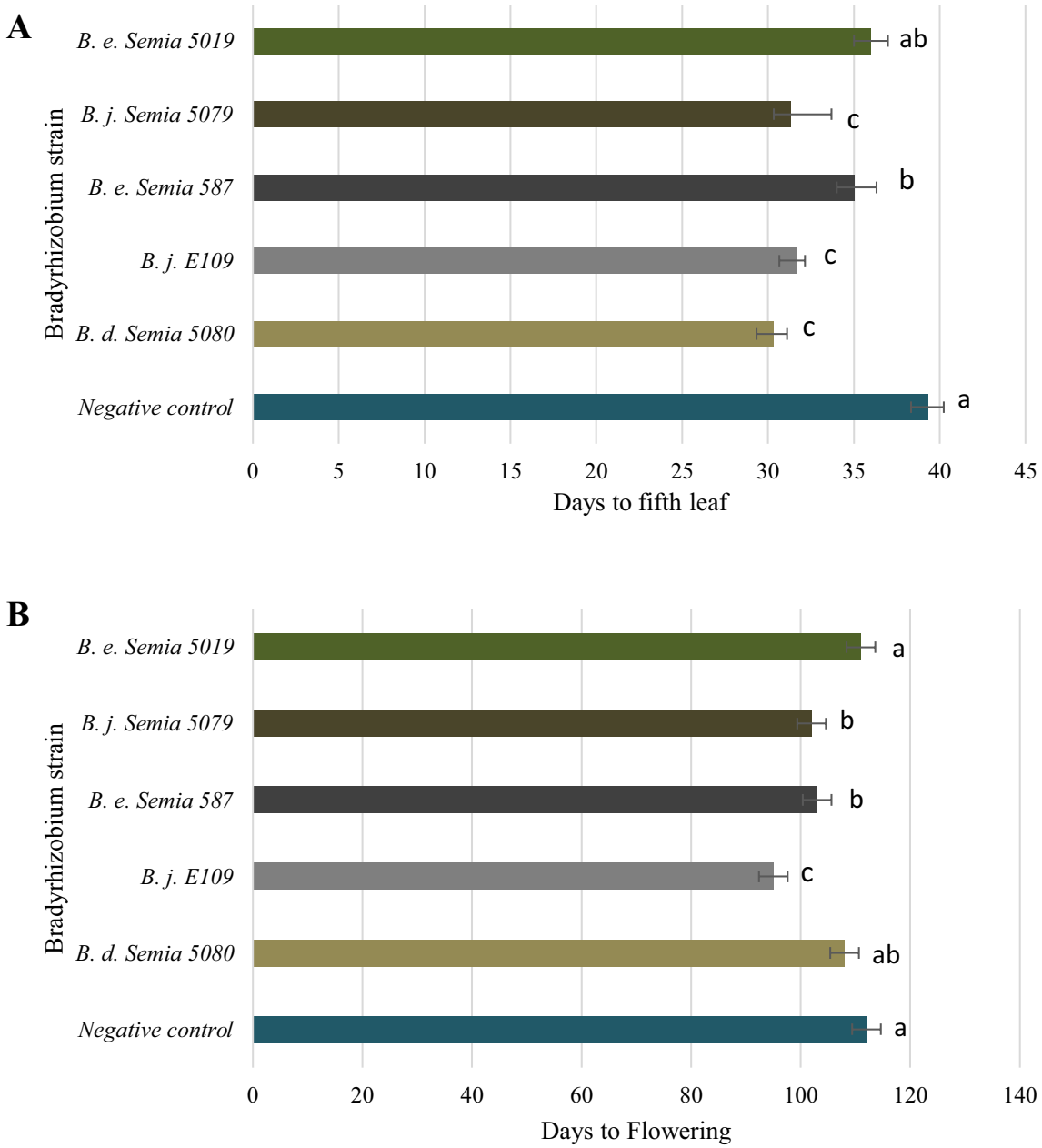

Fig. 2 Length of the period of time (days) required by rice plants cv Baldo non-inoculated (Negative control) and inoculated with *B. elkanii* SEMIA 5019, *B. japonicum* SEMIA 5079, *B. elkanii* SEMIA 587, *B. japonicum* E109, *B. diazoefficiens* SEMIA 5080 to reach the fifth leaf stage (**A**) and the flowering stage (**B**). Similar letters indicate that differences were not statistically significant

