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Rhizobia Enhance Growth in Rice Plants Under Flooding Conditions

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Abstract: In lowland areas rhizobia can fix nitrogen in legume association and colonize the rice plants in rotation. Recent studies have shown that rhizobia can promote plant growth by colonizing rice roots, stems and leaves. The production of phytohormones, mainly indole acetic acid (IAA) is possibly the main mechanism of growth promotion of rice by rhizobia. The objectives of this study were to check the responsiveness of different rice cultivars to inoculate with rhizobia; to study the colonization pattern of rice plants by rhizobia and legumes; and to genetically evaluate the biosynthesis of auxin by rhizobia. Several experiments were conducted in the greenhouse and in the laboratory with rice plants inoculated with different rhizobia. The rice cultivars tested IRGA424 proved to be more responsive to inoculation with rhizobia. Rhizobial strains marked with the *gus*A gene confirmed the colonization of rice. The rhizobia tested are able to produce IAA and there is evidence that the biosynthesis occurs by the route of indole-3- acetonitrile (IAN).

Key words: Interaction plant-microorganism · Colonization · Indole acetic acid

INTRODUCTION

In southern Brazil, rice is cultivated in plain areas with flooding irrigation and demand high doses of input nitrogen fertilizers. The high doses of nitrogen contribute nitrogen leaching from the soil-plant system, are responsible for the contamination of water resources and the increment of the greenhouse effect [1].

A common practice in rice production is rotation with cattle during winter. Due to the topography and the wet climate of the winter season, pasture establishment is challenging [2]. Fortunately, leguminous crops such as the genera *Trifolium* and *Lotus* have acquired resistance to flooding [3] and thus can be rotated with rice. Nitrogen fixation performed by adapted rhizobia symbionts with these legumes can decrease the need for nitrogen fertilization in rice cultivation and consequently production costs and environmental pollutants [4].

Although rhizobia form nodules in leguminous plants and thus fix nitrogen, studies have shown that they can endophytically colonize stems and leaves of non-leguminous plants such as rice, corn, wheat and canola [5-9]. In cases of endophytic growth, rhizobia can improve plant growth by fixing N_2, producing phytohormones and siderophores, dissolving phosphorus and help in disease control [5, 10-14]. It is known that rhizobia penetrate rice cell walls through radicular apertures in the secondary roots [7] and in this system, bacteria can move through xylem to the plant aerial portion [15, 16]. Specifically in rice, rhizobia can improve seed germination rates, stimulate the radicular growth and the aerial portion and increase the grain production [5, 16, 17, 18]. The main plant growth-promoting feature in rice is the production of indole acetic acid (IAA) by four different routes: indole-3-acetamide (IAM), indole-3-piruvate (IPyA), triptamide (TAM) and indole-acetonitrile (IAN) routes.

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Tryptophan is the main precursor of IAA in all known routes [19]. The route IPyA, predominant in plants, has been identified in bacteria of the genus *Bradyrhizobium* [20]. The enzyme tryptophan decarboxylase, present in the route TAM was identified in *Mesorhizobium loti* [21]. Nitrile hydratase, possibly involved in IAN route have been identified in *Rhizobium etli* [22], *Rhizobium leguminosarum* [23], *Bradyrhizobium* sp. [20] and *Sinorhizobium meliloti* [24].

Studies have searched for rice varieties enhanced by plant growth promoting microorganisms [25]. Plant growth promotion seems to be specific between microorganism species and plant variety or genotype [26]. Considering that rice varieties are used in rotation with lotus and clover in southern Brazil, the objectives of this work were to (a) evaluate the response of rice cultivars to rhizobial inoculation; (b) identify the production indole acetic pathway in these rhizobia and (c) identify the rhizobial localization in rice, lotus and clover plants.

MATERIALS AND METHODS

Host Plants, Rhizobial Isolates and Rice Varieties: Isolates, corresponding sampling sites and trap hosts are listed in Table 1. Four varieties of rice - IRGA409, IRGA417, IRGA422CL and IRGA424 - were used due their use for human consumption in southern Brazil. Additionally they can be cultivated under clover or lotus consortium under flooding conditions. The rhizobia were isolated on yeast mannitol (YM) agar [27] from fresh root nodules collected from potted trap host plants with soil from the different sampling sites and were purified by repeated streaking [28].

