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Indoleacetic Acid Producing Rhizobia Promote Growth of Tanzania grass (*Panicum maximum*) and Pensacola grass (*Paspalum saurae*)

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Abstract

Production of phytohormones, such as the indoleacetic acid (IAA), is possibly the main mechanism of plant growth promotion by rhizobia. The greenhouse present study aimed to quantify the production of IAA by rhizobia and evaluate the effect of inoculation with rhizobia on four forage grasses i.e., Tanzania grass (*Panicum maximum*), Pensacola grass (*Paspalum saurae*), brachiaria grass (*Brachiaria decumbens*) and ryegrass (*Lolium multiflorum*). The work also aimed to characterize genotypically the rhizobia strains studied in this work. All rhizobia were able to produce IAA and promote plant growth in at least one of the grasses studied. Tanzania grass plants inoculated with rhizobia SEMIA816, Lc134, Lc323, Lc348, Lc510 and Lc524 showed increased shoot and root dry mass as compared to control or non-inoculated plants. The inoculation with Lc336 only increased the root dry mass in Tanzania grass. In Pensacola grass increases in shoot and root dry mass were observed with inoculation of SEMIA816, Lc134, Lc336 and Lc394. The inoculation with Lc323 and Lc348 only increased the root dry mass in Pensacola grass. On the other hand, no increases on shoot and root dry mass was observed in plants of brachiaria grass and ryegrass by inoculation with the studied rhizobia. The rhizobia SEMIA 816 and Lc134 promoted the plant growth in both Tanzania grass and Pensacola grass. The rhizobia SEMIA 816, Lc336, Lc348 and Lc524 also increased the number of panicles in brachiaria grass. A higher accumulation of total nitrogen was noted in the shoots of Tanzania grass inoculated with the UFRGS Lc323, UFRGS Lc336 and SEMIA 816. After characterization of rhizobia genomic DNA by BOX PCR and 16S rRNA sequencing the unidentified rhizobia were grouped together in a dendrogram of similarity and found belonging to the genus *Mesorhizobium* or *Bradyrhizobium*.

Keywords: *Mesorhizobium*; *Bradyrhizobium*; Rhizobacteria; Phytohormones; 16S rRNA

Introduction

Livestock farming in Uruguay, Argentina and southern Brazil is heavily based on the use of native forage species, grasses and legumes, as main sources of food for the herd of cattle and sheep. However, during the colder seasons of the year, natural pastures face serious difficulties in establishment. The forage supply during this period is insufficient to meet the demand of forage for animals, both in qualitative and quantitative terms.

The use of forage legumes species is an alternative to increase the inflow of nitrogen in soils of pastures due to the well-known symbiotic nitrogen fixation by rhizobia symbionts. Thus, the use of inoculants containing rhizobia can increase the supply of nitrogen for forage legumes and reduce the need for use of nitrogen fertilizers in the pastures. Besides symbiotic nitrogen fixation it has also been demonstrated that rhizobia produce phytohormones such as auxins (Biswas *et al.*, 2000; Erum and Bano, 2008), cytokinins (Persello-Cartieaux *et al.*, 2003), gibberellins (Yanni *et al.*, 2001; Erum and Bano, 2008), abscisic acid (Dangar and Basu, 1991), and vitamins (Dakora, 2003).

The capacity of rhizobia to promote the growth of non-legume plants by phytohormone production and other mechanisms had been demonstrated by previous works (Yanni *et al.*, 2001; Chen *et al.*, 2005; Schlindwein *et al.*, 2008). The synthesis of auxins by rhizobia, specially the indoleacetic acid (IAA), promotes root growth and root hair density improving the absorption of water and nutrients from the soil and therefore improving the development of plants infected (Caballero-Mellado *et al.*, 2006).

Among the forage legume species, the birdsfoot trefoil (*Lotus corniculatus* L.) presents interesting characteristics of adaptation under prevailing edaphic-climatic conditions in the southern Brazil, such as soil acidity and poor soil fertility tolerance (Paim, 1988). Associated with its great

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nutritional value (López *et al.*, 1965) and its characteristic of not causing tympanism (Paim, 1988), birdsfoot trefoil is a forage species that can be highly exploited in the soil. When inoculated with efficient rhizobia, birdsfoot trefoil proved to be alternative forage species with great potential to be used for grasses intercropping or succession systems, promoting economic and environmentally sustainable pasture systems.

The rhizobia also have the ability to penetrate and colonize grasses by rupturing the root epidermis and fissures created during the emergence of lateral roots (Webster *et al.*, 1997; Yanni *et al.*, 1997). Therefore they can establish endophytic associations with maize, radish, canola, lettuce, rice, sugar beet and wheat (Antoun *et al.*, 1998; Biswas *et al.*, 2000; Gutierrez-Zamora and Martinez-Romero, 2001; Matiru and Dakora, 2004; Singh *et al.*, 2005; Perrine-Walker *et al.*, 2007).

To improve the forage biomass production of cultivated pastures, besides the selection of rhizobia effective in nitrogen fixation in symbiosis with forage legumes it is also necessary the selection of rhizobia capable of promoting the growth of forage grasses and the study of the plant growth promoting mechanisms and to study the biodiversity of these native rhizobacteria. The species *Brachiaria decumbens*, *Lolium multiflorum*, *Panicum maximum* and *Paspalum saurae* are plants belonging to the family Poaceae and are commonly explored in pastures in Brazil due to the great forage potential (Kissmann, 1997). However, forage production for these grasses is dependent on soil fertility and especially the supply of nitrogen from the soil (Beaty, 1974; Alvim *et al.*, 1987; Soares *et al.*, 2001; Soria *et al.*, 2003; Benett *et al.*, 2008). The growth promotion by inoculation with rhizobia in non-legume plants can be related with the increasing of the root system, the root hair proliferation and the absorption of water and nutrients from the soil and therefore with the development of the infected plants and more efficient use of nitrogen and other nutrients (Yanni *et al.*, 1997; Biswas *et al.*, 2000); phosphate solubilization (Rodríguez and Fraga, 1999).