Table 1 Average values for days to flowering, plant height, culm number and leaf number at flowering stage after the inoculation

Strain	Days to flowering	Plant height (cm)	Culm number	Leaf number
Negative control	112 ± 1.25 (a)	34.0 ± 0.45 (b)	2 ± 0.006 (a)	7 ± 0.047 (c)
B. diazoefficiens SEMIA5080	108 ± 1.39 (ab)	40.7 ± 1.37 (a)	2 ± 0.012(a)	8 ± 0.067 (b)
B. japonicum E109	95 ± 2.36 (c)	40.7 ± 0.08 (a)	1 ± 0.034 (b)	8 ± 0.081 (b)
B. elkanii SEMIA587	103 ± 1.08 (b)	37.3 ± 2.94 (ab)	2 ± 0.007 (a)	9 ± 0.069 (a)
B. japonicum SEMIA5079	102 ± 0.97 (b)	43.0 ± 1.11(a)	2 ± 0.008 (a)	8 ± 0.015 (b)
B. elkanii SEMIA5019	111 ± 2.47 (a)	38.7 ± 2.32 (ab)	2 ± 0.075 (a)	8 ± 0.054 (b)

Table 2 Spectrophotometric determination of absorbance for Chlorophyll a (Ch a), Chlorophyll b (Ch b) and Carotenoids with methanol solvent and the average concentrations (µg/mL) of Ch a, Ch b and Carotenoid detected in the inoculated seedlings of Baldo rice at three growth stages (2 weeks stage, 5th leaf stage and flowering stage)

Stage of growth	2 weeks stage						5th leaf stage						Flowering stage					
Treatment	NC	*B1*	*B2*	*B3*	*B4*	*B5*	NC	*B1*	*B2*	*B3*	*B4*	*B5*	NC	*B1*	*B2*	*B3*	*B4*	*B5*
Ch a	0.035	0.909	0.35	0.398	0.869	2.031	12.299	20.526	16.22	18.873	20.073	15.069	25.601	25.425	20.371	25.652	27.071	25.89
Ch b	0.026	0.381	0.282	0.234	0.716	0.571	4.256	4.946	4.671	4.644	7.812	4.641	5.965	8.004	5.071	11.347	14.641	11.892
Total *Ch* (a+b)	0.061	1.29	0.631	0.633	1.585	2.602	16.555	25.472	20.891	23.517	27.885	19.71	31.566	33.429	25.442	36.999	41.712	37.782
Carotenoid (x+c)	0.103	0.441	0.467	0.275	0.449	0.682	3.151	6.235	4.786	5.479	4.698	4.108	6.426	4.646	4.847	3.375	4.985	5.162
a/b	14.742	2.688	1.443	1.7	1.243	3.625	2.892	4.16	3.477	4.079	2.576	3.246	4.274	3.181	4.036	2.289	2.052	2.39
(a+b)/(x+c)	0.928	3.041	1.353	2.353	3.554	3.837	5.378	4.084	4.356	4.286	5.847	4.831	4.939	7.183	5.247	11.162	9.037	7.384

B1: *B. diazoefficiens* SEMIA 5080, B2: *B. japonicum* E109, B3: *B. elkanii* SEMIA 587, B4: *B. japonicum* SEMIA 5079, B5: *B. elkanii* SEMIA 5019

putative *Bradyrhizobium* was amplified with *nifH* gene primers, generated amplicons confirmed the presence of the gene within the bacterial genome, indicating that the bacteria re-isolated from the shoot of rice plants were nitrogen-fixing *Bradyrhizobium*.