Plant Assays: Experiment 1 evaluated the effect of the rhizobial inoculation on rice seed germination. Seeds were surface sterilized [16] and inoculated with 5 mL of each rhizobial isolate cultured in YM broth for 24h [27]. Plates were incubated for 24h at 28°C and germinated seeds were counted every 24h through a 6-days period. Initial germination was calculated by the ratio between the number of germinated seeds in the first evaluation and the final number of germinated seeds after 6 days.

Experiment 2 was performed in a greenhouse using sterilized vermiculite-sand as a substrate for rice growth. Seeds were surface sterilized [16] and pots were filled with a sterile mixture of vermiculite and sand (2:1) and were sowed with six seeds. After emergence, seedlings were thinned to two plants per pot; after 7 days plants were inoculated with 5 mL of bacterial culture grown in YM broth or 5mL of sterile YM medium, in the uninoculated control. Plants were grown in a greenhouse and watered with nutritive solution 50% [33]. Thirty-seven days after sprouting, plants were harvested and dried to a constant weight at 65°C. The dry matter was determined and the data was subjected to ANOVA and Scott Knott test ($p \leq 0.05$) using the software Sysvar 4.6 [34].

Table 1: Rhizobia isolates used in the study

Isolate	Sampling site	Trap host	Reference
Lc336	HulhaNegra, RS	*Lotus corniculatus*	[29]
Lc348	HulhaNegra, RS	*L. corniculatus*	[29]
Lg111	Porto Alegre, RS	*L. glaber*	[30]
EEL-1183U	Lages, SC	*L. uliginosus*	[31]
1TV	Dom Pedrito, RS	*Trifoliumvesiculosum*	[32]
VP16	Veranopolis, RS	*T.repens*	[32]

Experiment 3 was conducted in a greenhouse with plastic pots containing soil sampled from both a flooded and native field area. The soil was mixed with simple super phosphate and potassium chloride at 45 and 25 mg kg^{-1} of P_2O_5 and K_2O, respectively. Soil was flooded 16 days prior the sprouting, with a water depth of 5 cm above the soil level. Seeds were surface sterilized [16]. Before sprouting, seeds were sunk into a rhizobia culture grown in YM broth for 10 to 15 minutes or sterile YM broth in the uninoculated control. After 10 days, plantlets were thinned to two per pot and received a urea solution equivalent to 40 kg ha^{-1} of nitrogen. Two non-inoculated treatments were performed with 40 and 80 kg ha^{-1} of nitrogen. After the nitrogen fertilization, the flooding conditions were resumed. Nitrogen was added again 30 days after sprouting as described above. Sixty days after sprouting, the number of tillers per plants was counted and plants were harvested and dried to constant weight at 65°C. The dry weight was determined and the data submitted to ANOVA and Scott Knott ($p \leq 0.05$ and $p \leq 0.1$) using the software Sysvar 4.6 [34]. The relative efficiency of the rhizobia inoculation in rice varieties was analyzed using a modified version of the equation for symbiotic efficiency of rhizobia in legume [35].

DNA Isolation and Genotypic Characterization: The isolates Lc336, Lg111 and 1TV were grown in YM broth for seven days at 28°C at 128 rpm. Genomic DNA was isolated using CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad) following the manufacture instructions. The PCR amplification of 16S rRNA gene was performed using

Table 2: Primers utilized to identify IAA production pathway genes

Primer	Sequence	Fragment size (bp)	Target	Original organism
TM1F	CTTTCGCCTTCGACGACTGG	550	Tryptophan monoxigenase	*Pseudomonas fluorescens*
TM1R	GTAGAAGGTGCGGTCGTCCC			
PHYTM1F	TCACAAAGTTCATCACCGAC	700	Tryptophan monoxigenase	*Burkholderia phymatum*
PHYTM1R	TTGATAGACAGGCAGAAAGC			
TRIPBF1	TACTTCGGCTTCGTSATYGG	350	Triptamide	*Burkholderia xenovorans*
TRIPBF2	GAAGCCGAGCACGCGCAGCG			
IPDCF4	GCAGTTCCAGGTGTTCAAGG	800	Indole pyruvate descarboxilase	*Azospirilum brasiliense*
IPDCR4	ATGGCGGTGAACAGGCAGTC			
ipdCRhi-1F	CGCGAGATCTTCGGCATTCC		Indole pyruvate descarboxilase	
ipdCRhi-1R	CACGTCCACCACGATCACCG			
ipdCRhi-2F	CCGCTCTATCTCGAATTCCC		Indole pyruvate descarboxilase	
ipdCRhi-2R	GTATCCGACAGGATCACGCC			
nthAF	GTGTGCACGCTGTGCTCCT	800	Nitrile hydratase	
nthAR	ACCGTGTAGAGCCATTGCG			
nthBR	CGGCGGTGGAATCCCAGAC			