Thus, this work was aimed to quantify the production of IAA by rhizobia symbiont on birdsfoot trefoil evaluate the effect of rhizobia inoculation on the plant growth promotion of forage grasses and to characterize genotypically the studied rhizobia.

Materials and Methods

Rhizobia Cultures Studied

The rhizobia UFRGS Lc134, UFRGS Lc323, UFRGS Lc336, UFRGS Lc348, UFRGS Lc394, UFRGS Lc443, UFRGS Lc510, UFRGS Lc522 and UFRGS Lc524 were obtained from the UFRGS collection of rhizobia at the Soil Microbiology Laboratory, Department of Soils, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. These rhizobia isolated from root nodules of birdsfoot trefoil plants cultivated on soil samples from pasture fields in different municipalities in South Brazil and selected based on the efficiency on symbiotic nitrogen fixation (Frizzo, 2007). The rhizobium SEMIA 816, authorized by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) for use in the production of commercial inoculants for birdsfoot trefoil plant in Brazil, was obtained from the SEMIA Culture Collection of rhizobia of the Agricultural Research Foundation of Rio Grande do Sul (FEPAGRO).

Quantification of IAA Production

The capability of the studied rhizobia to produce IAA was evaluated in cultures growing in yeast mannitol (YM) liquid medium (Vincent, 1970) enriched with tryptophan and in medium without tryptophan using the method described by Asghar *et al.* (2002).

In order to produce the bacterial inoculum the rhizobia were inoculated in tubes with YM agar (pH 6, 8) and incubated at 28°C for 15 days. After, each rhizobia culture was inoculated in two Erlenmeyer flasks, one with 80 mL of YM liquid medium enriched with tryptophan (50 mg L^{-1}) and other with YM liquid medium without tryptophan. The rhizobial cultures were incubated in orbital shaker at 28°C and 120 rpm for 48 h. The number of rhizobia cells was determined by counting in a Neubauer chamber (Moura *et al.*, 1987) and was around 10^8 cells mL^{-1}. The production of IAA by rhizobia was quantified in triplicate samples using a spectrophotometer at wavelength of 530 nm (Gordon and Weber, 1951). The IAA concentration in the rhizobia broth samples was calculated by comparing the readings with a standard curve with 0, 0.2, 1, 2, 3, 6, 11, 20, 45, 100, 200 and 300 µg of synthetic IAA mL^{-1}. The data were subjected to analysis of variance and mean test (Scott Knott, 5%), using the software SISVAR (Ferreira, 2000).

Evaluation of the Inoculation of Rhizobia on Plant Growth of Forage Grasses

Aiming to evaluate the effect of rhizobia inoculation, four experiments were carried out during a period of 60 days in the greenhouse of the Department of Soils of the Agronomy School of Federal University of Rio Grande do Sul.

Species of grasses chosen for the experiments commonly growing in pastures in southern Brazil were: Tanzania grass (*Panicum maximum*), Pensacola (*Paspalum saurae*), brachiaria grass and ryegrass. The forage grasses were cultivated in plastic pots of different volumes due to the characteristic plant size of each species. Experiments with Tanzania grass and brachiaria, which can reach one meter high (Kissmann, 1997), were conducted in pots with capacity for 4 and 8 L, respectively. Smaller plants like ryegrass and Pensacola were cultivated in pots of 2 L. The pots were previously flamed with ethyl alcohol 99%. It was used as substrate a mixture of vermiculite and sand (2:1), previously sterilized by autoclaving. Each experiment was

conducted in a completely randomized design with 10 treatments and four replications. The treatments were: eight treatments, where the cultivated plants received rhizobia inoculation and two non-inoculated control treatments. One of the controls (control NI 50) received fertilization with nitrogen at a dose equivalent to 50 kg ha^{-1} and the other (control NI 100) received a dose equivalent to 100 kg ha^{-1}. Nitrogen was weekly added using aliquots of NH_4NO_3 solution with concentration of 7.14 g L^{-1} and the volume of aliquots was adjusted according to the volume of the pots in order to add the corresponding dose of N.

The rhizobia inoculum were produced by inoculation in 250 mL flasks with 80 mL of YM liquid medium and maintained in an orbital incubator at 28°C at 120 rpm for six days. The seeding was made by placing six sterilized and pre-germinated seeds in each pot. The rhizobia inoculation over the pre-germinated seeds was performed using aliquots of 2 mL of rhizobia broth containing about 10^8 colony forming units (CFU) mL^{-1}. The seeds of the forage grasses species presented similar size and to avoid any influence of the inoculum size it was used the same volume of rhizobia broth as inoculum to all the grasses. The thinning was performed 15 days after the plant emergence, leaving two plants for each pot, except in the experiment conducted with brachiaria, which was carried out with only one plant per pot due to poor seed germination. The plants were cultivated by a period of 60 days. At the end of the period the number of tillers was determined and the plants were harvested. The roots and the aerial part of the plants were separated and stored in paper bags and placed to dry at 65°C. Plant dry mass was evaluated and the N contents of aerial parts (Tedesco *et al.*, 1995). Data were analyzed by ANOVA and the mean comparison by Scott Knott test (5%) using the statistic software SISVAR (Ferreira, 2000).