Principal Component Analysis (PCA) and Hierarchal Cluster Analysis (HCA)

PCA and HCA were performed to further confirm and describe how bacteria are clustered based on their effect on growth at the different developmental stages examined. The PC analysis showed that four PCs accounted for more than 96% of the whole variation. Table 3 reports the characters that contributed positively or negatively to each PC as well as their percentages of variation. Shoot, root and total length, root weight, *Ch b* and carotenoid content at the 2-weeks stage, days to the fifth leaf, days to flowering, leaf number, carotenoid content at flowering stage were included in PC1 explaining 45.3% of the total variation.

In HCA, the five commercial *Bradyrhizobium* strains were grouped into four main clusters formed at the rescaled distance of 5. The clustering was performed according to all vegetative and physiochemical characters measured at the 2-weeks, fifth leaf and flowering developmental stages of *Baldo* rice plants, which were separately inoculated with five *Bradyrhizobium* strains (Fig. 3). The dendrogram obtained using the Wards Linkage method is shown in Fig. 3. Baldo rice plants inoculated with strains *B. diazoefficiens* SEMIA 5080 (BRD83), *B. japonicum* SEMIA 5079 (BRD86), *B. elkanii* SEMIA 587 (BRD85) and *B. elkanii* SEMIA 5019 (BRD87) responded similarly, but plants inoculated with *B. japonicum* E109 (BRD84) was clustered alone, like uninoculated control plants showing that they each had a unique and different performance.

Correlation Analysis

We correlated several vegetative and physicochemical traits and the results are presented in Table 3. Statistically positive and negative correlations at $P < 0.1$ and $r > 0.3$ were identified. The length period it took the plant to reach the 5th leaf stage was positively correlated with root weight at 2 weeks stage, days to flowering, *Ch a* content and carotenoid content at flowering stage. But it was negatively correlated with shoot length, root length, total length, *Ch b* and carotenoid content at 2 weeks stage, plant height at fifth leaf stage, leaf number and plant height at flowering stage. Furthermore, days to flowering positively correlated with root weight, chlorophyll content at 2 weeks stage, days to fifth leaf, *Ch a*, total chlorophyll and carotenoid content at flowering stage. We tested the effect *Bradyrhizobium* strains inoculation had on the early

Table 3 Principal components, percentage of variances and variables belonging to each PC

PC	% of variance	Cumulative %	Variables belonging for each PC and composition of characters
1	45.336	45.336	SL2 (0.897), RL2 (0.949), TL2 (0.961), PH (0.938), DFL (−0.881), RW2 (−0.941), CrtnF (−0.871), Chb2 (0.727), Crtn2 (0.715), DF (−0.648), LN (0.584)
2	32.244	77.581	TChF (0.979), ChbF (0.928), ChaF (0.889), SW2 (−0.835), CN (0.811), TW2 (−0.722), PHL (−0.663)
3	13.217	90.798	Cha2 (0.703)
4	5.800	96.598	TCh2 (0.671)
Total variance	96.598%		

2 weeks stage data for shoot length (SL2), root length (RL2), total length (TL2), shoot weight (SW2), root weight (RW2), total weight (TW2), *Chlorophyll a* content (*Cha*2), *Chlorophyll b* content (*Chb*2), Total *chlorophyll* content (*TCh*2), carotenoid content (Crtn2), days to fifth leaf (DFL), plant height at fifth leaf stage (PHL), days to flowering (DF)

Flowering stage data for plant height (PH), leaf number (LN), culm number (CN), *Chlorophyll a* content (*Cha*F), *Chlorophyll b* content (*Chb*F), Total *Chlorophyll* content (*TCh*F), carotenoid content (CrtnF)