the primers 616V and 630R [36]. The PCR products were cloned and sequenced. Sequences were analyzed using the BLAST tool in the NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Indole Acetic Acid Production and Quantification: Rhizobial isolates Lc336, Lg111 and 1TV were chosen to evaluate the production and quantification of indole acetic acid [37]. Bacterial cells previously grown in PY medium (5 g peptone, 3 g yeast extract and 0.7 g $CaCl_2$ per liter) were washed with 10 mM $MgSO_4$ and the OD determined at 600 nm. Reaching an OD of 0.2, one milliliter of the culture was inoculated in the indole determination medium with and without tryptophan added. Identification of routes for the production of indole acetic acid were performed with PCR primers that amplify genes involved in biosynthesis of IAA in *Azospirillum*, *Bulkholderia* and *Pseudomonas* (Table 2). Based on the sequences of bacteria belonging to the order Rhizobiales, specific primers were designed to amplify the genes for indole pyruvate decarboxylase (IPDC) and nitrile hydratase (Table 2).

Bacterial Localization in Rice, Lotus and Clover: The *gus*A reporter gene was inserted in the bacteria to monitor the bacteria localization in the plant tissue. The isolate Lc336 was grown in yeast extract/mannitol broth (YM medium) and the isolate Lg111 was grown in peptone/yeast broth media (PY medium) both supplemented with nalidixic acid (20 μg mL^{-1}). The isolate 1TV was grown in PY medium supplemented with spectinomycin (50 μg mL^{-1}). *Escherichia coli* donor and intermediate were grown in LB medium (1 g peptone, 0.5 g yeast extract and 1 g NaCl per liter) supplemented with kanamycin (50 μg mL^{-1}). After the conjugation and 48 hours of incubation, the colonies were inoculated in YM agar containing X-Gluc. The blue colonies were selected for comparison with the wild type, by PCR for amplification with primers nodAB1 and nodAB2. The colonies that showed the same band profile as the wild type were inoculated into plants.

Seeds of *Oryza sativa*, *Trifolium vesiculosum* and *Lotus corniculatus* were inoculated with the isolates Lc336, Lg111 and 1TV marked with the *gus*A reporter gene as described above. Legume inoculation and colonization were evaluated with surface sterilized seeds [16] and sown in 100 mL tubes containing 35 mL of N-free nutrient solution [38] and 0.75% agar. Rice seeds were sown in flasks containing 210 mL of nutrient solution and 0.75% agar. The agar-containing portion was covered with a dark paper to encourage the development of roots. Bacteria containing the *gus*A gene were grown in 5 mL PY broth per 24h. The number of cells in each inoculum was adjusted by determining the turbidity cell in spectrophotometer at 600 nm. The rhizobial inoculation was performed 24h after sprouting.

After two weeks (for rice) and three weeks (for legumes) sprouting, seedlings were removed from the nutrient, placed in 15 mL falcon tubes submerged in Na_2HPO_4/NaH_2PO_4 50mM buffer containing X-Gluc 2mM and incubated at 37°C for 48 hours to stain the bacteria containing the *gus*A gene.

RESULTS

Experiment 1 - Response of the Rice Varieties to the Rhizobial Inoculation: Differences in the speed of seed germination were observed among the rice varieties when inoculated with different rhizobial isolates.

Table 3: Percentage of germinated seeds of rice varieties inoculated with native rhizobial isolates after 24h of germination

Isolates	IRGA 409	IRGA 417	IRGA 422CL	IRGA 424
1TV	20.9 c*	26.7 a	55.6 b	85.8 a
VP16	17.8 c	23.0 b	53.9 b	80.4 b
Lg111	40.3 a	28.0 a	53.6 b	91.4 a
Lc336	29.4 b	23.0 b	45.4 c	88.7 a
Lc348	17.6 c	29.3 a	61.6 a	91.2 a
EEL1183	16.6 c	26.0 a	62.5 a	80.4 b
Uninoculated seeds	20.8 c	22.7 b	44.6 c	67.4 c

* values in the same column followed by the same letter did not differ statistically at $P \leq 0.05$ (Scott Knott test).