For each inoculated treatment, relative efficiency (RE) index that estimates the bacterial efficiency on symbiotic nitrogen fixation (Brockwell *et al.*, 1966) was measured with the following formula and used to estimate the bacteria efficiency on plant growth promotion:

$$\mathrm{RE}(\%) = \frac{(\text{DM Inoculated} - \text{DM Control NI 50})}{(\text{DM Control NI 100} - \text{DM Control NI 50})} \times 100$$

Where:

RE (%) = Relative Efficiency index;

DM Inoculated = total dry mass of plants from the inoculated treatment with the addition of nitrogen equivalent to 50 kg de N ha^{-1};

DM Control NI 50 = total dry mass of plants from non-inoculated treatment with the addition of nitrogen equivalent to 50 kg de N ha^{-1};

DM Control NI 100 = total dry mass of plants from non-inoculated treatment with the addition of nitrogen equivalent to 100 kg de N ha^{-1}.

Characterization of Rhizobia Genomic DNA

The genomic DNA of rhizobia SEMIA 816, UFRGS Lc134, UFRGS Lc336, UFRGS Lc394, UFRGS Lc510, UFRGS Lc522 and UFRGS Lc524 was extracted using the PROMEGA's Wizard Genomic DNA Purification Kit Protocol (Promega Corp. Madison, USA) according to manufacturer's specifications. The cells were obtained from rhizobia culture growing in tubes containing YM medium (Vincent, 1970) and incubated at 28°C for 48 h under constant agitation of 120 rpm. The rhizobia genomic DNA was characterized by PCR amplification with primer BOX A1, according to Versalovic *et al.* (1994), with modifications by Giongo (2007). The conditions applied for the PCR reaction were the following: an initial cycle at 95°C for 7 min, 30 cycles of denaturalization at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min and a final cycle extension at 65°C for 16 min. Amplifications were visualized on agarose gel electrophoresis at 1.5% horizontally, with molecular weight marker of 400 bp. The agarose gel was visualized on photodocumenter KODAK G2200.

The fragments profile was transformed into a binary matrix, where zero (0) indicates the absence of bands and one (1) their presence. The genetic similarity was calculated using Jaccard's coefficient and the dendrogram constructed based on Tocher's grouping method using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm run in the program NTSYS 2.0.

Identification of Rhizobia by 16S rRNA Sequencing

The genomic DNA obtained from rhizobia cultures was also used as template for amplification of the 16S rRNA gene. Nearly full-length 16S rRNA genes were amplified by PCR using primer 8F (5'- AGAGTTTGATCCTGGCTCAG-3') target to bacteria (Edwards *et al.*, 1989) and 1492R (5'-GGTTACCTT GTTACGACTT-3') universal primer (Stackebrandt and Liesack, 1993). The PCR cycles were the following: an initial cycle at 95°C for 3 min, 35 cycles of denaturalization at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension cycle at 72°C for 3 min. Once the fragments were sequenced, the homologous nucleotides sequences were verified on GenBank (www.ncbi.nlm.nih.gov) using the program BLASTn (Altschul, 1997) and the fragments analyzed by the algorithm Megablast. The selected sequences were aligned using ClustalW algorithm and phylogenetic trees were achieved using the Neighbour-joining method using the software MEGA 4.0 (Tamura *et al.*, 2007).

Results

Rhizobia Selection Based on IAA Production

The capacity to produce IAA was observed in 9 out of 10 bacteria studied when cultivated in YM medium, even without the addition of tryptophan. In medium without tryptophan, the average values for IAA produced by

bacteria varied between 0.2 and 4.0 µg of IAA mL^{-1} and the higher value of IAA (4 µg mL^{-1}) was produced by SEMIA 816 followed by Lc394 that produced 3.2 µg of IAA mL^{-1}. In YM culture media enriched with 50 mg L^{-1} tryptophan, all rhizobia were stimulated to produce IAA except Lc394. Among the rhizobia, IAA production Lc348 presented the higher value 60.7 µg mL^{-1} followed by SEMIA816 that produced the amount of 50.1 µg mL^{-1}.

Effect of Rhizobia Inoculation on the Growth of Forage Grasses Plants

The plant shoot and root dry mass produced by plants of Tanzania grass inoculated with SEMIA816, UFRGS Lc134, UFRGS Lc323, UFRGS Lc348, UFRGS Lc510 and UFRGS Lc524 was higher than that in non-inoculated plants (Table 1), and inoculation with UFRGS Lc336 only increased the root dry mass. In plants of Pensacola grass inoculated with SEMIA816, UFRGS Lc134, UFRGS Lc336 and UFRGS Lc394 the plant shoot and root dry mass was also increased in comparison to non-inoculated plants of treatment control NI 50 and the inoculation with UFRGS Lc323 and UFRGS Lc348 only increased the root dry mass. The rhizobial inoculation on plants of brachiaria and ryegrass did not increase in plant shoot and root dry mass (Table 1). Interestingly the SEMIA 816 and UFRGS Lc134 stimulated the plant growth in both Tanzania grass and Pensacola grass. UFRGS Lc323, UFRGS Lc348, UFRGS Lc510 and UFRGS Lc524 increased plant shoot dry mass only in plants of Tanzania grass and the UFRGS Lc336 and UFRGS Lc394 promoted growth only in plants of Pensacola grass (Table 1).

The inoculation with SEMIA 816, UFRGS Lc336, UFRGS Lc348 and UFRGS Lc524 increased the number of panicles in brachiaria grass while control plants produced no panicles (Table 1). Increases in the root volume were observed in plants of Tanzania grass inoculated with all rhizobia except UFRGS Lc394 and in Pensacola grass only in plants inoculated with SEMIA 816 and UFRGS Lc394 and in plants of ryegrass only in those inoculated with UFRGS Lc323. On the other hand, there was no increase on root volume in plants of brachiaria grass.