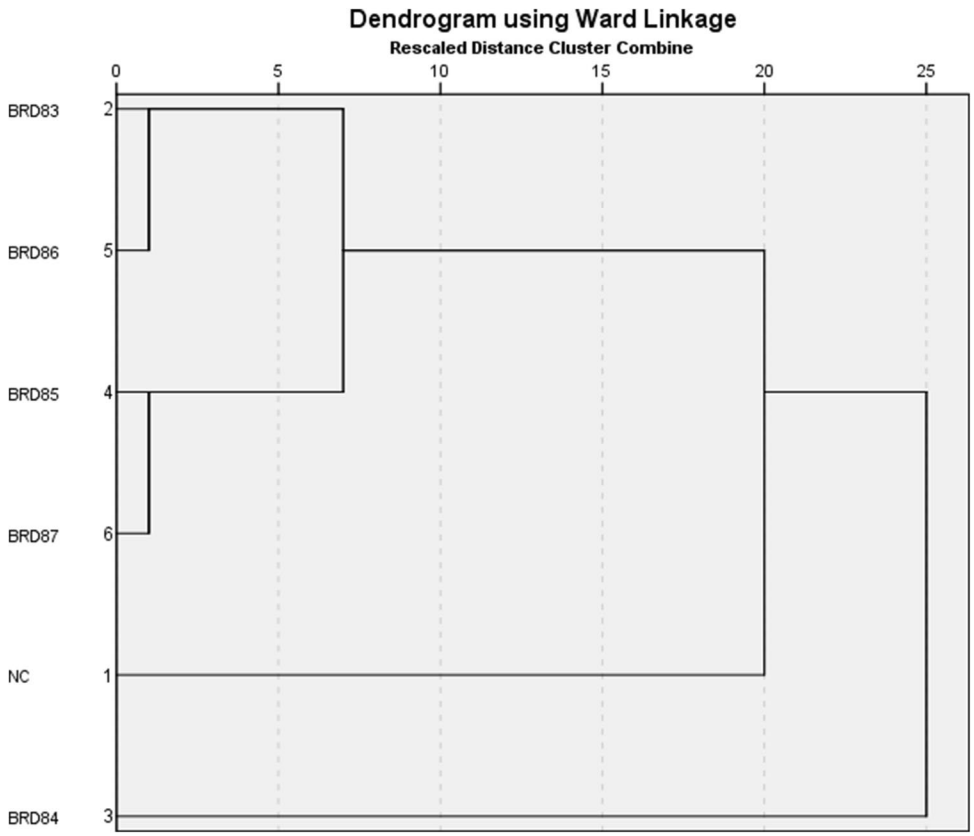

Fig. 3 Cluster analysis of the effect of Bradyrhizobium inoculation on Rice plants cv Baldo at three different plant developmental stages: 2 weeks, fifth leaf and flowering stage. (BRD83: *B. diazoefficiens* SEMIA 5080, BRD84: *B. japonicum* E109, BRD85: *B. elkanii* SEMIA 587, BRD86: *B. japonicum* SEMIA 5079, BRD87: *B. elkanii* SEMIA 5019)

flowering Italian variety Baldo in in vitro experiments. Filella et al. [31] and Moran et al. [32] stated that chlorophyll content might be an indirect measurement useful to predict the plant nutrient status since available N is rapidly incorporated to chlorophyll. Furthermore, the ratio of *Ch a* to *Ch b* in land plants is widely used as an indicator of response to shade and as an early indicator of senescence [33]; because of these reasons we measured chlorophyll content [31]. Carotenoid pigments can be located in chromoplasts or in chloroplasts and together with chlorophylls are involved in the photosystem and have an impact on its activity [34]. The ratio between chlorophyll and carotenoids is a sensitive indicator for the distinction of natural full-term senescence and senescence due to environmental stress [35, 36]. In this study it is shown that *Bradyrhizobium* inoculated plants had a higher chlorophyll content compared to uninoculated plants, suggesting that inoculation affected plant nitrogen content as well as crop productivity. Future studies should analyse if the life cycle of the plant is modified by the higher availability of N.

Bacteria and plants can establish a wide array of different interactions. Plant-associated bacteria colonize tissues

internally that is the apoplast of tissues becoming in this way endophytic bacteria (endophytes), or they basically might live within the phyllosphere (epiphytes) and also within the rhizosphere (rhizobacteria), being each of these a completely different environment [37]. Endophytes have been isolated from leaves, fruits, flowers, stems, seeds and roots of various plant species [38]. Host plants provide a shelter to endophytes that are protected from environmental stresses and microbial competitions. Concomitantly these endophytic bacteria beneficially affect the plant since among many other things it can induce mechanisms of resistance to plant pathogens [39, 40], nitrogen fixation [41, 42] and plant growth promotion [43].