Table 4: Production of aerial and roots dry matter yields of rice cultivars cultivated in vases with axenic substrate and inoculated with rhizobial isolates

	Rice cultivar			
Rhizobial isolates	IRGA 409	IRGA 417	IRGA 422CL	IRGA 424
	Aerial dry matter (mg plant^{-1})			
1TV	271 a*	273	326 a	308 b
VP16	254 a	273	331 a	264 c
Lg111	264 a	304	245 b	325 b
Lc336	271 a	326	323 a	383 a
Lc348	269 a	302	283 b	324 b
EEL1183	284 a	322	345 a	260 c
Control	205 b	269	297 b	256 c
	Roots dry matter (mg plant^{-1})			
1TV	295 b	634 a	528 b	381 b
VP16	378 b	508 a	813 a	373 b
Lg111	289 b	331 b	554 b	482 a
Lc336	516 a	337 b	382 c	462 a
Lc348	261 b	360 b	353 c	413 b
EEL1183	334 b	339 b	693 a	392 b
Control	213 b	373 b	318 c	375 b

* values in the same column followed by the same letter did not differ statistically at $P \leq 0.05$ (Scott Knott test).

Germination started after 48h in the rice cultivars IRGA409, IRGA417 and IRGA424 and 72h in the variety IRGA422CL. The number of germinated seeds of the varieties IRGA409 and IRGA424 remained constant after four days, as opposed to the rice cultivars IRGA417 and IRGA422CL, which took six days to interrupt germination (Figure 1).

Each rhizobial isolate had a specific impact on seed germination in the different rice cultivars. Isolates 1TV, Lg111 and Lc348 increased the germination speed seeds in two rice varieties (Table 3). The isolate Lg111 promoted an increase of 93.75%, 23.35% and 35.6% in the germination speed of cultivars IRGA409, IRGA417 and IRGA424, respectively (Table 3).

Experiment 2 - Growth Promotion in Axenic Conditions: The inoculation with rhizobial strains promoted growth in all the rice cultivars tested under axenic conditions except by the rice cultivar IRGA417. Cultivar IRGA409 had a significant dry matter yield when inoculated with the tested rhizobial strains. Cultivar IRGA422CL responded only to the isolates 1TV, VP16, Lc336 and EEL1183. The isolates 1TV, Lg111, Lc336 and Lc348 stimulated dry matter yields in the cultivar IRGA424 (Table 4).

The radicular system of rice was increased when inoculated with rhizobial strains. Cultivar IRGA409 increased 142% of the root dry matter when inoculated with the isolate Lc336 when compared with the control. Isolates VP16 and EEL1183 increased the root dry matter in cultivar IRGA422CL in 156% and 118% when compared with the uninoculated treatment (Table 4).

Experiment 3 - Growth Promotion in Non-axenic Conditions: The rice cultivar IRGA424 was the only cultivar to show a positive response when inoculated with rhizobial strains under non-axenic conditions, compared to the control. Inoculation with isolate Lc348 was equally efficient compared to the positive control inoculated with nitrogen (Table 5). In addition, isolate Lc348 presented a relative efficiency of 88.4% when compared to the treatment without inoculation. Other isolates presented a relative efficiency of more than 47% when inoculated in the rice cultivar IRGA424 (Figure 2).

Table 5: Production of aerial dry matter and tillers number of rice cultivars cultivated in vases with soil and inoculated with rhizobial isolates

	Rice cultivars			
Isolate	IRGA 409	IRGA 417	IRGA 422CL	IRGA 424
	Aaerial dry matter (mg plant^{-1})			
1TV	2.669 b*	2.329 b	1.985 b	2.264 b
VP16	2.287 c	2.246 b	2.358 b	2.466 b
Lg111	2.651 b	2.405 b	2.140 b	2.245 b
Lc336	2.461 c	2.395 b	2.029 b	2.265 b
Lc348	2.391 c	2.199 b	2.325 b	2.635 a
EEL1183	2.619 b	2.177 b	2.109 b	2.395 b
Control (40 kg N ha^{-1})	2.647 b	2.315 b	2.200 b	1.794 c
Control (80 kg N ha^{-1})	3.116 a	2.792 a	2.730 a	2.745 a
	Number tillers plant^{-1}			
1TV	5,22 b	5,22 b	5,22 b	7,11 b
VP16	5,22 b	4,67 b	5,56 b	7,89 a
Lg111	6,00 a	4,78 b	5,56 b	8,22 a
Lc336	5,00 b	4,78 b	6,22 a	7,67 a
Lc348	5,00 b	5,33 b	4,78 b	8,56 a
EEL1183	5,00 b	5,00 b	5,89 a	8,78 a
Control (40 kg N ha^{-1})	5,22 b	5,00 b	5,22 b	5,78 c
Control (80 kg N ha^{-1})	6,33 a	6,00 a	6,78 a	8,89 a