Results showed that despite not being able to biologically fix atmospheric nitrogen, plants of Tanzania grass inoculated with SEMIA 816, UFRGS Lc323, UFRGS Lc336 presented higher accumulation of total nitrogen in shoots (Table 1) in comparison with non-inoculated plants that received a dose of nitrogen equivalent to 50 kg ha^{-1}.

The relative efficiency index (RE) of the plant growth promotion by rhizobia inoculation in all the grass species is shown in Fig. 3A–D, respectively. In Tanzania grass, all rhizobia, except UFRGS Lc394, manifested RE index >100%, outperforming the non-inoculated plants in control treatment NI100. Pensacola grass inoculated with SEMIA 816, UFRGS Lc394, UFRGS Lc134 and UFRGS Lc336 revealed RE index >80%, while the rhizobia UFRGS Lc348, UFRGS Lc510 and UFRGS Lc524 were inefficient (Fig. 3B). However, no rhizobia were efficient on plant growth promotion of brachiaria grass (Fig. 3C) and in ryegrass (Fig. 3D) showing RE index <40%.

Genotypic Characterization

The amplification of the genomic DNA of rhizobia by BOX PCR exhibited electrophoretic band profile, which showed a similarity dendrogram where could be observed 28 OTUs. The similarity coefficient from the PCR reaction with the primer BOX ranged between 12 and 35% (Fig. 1). By sequencing the amplification of the 16S rDNA region with primers 8F and 1492R (Edwards *et al.*, 1989), sequences from 893 to 1169 base pairs were analyzed through the Neighbour-joining method and a phylogenetic tree was constructed (Fig. 2). Comparing the results with sequences in the database, it was known that the rhizobia studied here belonged to the bacterial genera: *Mesorhizobium* (rhizobia cultures UFRGS Lc134, UFRGS Lc510, UFRGS Lc522, UFRGS Lc524 and SEMIA 816), and *Bradyrhizobium* (rhizobia cultures UFRGS Lc336 and UFRGS Lc394).

Discussion

The data of IAA production by the rhizobia cultures shows that even in an environment with small concentration of tryptophan, naturally present in the culture medium, most rhizobia could produce IAA. In medium supplemented with tryptophan the rhizobia Lc348 produced the highest amount of IAA (60.7 µg mL^{-1}) but this rhizobia culture only stimulated the plant growth in Tanzania grass. Higher values of IAA production were recorded from rhizobia. The production of IAA (171.1 µg mL^{-1}) was higher in isolate TV-13 of *Rhizobium leguminosarum* bv. *trifolii*, in which high production of IAA was injurious to the development of lettuce seedlings (Schlindwein *et al*., 2008). It is reported that high yields IAA by rhizobacteria were detrimental to plant growth, however small productions increased seedling vigor compared to control treatments without inoculation (Barazani and Friedman, 1999; Schlindwein *et al*., 2008). In wheat and rice seedlings *in vitro* conditions, higher amounts of IAA produced by *Azospirillum* strains reduced the length of roots and stems in the presence of tryptophan (Radwan *et al.*, 2004). Other mechanisms may be related with plant growth inhibition by rhizobia as some genes on certain plasmids of *R. leguminosarum* bv. trifolii that affect rice growth, development and root morphology (Perrine *et al.*, 2001). Wild-type *Sinorhizobium meliloti* strains Sm1021 and Rm2011 inhibited rice growth producing short lateral roots and yellow shoots at 21 days (Perrine *et al.*, 2005). The inhibition of rice seedling growth may be due to nitric oxide (NO) toxicity with the reduction of nitrate by *Rhizobium leguminosarum* bv. *trifolii* strain instead of high IAA production (Perrine-Walker *et al.*, 2007).

On the other hand, the inoculation with rhizobia with

Table 1: Plant shoot and root dry mass production, root volume, total nitrogen and number of panicles per plant of Tanzania (*P. maximum)*, Pensacola (*P. saurae*), brachiaria (*B. decumbens*) and ryegrass (*L. multiflorum*) inoculated with rhizobia and evaluated after cultivation by a period of 60 days

	Treatments										
Plants	Cont. NI 100	816	Lc134	Lc323	Lc336	Lc348	Lc394	Lc510	Lc524	Cont. NI 50	CV (%)
	Shoot dry mass per plant (g)										
P. maximum	3.4 a	3.3 a	3.3 a	3.3 a	3.1 b	3.2 a	2.9 b	3.4 a	3.2 a	2.8 b	7.1
P. saurae	2.1 a	2.0 a	2.1 a	1.8 b	2.0 a	1.6 b	2.2 a	1.8 b	1.6 b	1.7 b	9.7
B. decumbens	13.8 a	10.4 b	9.5 b	10.0 b	10.3 b	10.6 b	9.8 b	9.6 b	10.6 b	9.5 b	9.5
L. multiflorum	3.2 a	1.8 b	1.3 b	2.0 b	1.9 b	1.5 b	1.6 b	1.5 b	1.7 b	1.7 b	15.1
	Root dry mass per plant (g)										
P. maximum	1.7 b	1.9 a	1.8 a	1.9 a	2.0 a	2.1 a	1.6 b	1.9 a	1.8 a	1.5 b	14.2
P. saurae	1.6 b	1.8 a	1.6 b	1.6 b	1.6 b	1.5 b	1.8 a	1.3 c	1.2 c	1.3 c	9.8
B. decumbens	10.0*	7.9	9.7	7.6	8.5	9.4	10.4	8.8	9.3	9.2	14.3
L. multiflorum	3.0*	1.4	1.6	2.3	1.7	2.0	1.6	1.4	1.9	1.6	29.2
	Root volume (cm^3)										
P. maximum	31.0 a	32.0 a	29.0 a	34.0 a	34.0 a	35.0 a	24.0 b	31.0 a	30.0 a	25.0 b	13.2
P. saurae	20.5 b	27.0 a	19.0 b	17.5 b	20.0 b	15.5 b	26.5 a	15.5 b	18.0 b	16.5 b	14.5
B. decumbens	148.5*	126.0	192.3	105.8	137.3	141.8	149.8	137.3	153.0	131.8	21.6
L. multiflorum	26.4 a	18.4 b	18.4 b	26.7 a	17.4 b	19.2 b	19.7 b	18.4 b	21.3 b	19.2 b	10.8
	Total nitrogen in shoot (mg)										
P. maximum	50.0 a	37.4 b	30.8 c	38.8 b	34.7 b	32.2 c	31.2 c	31.5 c	33.0 c	30.6 c	9.0
P. saurae	55.1 a	35.6 b	34.5 b	33.0 b	38.0 b	32.9 b	37.2 b	37.6 b	32.9 b	33.8 b	18.9
B. decumbens	186.5 a	84.2 c	75.2 c	96.7 b	91.9 b	97.8 b	77.4 c	81.3 c	91.1 b	100.0 b	13.1
L. multiflorum	12.2 a	5.5 b	3.9 b	6.0 b	5.7 b	5.3 b	4.9 b	4.3 b	5.5 b	5.0 b	14.5
	Number of panicles per plant										
B. decumbens	0.8 b	0.8 b	0	0	2.0 a	2.0 a	0	0	1.3 a	0	25.4