Field Trials

Bradyrhizobium promoted growth of rice in field trials, although with differences between the two rice varieties tested, Guri and Yerua', and the strains used for the inoculation. We observed that *B. elkanii* had a more pronounced growth-promoting effect on both the varieties than the other strains, although it was not the case in preliminary laboratory experiments in which the Italian rice variety Baldo was used. In addition, *B. elkanii* incidentally can synthesize a toxin, rhizobitoxine that in soybeans provokes chlorosis in plants [44] and could have affected the early stages of rice development. Although at this moment we do not know the reason for such differences, both results, those obtained in preliminary experiments and those of field trials, suggest that *Bradyrhizobium* promote the growth of rice. In ¨in vitro¨ assays performed in laboratory conditions, inoculated rice produced more dry matter in a shorter period of time compared to uninoculated rice early during plant growth (Fig. 1). Such promotion of growth was confirmed by the fact that inoculation reduced the length of plant life cycle, that is to say, plants reach the fifth leaf and flowering stage in a shorter period (Fig. 2), which confirms the faster growth of plants.

In field conditions we could not confirm statistically this behaviour due to high variability (Fig. 4), although the average values suggest some differences in term of length (Fig. 4A) and dry weight (Fig. 4B) of roots and aerial parts of rice plants. Though differences between treatments remained they were not significant as plants develop and complete their life cycle. These differences decreased as the crop progressed, until they became minimal and not significant at harvesting, as shown in Fig. 5 for dry weight. The results were not so clearly related with the *Bradyrhizobium* species inoculated unlike what was observed at earlier stages of plant development, where differences between the *Bradyrhizobium* species inoculated were clear and consistent. Interestingly, inoculated plants yielded consistently more than uninoculated ones, whether they were inoculated with *B. japonicum, B. diazoefficiens* or *B. elkanii.* Cultivar Yeruá yielded significantly more when it was inoculated with *B. diazoefficiens* SEMIA5080, *B. japonicum* E109 and *B. elkanii* SEMIA 587 than uninoculated rice, the former ones yielded between 23 and 27% more than the latter one (Fig. 5). On the contrary, plants of cultivar Guri had a significant increase of yield only when inoculated with *B. elkanii* SEMIA 587, compared to the other inoculated plants as well as the uninoculated controls (Fig. 5). Both results of cultivar Yeruá and Guri suggest that the effect of inoculation is related with the bacterial species and/or strains but also with cultivars genetic background, which confirms other findings regarding the effect of plant genotype in plant–microbe interactions [45]. In a way cultivar Gurí behaved similarly at the yield level. It is important to highlight the low yields observed in the field trials, which might be related to two reasons: one is that the plot was not fertilized and the other one that the experiment was seeded late in the season, shortening in this way plants life cycle, growth and consequently yield. The thousand seed weight was different in the two cultivars because of the characteristic of each cultivar, but was the same within the same cultivar whether plants were inoculated or not suggesting that yield differences can be explained by more tillers carrying more panicles or more seeds (Fig. 5). In this regard, more experiments including several cultivars should be performed to confirm this hypothesis. *B. elkanii* SEMIA 587 was the one that affected plant the most since it promoted plant growth of both varieties and in the field. It would be interesting to evaluate the effect of inoculation under a management system that include fertilization and early seedling, conditions where average yields should range between 7000 and 10,000 kg/ha under a fertilized, flooding and earlier sowing management.

Conclusions

Inoculation with *B. japonicum* E109 promoted the growth of the Italian rice variety *Baldo* very early within the plant life cycle and this was also related with a reduction in plant life cycle. Days to fifth leaf stage and flowering were reduced in plants inoculated with *Bradyrhizobium* and mostly with *B. japonicum* E109. In addition, *Bradyrhizobium* representatives inoculated at seeding showed the ability to colonize rice shoots.

Not all *Bradyrhizobium* species had the same effect on rice growth since strains provoked different increments in growth and yield. *B. elkanii* SEMIA 587 gave the highest yield increment in both rice varieties in the field.