* values in the same column followed by the same letter did not differ statistically at P ≤ 0.05 (Scott Knott test)

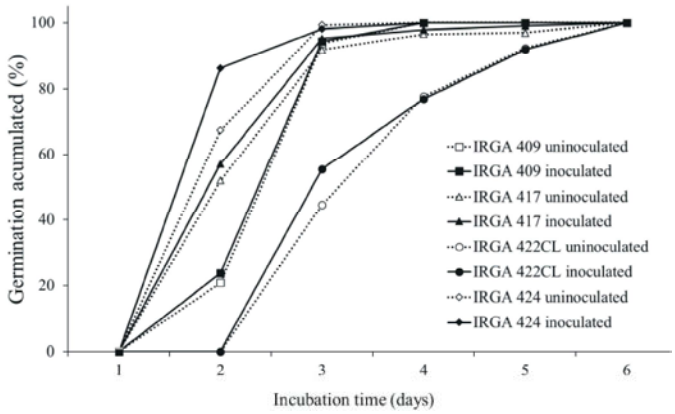

Fig. 1: Effect of inoculation with rhizobia isolates under the seed germination of rice cultivars IRGA409, IRGA417, IRGA422CL e IRGA424

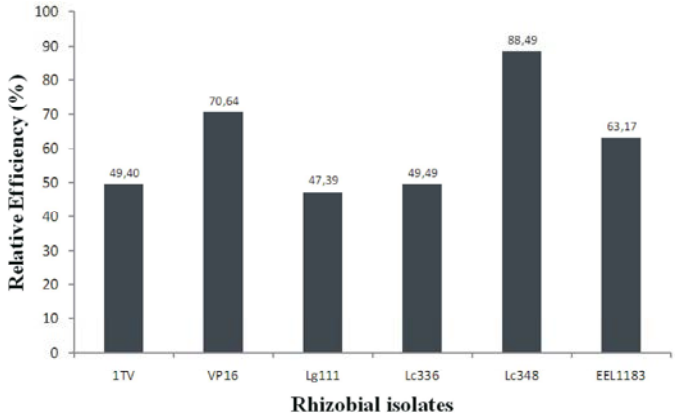

Fig. 2: Relative efficiency of native rhizobia when inoculated in the cultivar IRGA424 under non-axenic conditions in greenhouse conditions