Values are the average of four replicates with two plants per replication. Cont. NI 50: non-inoculated control treatment with the addition of 50 Kg of N ha^{-1}; Cont. NI 100: non-inoculated control treatment with the addition of 100 Kg of N ha^{-1}; All inoculates were treated with the addition of N, equivalent to 50 kg ha^{-1}; Values with same letter in the row do not differ according to the Scott Knott test (5%). * = no significant differences

low production of IAA (UFRGS Lc336 and UFRGS Lc323) or no production in a non-enriched medium with tryptophan (UFRGS Lc134) stimulated plant growth in Tanzania and Pensacola grasses, probably due to the production of other phytohormones by rhizobia that were not studied here, such as cytokinins (Persello-Cartieaux *et al*., 2003), gibberellins (Yanni *et al.*, 2001; Erum and Bano, 2008), abscisic acid (Dangar and Basu, 1991) and lipo-chitooligosaccharides (Miransari and Smith, 2009). Other authors also observed that rhizobia can promote the grasses and other non-legumes growth (Prithiviraj *et al*., 2003; Etesami *et al*., 2009; Osorio Filho, 2009).

Interestingly the inoculation of rhizobia SEMIA 816 and UFRGS Lc134 stimulated the plant growth in both Tanzania and Pensacola grass showing that those are promising rhizobia to further studies. Also these rhizobia do not promoted the plant growth in brachiaria grass and ryegrass. Besides some rhizobia some strains (UFRGS Lc323, UFRGS Lc348, UFRGS Lc510 and UFRGS Lc524) increased plant shoot dry mass of Tanzania grass and other rhizobia (UFRGS Lc336 and UFRGS Lc394) brought such increase in Pensacola grass (Table 1). Those results suggested that there is some degree of compatibility between rhizobia and grasses, as well as different responses of grasses to inoculations.

The inoculation of Pensacola grass with UFRGS Lc394 revealed a higher relative efficiency, surpassing the control NI 100 (Fig. 3B). However the rhizobial strain UFRGS Lc394 was not effective in promoting the growth of Tanzania grass, presenting an underperformance in relation to all other inoculated treatments (Fig. 3A). This highlights the need for further investigation on compatibility of rhizobia with grasses of agricultural interest that can be potentially used in succession/intercropping system field in order to better exploit beneficial interactions occurring naturally between diazotrofic bacteria and plants. The association of the forage species Tanzania and the Pensacola grasses with the studied rhizobia presented the plant growth stimuli most significant. Therefore, the interactions between these two species and the compatible rhizobia should be evaluated based on the field performance, in order to reduce the use of nitrogen fertilizer in pastures with these two forage species by identifying the relation between different rice cultivars and rhizobia, which were the most responsive to inoculation with some bacteria (Osorio Filho, 2009). Thus, studies with rhizobia compatible with cultivar are of great importance and an alternative to improve economic and ecological sustainability of pastures and reducing the need for chemical fertilizers with no yield losses.

As regards genotypic characterization, the similarity coefficient of rhizobia in this study was low ranging from 12 to 35%. Working with rhizobia isolated from birdsfoot trefoil plants, Frizzo (2007) determined 40% similarity coefficient. In other study with rhizobia isolated from *Lotus* sp. the genetic similarity between rhizobial strains, leading to the formation of two major groups presenting similarity around 15% (Tonon, 2008). The genotypic characterization based on the band profile fingerprint revealed that the rhizobia cultures in this study were genotypically different from strain SEMIA816 currently recommended for

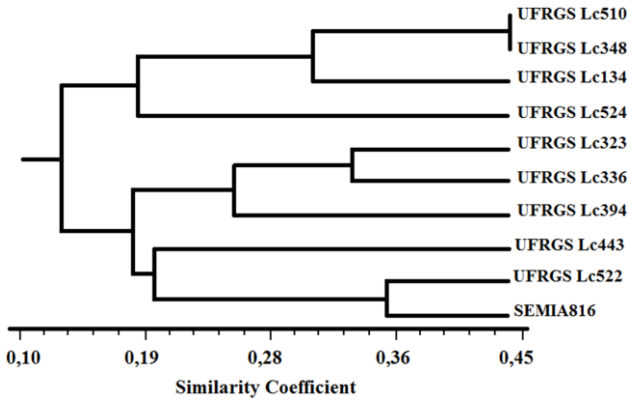

Fig. 1: Dendrogram of similarity of rhizobia based on fingerprint of bands of genomic DNA amplified by BOX A1 PCR. Grouping was obtained by UPGMA using Jaccard's coefficient