Future work should be aimed at evaluating several rice cultivars and their response to inoculation as well as the role of the *Bradyrhizobium* isolates in improving growth of rice. Most probably, a selection of *Bradyrhizobium*

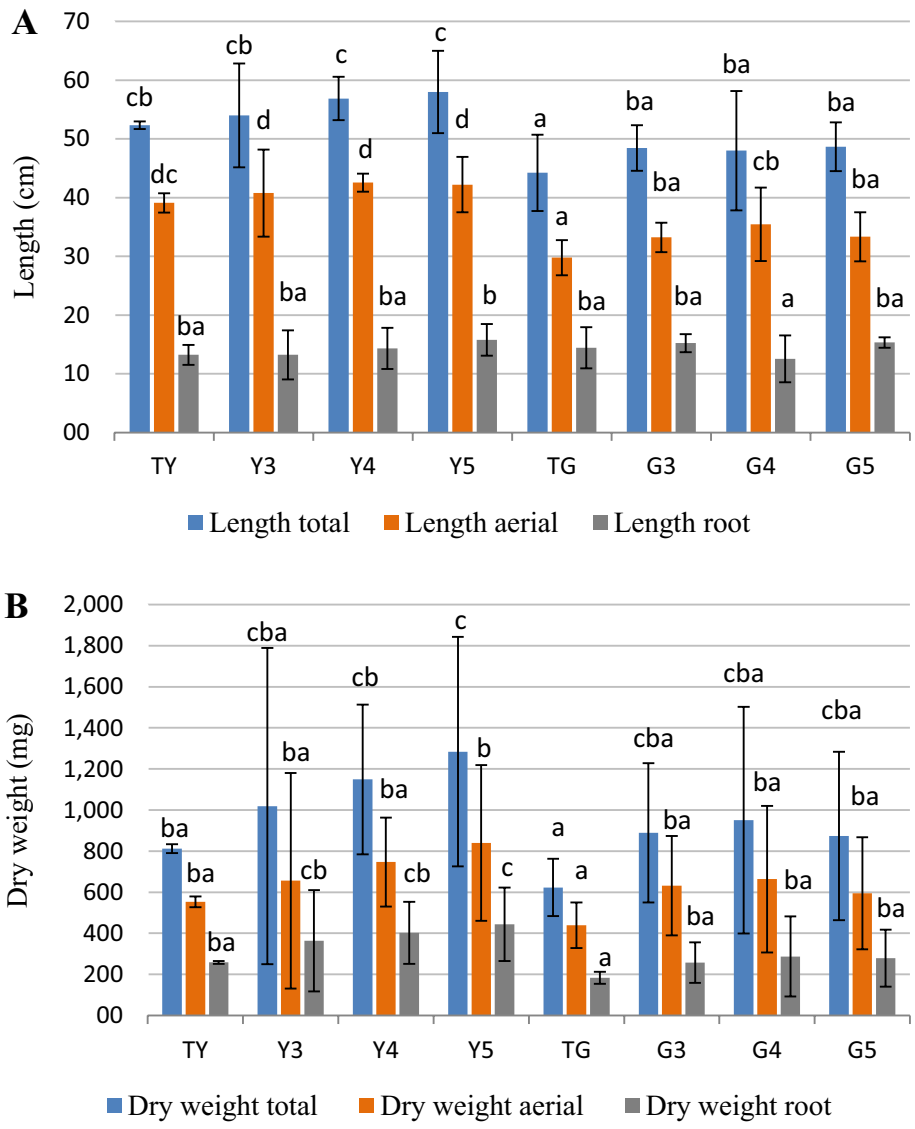

Fig. 4 Growth and yield of rice plants of cultivars Yerua' (Y) and Guri (G) inoculated with *B. diazoefficiens* SEMIA 5080, *B. japonicum* E109 and *B. elkanii* SEMIA587. Results of field trials after 2 months (see Materials and methods). **A** Average length of entire plant (total), of aerial part and of root expressed in centimeters. **B** Average weight of entire plant (total), of aerial part and of root expressed in milligrams. Legend: T = non-inoculated; Y = Yeruá; G = Gurí; 3 = BRD83, *B. diazoefficiens* SEMIA 5080; 4 = BRD84, *B. japonicum* E109; 5 = BRD85, *B. elkanii* SEMIA 587. Similar letters indicate differences were not statistically significant ($P \leq 0.05$)