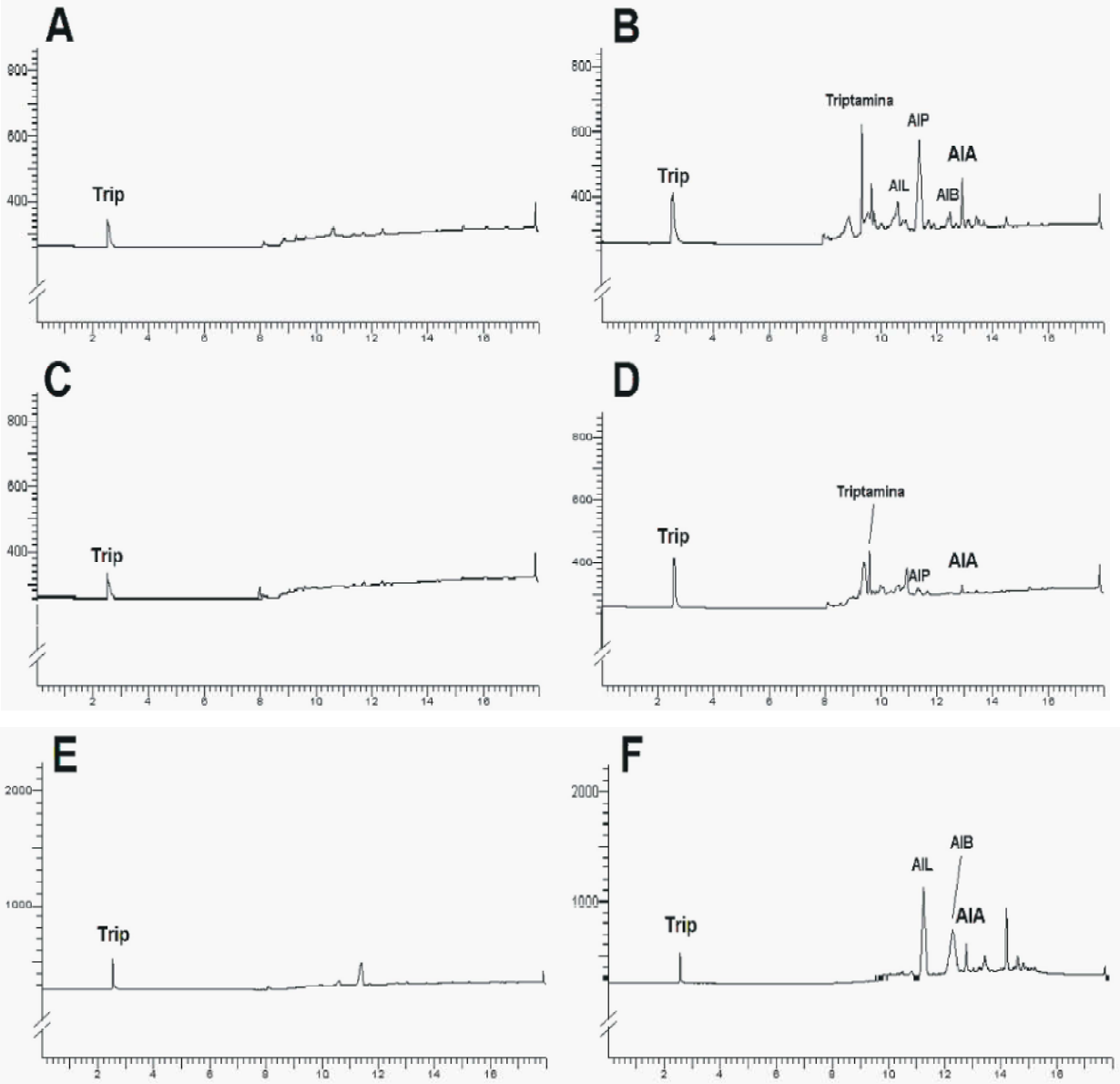

Fig. 3: Chromatograms of the production of indoles - indole acetic acid (IAA), indole lactic acid (ILA), indole butyric acid (IBA), indole pyruvic acid (IPA) and triptamidaby rhizobia in the absence and presence of tryptophan (Trip). A) *Bradyrhizobiumjaponicum* Lc336 without tryptophan; B) *B. japonicum* Lc336 with tryptophan; C) *Mesorhizobiumamorphae* Lg111 without tryptophan, D) *M. amorphae* Lg111 with tryptophan; E) *Rhizobium leguminosarumbv. viciae* 1TV without tryptophan, F) *R. leguminosarumbv. viciae* 1TV with tryptophan

Indole Acetic Acid Production and Quantification: According to the sequence analysis of the 16S rDNA partial sequences, the isolate Lc336 showed a high genetic similarity (99% homology) with *Bradyrhizobium japonicum*, Lg111 showed 99% of homology with *Mesorhizobium amorphae* and the isolate 1TV showed high homology (99%) to *Rhizobium leguminosarum* bv. *viciae*.

The production of IAA was confirmed by liquid chromatography. Since no significant IAA production is observed in the absence of tryptophan, the pathway for the production of IAA in the rhizobia isolates is considered dependent on this amino acid (Figure 3). Primers ipdCRhi-1F, ipdCRhi-1R, ipdCRhi and ipdCRhi-2F-2R, designed to amplify the gene for indole-pyruvate decarboxylase, were not effective for the three rhizobia