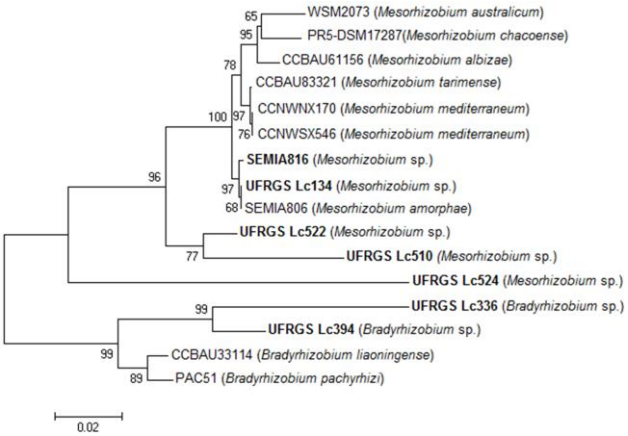

Fig. 2: Phylogenetic tree based on the analysis of 16S rRNA of strain SEMIA 816 and sequenced isolates obtained through the Neighbour-joining method. The numbers in the branches represent the bootstrap values

inoculating the forage legume birdsfoot trefoil plant (Machado, 2011). These results indicated that those rhizobia are not re-isolation of the rhizobia strain SEMIA816 and that they should be used in rhizobia strains selection programs regarding the plant growth promotion in forage grasses (Fontoura, 2007; Frizzo, 2007; Tonon, 2008).

From the sequencing of the 16S rDNA region with primers 8F and 1492R using Neighbour-joining method the phylogenetic relations were prepared (Fig. 2). It was noted that 16S rDNA sequences were placed in different groups through the phylogenetic tree. In one group, the sequences of SEMIA 816 and UFRGS Lc134 were grouped together and identified as *Mesorhizobium sp.*; so was the sequence from UFRGS Lc524. Also the rhizobia UFRGS Lc510 and UFRGS Lc522 were grouped together with sequences of other *Mesorhizobium* bacteria, while sequences of the rhizobia UFRGS Lc336 and UFRGS Lc394 were placed on other group with sequences from *Bradyrhizobium*. These results indicated that the rhizobial cultures SEMIA 816, UFRGS Lc134, UFRGS Lc510, UFRGS Lc522 and UFRGS Lc524 belonged to the genus *Mesorhizobium* and UFRGS Lc336 and UFRGS Lc394 was from the genus *Bradyrhizobium*. The classification of rhizobia SEMIA 816

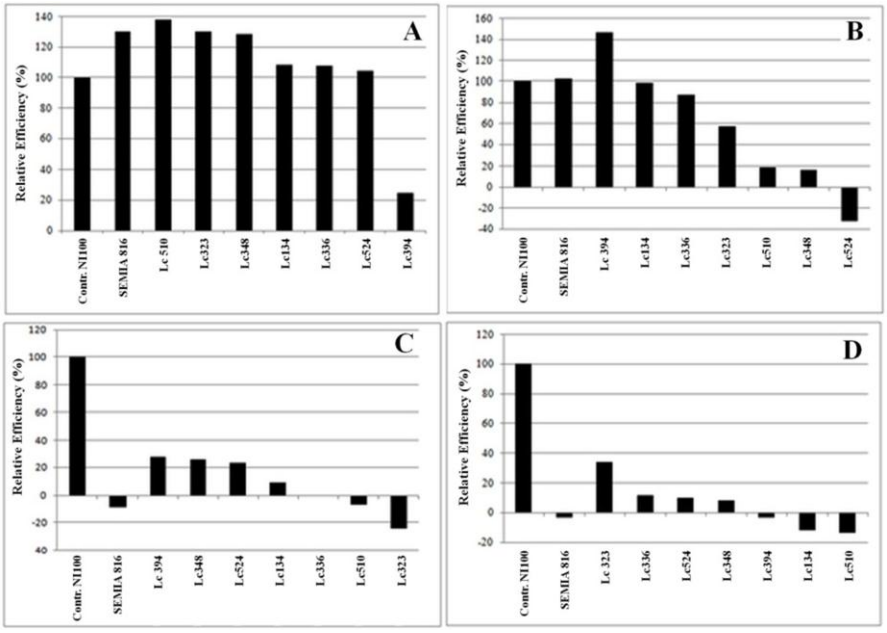

Fig. 3: Relative efficiency index (%) of rhizobia inoculation on dry mass increasing of plants of tanzania (*P. maximum*) (A), pensacola (*Paspalum saurae*) (B), brachiaria (*B. decumbens*)(C) and ryegrass (*L. multiflorum*) (D)

belonged to the genus *Mesorhizobium* as reported by Menna *et al.* (2006) and Toledo (2008).

In conclusion, the genotypic diversity among the rhizobia isolated from birdsfoot trefoil plants was high and those rhizobia cultures were different from strain SEMIA 816, recommended in Brazil for inoculant production. It is possible to select rhizobia for increased forage production among a group of previously selected rhizobia based on nitrogen fixation efficiency in legumes. These results open new avenues for the rhizobia strains selection programs and possibilities to reduce the nitrogen fertilizer applied on pastures due to the combined use of the same rhizobial strain both in forage legumes and grasses.