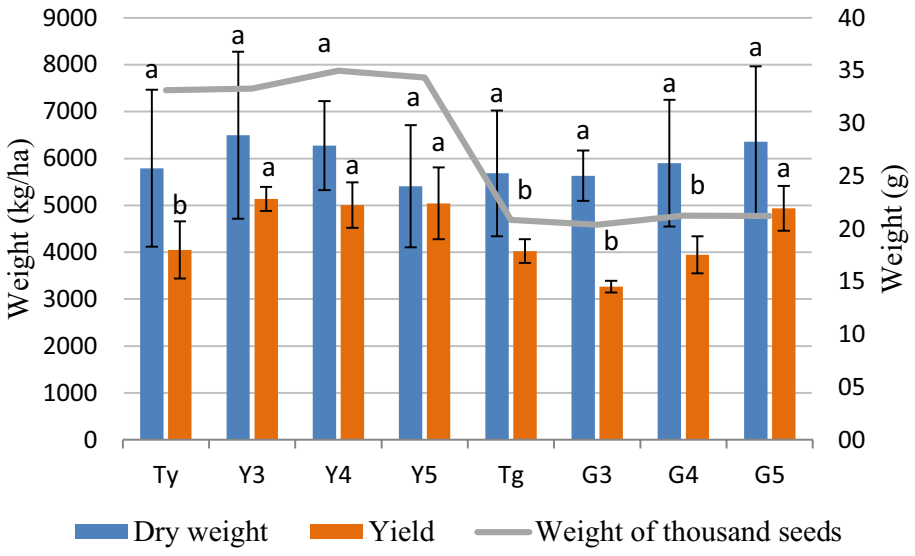

Fig. 5 Results of field trials at harvesting (see Materials and methods): average dry weight of aerial part of rice plants (kg/ha); yield of rice seeds (kg/ha); average weight of thousand seeds (grams). Values with a common letter are not significantly different ($P \leq 0.05$). Legend: T = non-inoculated; Y = Yeruá; G = Gurí; 3 = BRD83, *B. diazoefficiens* SEMIA 5080; 4 = BRD84, *B. japonicum* E109; 5 = BRD85, *B. elkanii* SEMIA 587. Similar letters indicate differences were not statistically significant ($P \leq 0.05$)

adapted to rice-growing conditions should be performed in order to develop rice inoculants in the future.

As an alternative to the fertilization of rice, exploration and usage of endophytes that, as shown here, can be incorporated at seeding to increase nutrient supply for plant growth either by providing more nutrients or by the effect of the microorganism active compounds should be considered. Endophytes reside in plant tissues and establish an ample array of relationships that range from symbiotic to slightly pathogenic can synthetize molecules which are already being used in fields such as modern medicine, agriculture and industry [46, 47]. Therefore, rice production might be increased by using microorganisms that promote growth as well as yield, studying if such effect might be exerted by a consortium of endophytic bacteria. Future studies should be able to identify the organisms that additionally might be incorporated at seeding. Another issue is to identify within such organism which are the active compounds.

Various studies conducted on plant growth-promoting bacteria (PGPR) determine that PGPR can be used as biofertilizer to promote sustainable agriculture. Their ability to solubilize inorganic phosphorous, fix nitrogen and excrete plant growth regulators such as IAA is very prominent in plant growth promotion [48]. With the influence of diazotrophic bacteria, plant receives the optimum amount of nutrients which enable the plant to perform better with a higher growth rate [49]. Plant-associated bacteria such as the *Bradyrhizobium* of this study interact with rice plants and seem to be involved in the biological processes that lead rice to growth promotion and plant productivity. The mechanisms of rice plant colonization and growth promotion, as well as the specificity between rice varieties and *Bradyrhizobium* strains, still remain to be investigated and clarified.

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Author Contributions DP performed the experiments in the laboratory; JMR and RB followed the field trials; SG, PAB and GD planned the experiments, elaborated the results and wrote the manuscript.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflicts of interest.

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