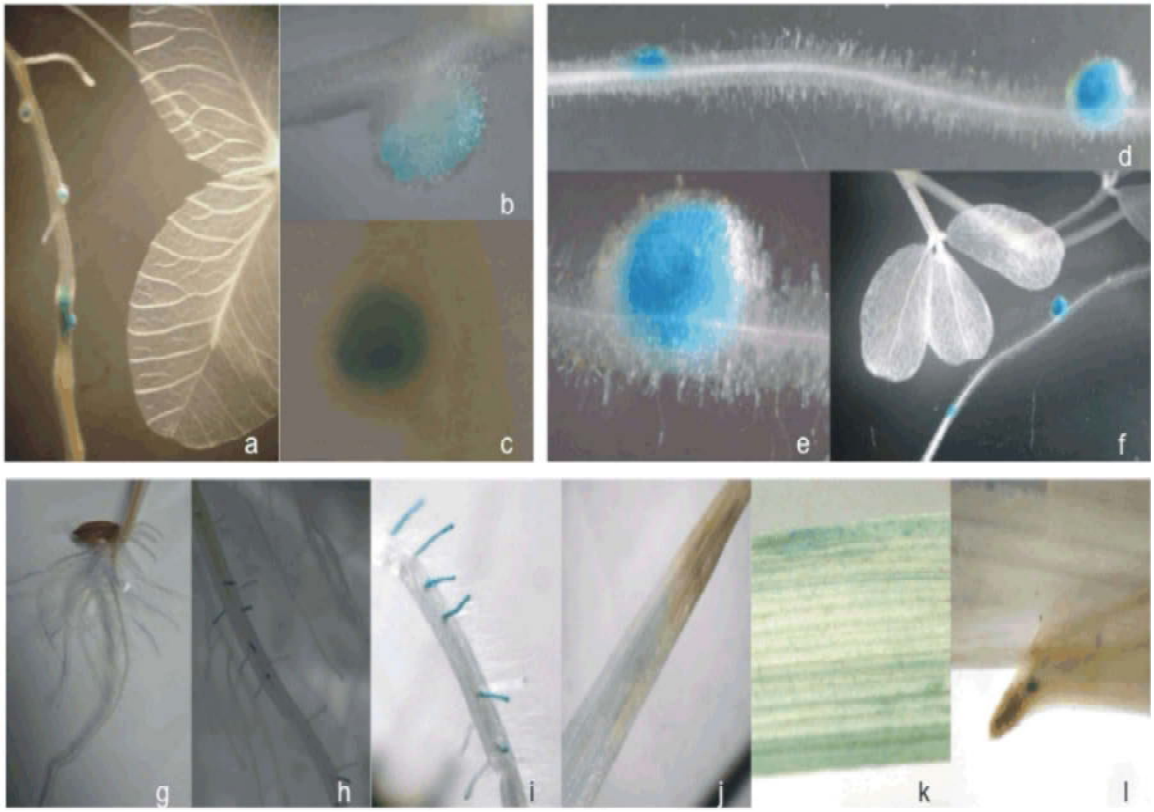

Fig. 4: Colonization of *Trifolium vesiculosum* by *Rhizobium leguminosarum* bv. *viciae* 1TV in (a) roots and leaves; (b,c) detail of the nodule; colonization of *Lotus corniculatus* by *Mesorhizobium amorphae* Lg111 in (d) root nodule and nodal primordial; (e) detail of node and (f) roots and leaves; colonization of rice plants by *Rhizobium leguminosarum* bv. *viciae* 1TV in (g) seed, root and stem, (h,i) main root with secondary roots; (j) leaf; (k) veins of the leaf; and (l) leaf primordial

studied. However, when primers nthAF and nthAR for the enzyme nitrile hydratase were used, amplified sequences were observed in these three bacteria. In *B. japonicum* Lc336, part of the amplified sequence corresponds to the alpha-subunit gene and another part to the beta subunit of nitrile hydratase [39]. In *M. amorphae* Lg111 the amplified sequence corresponds to genes related to the alpha and beta subunits of nitrile hydratase of *M. loti* [21]. Similarly, in *R. leguminosarum* 1TV, amplified genes were related to the alpha and beta subunits of nitrile hydratase, identical to those determined in *R. etli* [40] and *R. leguminosarum* [41].

Rhizobial Colonization in Rice, Lotus and Clover: The colonization of rhizobia on plants of *Trifolium vesiculosum*, *Lotus corniculatus* and *Oryza sativa* was confirmed by visualizing the presence of rhizobia containing the *gus*A gene and expressing the blue color in the plant tissue (Figure 4).

The isolate *R. leguminosarum* bv. *viciae* 1TV colonized the roots of *T. vesiculosum* preferentially in the region of root lumps and lacked colonization in the shoots or leaves (Figures 4a, 4b, 4c). In *L. corniculatus*, the bacteria *M. amorphae* Lg111 penetrated the roots, focusing on early nodal or nodules (Figures 4d, 4e, 4f). There was lack of rhizobial colonization in shoots of *L. corniculatus* (Figure 4f). In *O. sativa*, rhizobia were able to colonize roots and shoots (Figures 4g to 4l). In that case, rhizobia colonize the secondary roots in rice.

DISCUSSION

The ratio between seed germination in the negative control and in the seeds inoculated with rhizobial cultures demonstrated that inoculation with rhizobial cultures contributed to the speed of the germination in rice. The increase in germination speed is sought in the fields because it increases the uniformity of the crop;

especially in the panicle maturation that reduces loses in the harvest period. Additionally faster germination rates reduce the period of heterotrophism and reduce the chances of attack by soil pathogens [42]. In a work [43] was observed that rhizobia isolated from alfalfa increased the speed germination of rice seeds and stimulated the growth of rice seedlings. On the other hand, other researchers [44] observed that high doses of IAA produced by rhizobia could inhibit germination in lettuce seeds.