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References

Alvim, M.J., C.E. Martins, M.A. Botrel and A.C. Coser, 1987. Efeito da fertilização nitrogenada sobre a produção de matéria seca e teor de proteína bruta no azevém (*Lolium multiflorum* LAM.), nas condições da zona da mata de Minas Gerais. *Rev. Bras. Zootecn.*, l6: 606–614

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 25: 3389–3402

Antoun, H., C.J. Beauchamp, N. Goussard, R. Chabot and R. Lalande, 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effects on radishes (*Raphanus sativus* L.). *Plant Soil*, 204: 57–67

Asghar, H.N., Z.A. Zahir, M. Arshad and A. Khaliq, 2002. Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in *Brassica juncea* L. *Biol. Fert. Soils*, 35: 231–237

Barazani, O. and J. Friedman, 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *J. Chem. Ecol.*, 25: 2397–2406

Beaty, E.R., Y.C. Smith and J.D. Powell, 1974. Response of Pensacola Bahiagrass to irrigation and time of N fertilization. *J. Range Manage.*, 27: 394–396

Benett, C.G.S., O.M. Yamashita, P.S. Koga and K.S. Silva, 2008. Resposta da *Brachiaria brizantha* cv. Marandu a diferentes tipos de adubação. *Rev. Ci. Agro-Ambient.*, 6: 13–20

Biswas, J.C., J.K. Ladha, F.B. Dazzo, Y.G. Yanni and B.G. Rolfe, 2000. Rhizobial inoculation influences seedling vigor and yield of rice. *Agron. J.*, 92: 880–886

Brockwell, J., F.W. Hely and C.A. Neal-Smith, 1966. Some symbiotic characteristics of rhizobia responsible for spontaneous, effective field nodulation of *Lotus hispidus*. *Aust. J. Exp. Agric. Anim. Husb.*, 6: 365–370

Caballero-Mellado, J., 2006. Microbiología agrícola y interaciones microbianas con plantas. *Rev. Lat. Microbiol.*, 48: 154–161

Chen, X., J. Feng, B. Hou, F. Li, Q. Li and G. Hong, 2005. Modulating DNA bending affects NodD-mediated transcriptional control in *Rhizobium leguminosarum*. *Nucleic Acids Res.*, 33: 2540–2548

Dakora, F.D., 2003. Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. *New Phytol.*, 158: 39–49

Dangar, T.K. and P.S. Basu, 1991. Abscisic acid production in culture by some *Rhizobium* spp. of leguminous trees and pulses. *Folia Microbiol.*, 36: 527–532

Edwards, U., T. Rogall, H. Blöcker, M. Emde and E.C. Böttger, 1989. Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.*, 17: 7843–7853

Erum, E. and A. Bano, 2008. Variation in phytohormone production in *Rhizobium* strains at different altitudes of north areas of Pakistan. *Int. J. Agric. Biol.*, 10: 536–540

Etesami, H., H. Ali Alikhani and A. Ali Akbari, 2009. Evaluation of plant growth hormones production (IAA) ability by Iranian soils rhizobial strains and effects of superior strains application on wheat growth indexes. *World Appl. Sci. J.*, 6: 1576–1584

Ferreira, D.F., 2000. *Sistemas de Análise Estatística Para Dados Balanceados.* UFLA/DEX/SISVAR, Lavras, Brazil

Fontoura, R.A., 2007. *Seleção de rizóbios nativos, de solos do Rio Grande do Sul, para Lotus glaber e Lotus subbiflorus*. Graduate Program in Soil Science, UFRGS, Porto Alegre, Brazil

Frizzo, M.L.S., 2007. *Seleção e caracterização de rizóbios nativos, de solos do Rio Grande do Sul, para Lotus corniculatus L. e Lotus uliginosus Schkuhr*. Graduate Program in Soil Science, UFRGS, Porto Alegre, Brazil

Giongo, A., 2007. *Diversidade de Bradyrhizobium elkanii e B. japonicum que nodulam soja em solos do Rio Grande do Sul*. Graduate Program in Genetics and Molecular Biology, UFRGS, Porto Alegre, Brazil

Gordon, S.A. and P.R. Weber, 1951. Colorimetric estimation of indolacetic acid. *Plant Physiol*., 26: 192–195

Gutierrez-Zamora, M. and E. Martinez-Romero, 2001. Natural endophytic association between *Rhizobium etli* and maize (*Zea mays*). *J. Biotechnol.*, 91: 117–126

Kissmann, K.G., 1997. *Plantas Infestantes e Nocivas*, 2nd edition. BASF, São Paulo, Brazil

López, J., P.J.Q. Prestes and E. Magalhães, 1965. *A curva de crescimento e a composição em carboidratos solúveis, estruturais, lignina e proteína e a digestibilidade em cornichão.* In: Congresso Internacional de Pastagens, 9th edition. pp: 851-887. Alarico, São Paulo, Brazil

Machado, R.G., 2011. *Promoção de crescimento em gramíneas forrageiras por rizóbios isolados de Lotus corniculatus.* Graduate Program in Soil Science, UFRGS, Porto Alegre, Brazil

Matiru, V.N. and F.D. Dakora, 2004. Potential use of rhizobial bacteria as promoters of plant growth for increased yield in land races of African cereal crops. *Afr. J. Biotechnol.*, 3: 1–7

Menna, P., M. Hungria, F.G. Barcellos, E.V. Bangel, P.N. Hess and E. Martínez_Romero, 2006. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. *Syst. Appl. Microbiol.*, 29: 315–332

Miransari, M. and D. Smith, 2009. Rhizobial lipo-chitooligosaccharides and gibberellins enhance barley (*Hordeum vulgare* L.) seed germination. *Biotechnology*, 8: 270–275

Moura, R.A., C.S. Wada, A. Purchio and T.V. Almeida, 1987. *Técnicas de Laboratório*, 3rd edition. Atheneu. Rio de Janeiro, Brazil