Clearly, we observed differences between the rice cultivars and the bacteria tested regards to the growth stimuli of the aerial plant. In a similar study, two out of six rice cultivars responded to inoculation with growth promoting bacteria [17]. In this case, the length of the aerial portion was significantly increased by the rhizobial inoculation. Other authors [45] observed stimulation in the biomass production, root volume and nitrogen accumulation when four rice cultivars were inoculated with *R. leguminosarum* bv. *trifolii* SN10.

Different responses from strains when inoculated in rice cultivars were observed. Egyptian and American authors [17, 18] observed that different rice cultivars presented variation in stimuli depending on the rhizobial strains inoculated. They used four rice cultivars from Egypt and USA tested with eight isolates of rhizobia. They observed that the Egyptian variety Giza177 had spurred the shoots when inoculated with the isolate E11, but the root volume was not stimulated by inoculation with the same strain. However, the same isolate stimulated root growth and lacked a response to the aerial part of the American variety M202. Our studies also demonstrated that the strains that increase the aerial dry matter are not necessarily the same that stimuli the radicular system in the same cultivar. In a survey of microbial communities presented in 10 rice cultivars, researchers found that different rice cultivars selected specific communities, indicating that the genotype of the plant is a factor that might determine the interaction between rice and associated microorganisms [46]. The differences in the responses between plants and microorganisms observed in this work might be attributed to the genetic differences between cultivars. Others researchers [47] observed stimuli by two different varieties of rice when inoculated with two bacterial genera isolated from the rice rhizosphere. They found different responses in the production and nitrogen accumulation in rice tissues, especially when inoculated with *Rhizobium* and *Corynebacterium*.

The rice breeding process might influence different interactions between rice cultivars and rhizobial strains. Naturally, those plants may be adapted to low water and nutrient contents, or the presence to toxic elements. Although in a field experiment where water and nutrients are supplied to the plants, the microorganism-plant interaction might be underestimated and might not reflect the real symbiosis between them. On the other hand, the satisfactory performance observed by the studied rhizobia strains has showed that they can be efficient even in soils with a previous microbial community established.

The capacity of competing with the established soil microbial community is an important feature to be considered when using these strains for agricultural purposes [48]. In a work, the authors observed different responses from the inoculation of different rhizobia isolates in tobacco cultivar under axenic and non-axenic conditions [49]. When the inoculation with *Sinorhizobium meliloti* 1021 marked with the GFP gene was performed in axenic conditions, the bacterial colonization was more intense in roots, stems and in second and third leaves compared with the negative control. These authors attributed the increase in the colonization in axenic conditions to the high moisture and temperature provided by the cultivation method used.

Others researchers [50, 51] also found that the production of IAA was stimulated by the presence of tryptophan in the culture media. In addition, a work [52] detected production of IAA in *Azorhizobium caulinodans* culture grown in a medium supplemented with tryptophan. An accumulation of IAA in rice leaves inoculated with strains of *A. caulinodans* and *S. meliloti* was observed using liquid chromatography [53].

This observation agrees with studies reporting that the entrance of rhizobia occurs where the secondary roots emerge [54, 53, 7, 15]. Possibly, rhizobia penetrate these points and colonize the epidermis and cortex of the secondary root. In leaves, it was observed that colonization was ordered in the vascular bundles (Figure 4j and 4k). The colonization of the shoot of rice was only observed with the inoculation of *R. leguminosarum* bv. *viciae* 1TV. The bacteria that penetrate the roots of rice following the intercellular spaces, colonizing the epidermis, cortex and vascular tissues and aerenchyma, rising to the stem and leaves [54].

Our results suggest that rhizobia can colonize rice plants in a broad way. It differs from the *T. vesiculosum* and *L. corniculatus* colonization where the rhizobia

localize mainly inside the root nodules. This broad colonization reflects in different speeds of germination and in the plant growth speed, depending on the rice variety and the intrinsic microbial soil competition. The main mechanism of plant growth involved is probably the IAA production using the indole-3-acetonitrile (IAN).

These observations emphasize the relevance of the correct rhizobial inoculation in responsive rice varieties with the purpose of increase rice production in a more efficient economical and environmental points of view. Besides the inoculation benefits, this work claims the benefits of cattle raising-crop production system integration, with a rice-legume rotation. In this system, the rhizobia in the soil might fix nitrogen in legumes and stimuli growth in rice plants.

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