Osorio Filho, B.D., 2009. *Rizóbios eficientes em Lotus em condições de estresse hídrico e promotores de crescimento em arroz irrigado*. Graduate Program in Soil Science, UFRGS, Porto Alegre, Brazil

Paim, N.R., 1988. Research on *Lotus* spp. in Rio Grande do Sul, Southern Brazil. *Lotus Newslett.*, 19: 37–43

Perrine, F.M., J. Prayano, J.J. Weinman, F.D. Dazzo and B.G. Rolfe, 2001. *Rhizobium* plasmids are involved in the inhibition or stimulation of rice growth and development. *Aust. J. Plant Physiol.*, 28: 923–937

Perrine, F.M., C.H. Hocart, M.F. Hynes and B.G. Rolfe, 2005. Plasmid-associated genes in the model micro-symbiont *Sinorhizobium meliloti* 1021 affect the growth and development of young rice seedlings. *Environ. Microbiol.*, 11: 1826–1838

Perrine-Walker, F.M., J. Prayitno, B.G. Rolfe, J.J. Weinman and C.H. Hocart, 2007. Infection process and the interaction of rice roots with rhizobia. *J. Exp. Bot.*, 58: 3343–3350

Persello-Cartieaux, F., L. Nussaume and C. Robaglia, 2003. Tales from the underground: molecular plant-rhizobacteria interactions. *Plant Cell Environ.*, 26: 189–199

Prithiviraj, B., X. Zhou, A. Souleimanov, W.M. Khan and D.L. Smith, 2003. A host-specific bacteria-to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. *Planta*, 21: 437–445

Radwan, T.S.D., Z.K. Mohamed and V.M. Reis, 2004. Efeito da inoculação de *Azospirillum* e *Herbaspirillum* na produção de compostos indólicos em plântulas de milho e arroz. *Pesq. Agropec. Bras.*, 39: 987–994

Rodríguez, H. and R. Fraga, 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.*, 17: 319–339

Schlindwein, G., L.K. Vargas, B.B. Lisboa, A.C. Azambuja, C.E. Granada, N.C. Gabiatti, F. Prates and R. Stumpf, 2008. Influência da inoculação de rizóbios sobre a germinação e o vigor de plântulas de alface. *Rev. Ci. Rural*, 38: 658–664

Singh, R.K., R.P.N. Mishra and H.K. Jaiswal, 2005. Can rhizobial inoculation promote rice growth through nitrogen fixation? *Int. Rice Res. Notes*, 30: 28–29

Soares, W.V., E. Lobato, D.M.G. Sousa and L. Vilela, 2001. *Adubação fosfatada para manutenção de pastagem de Bachiaria decumbens no Cerrado*, Technical Communique 53, Embrapa. Embrapa Cerrados, Planaltina, Brazil

Soria, L.G.T., R.D. Coelho, V.R. Herling and V. Pinheiro, 2003. Resposta do capim Tanzânia a aplicação do nitrogênio e de lâminas de irrigação. I: Produção de forragem. *Rev. Bras. Eng. Agric. Amb.*, 7: 430–436

Stackebrandt, E. and W. Liesack, 1993. Nucleic acids and classification. *In*: *Handbook of New Bacterial Systematics*, p. 152–189. Goodfellow, M. and A.G. O'Donnell (eds.). Academic Press, London, England

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 24: 1596–1599

Tedesco, M.J., C. Gianello, C.A. Bissani, H. Bohnen and S.J. Volkweiss, 1995. *Análise de Solo, Planta e Outros Materiais*, 2nd edition, UFRGS. Department of Soil, Porto Alegre, Brazil

Toledo, B.F.B., 2008. *Identificação de estirpes de rizóbios por sequenciamento parcial dos genes 16S rDNA e nifH*. Graduate Program in Agronomy – Plant Breeding and Genetics, UNESP, Jaboticabal, Brazil

Tonon, B.C., 2008. *Compatibilidade simbiótica e caracterização de rizóbios de Lotus spp., isolados de solos do Rio Grande do Sul.* Graduate Program in Soil Science, UFRGS, Porto Alegre, Brazil

Versalovic, J., M. Schneider, F.J. de Bruijn and J.R. Lupski, 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Mol. Cell. Biol.* 5: 25–40

Vincent, J.M., 1970. *Manual for the Practical Study of Root Nodule Bacteria.* Blackwell Scientific, Oxford, UK.

Webster, G., C. Gough, J. Vasse, C.A. Batchelor, S.L. O´Callaghan, S.L. Kothari, M.R. Davey, J. Dénarié and E.C. Cocking, 1997. Interactions of rhizobia with rice and wheat. *Plant Soil*, 194: 115–122

Yanni, Y.G., E.Y. Rizk, V. Corich, A. Squartini, K. Ninke, S. Philip-Hollingsworth, G.G. Orgambide, F.J. De Bruijn, J. Stoltzfus, D. Buckley, T.M. Schmidt, P.F. Mateos, J.K. Ladha and F.B. Dazzo, 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. *Plant Soil*, 194: 99–111

Yanni, Y.G., R.Y. Rizk, F.K. Abd El-Fattah, A. Squartini, V. Corich, A. Giacomini, F. de Bruijn, J. Rademaker, J. Maya-Flores, P. Ostrom, M. Vega-Hernandez, R.I. Hollingsworth, E. Martinez-Molina, P. Mateos, E. Velazquez, J. Wopereis, E. Triplett, M. Umali-Garcia, J.A. Anarna, B.G. Rolfe, J.K. Ladha, J. Hill, R. Mujoo, P.K. Ng and F.B. Dazzo, 2001. The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots. *Aust. J. Plant Physiol.*, 28: 845–870

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