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Full Length Article

Rhizobia Symbionts of Legume Forages Native to South Brazil as Promoters of Cultivated Grass Growing

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Abstract

The aim of this study was to evaluate the effect of previously selected rhizobia on the yield of sudan grass, millet, aries grass and sorghum forage. The rhizobia studied: UFRGS Om57; UFRGS Om59 and UFRGS Om148, obtained from the collection of cultures of UFRGS Soil Microbiology Laboratory, the isolated adesmia EEL46210, donated by Epagri, and SEMIA929 and SEMIA6437 strains, released by the Ministry of Agriculture, Livestock and Food Supply (MAPA) for the production of inoculants for *Ornithopus micrantus* and *Adesmia latifolia*. In the experiments with each species of plant, in addition to the inoculated treatments, two control treatments without inoculation were conducted, one with the adding of nitrogen N dose equivalent to 100 kg $N.ha^{-1}$ and the other with a dose equivalent to 50 kg $N.ha^{-1}$. The study was arranged in a completely randomized design and lasted 45 days. The isolated UFGRS Os57, UFRGS Om148, EEL46.210, SEMIA929 and SEMIA6437 stimulate root dry mass of sudan grass. Isolated UFRGS Om57 induces higher plant height compared to Control N - at 15 and 30 days after emergence. In millet, the SEMIA 929 strain increased the shoot and root dry mass, height at 15 days, and the Relative Efficiency Ratio (RER%) is more than 150%. The inoculation with UFRGS Om57 isolate stimulates sorghum shoot and root dry mass. For this treatment the RER% is 179%. There were genotypic differences among isolates of this study and the strains currently recommended for composition of commercial inoculants of *Ornithopus micrantus* and *Adesmia latifolia.* © 2016 Friends Science Publishers

Keywords: Plant growth-promoting rhizobacteria (PGPR); Suppression of mineral nitrogen fertilization; Symbiosis

Introduction

Recent studies have shown that the presence of legume in intercropping/ succession systems with grasses is not only associated to the N supply to the soil, but also to the direct or indirect effect on the yield of grasses (Yanni *et al.*, 2001; Hahn *et al.*, 2014). In, Reddy *et al.* (1997) found that rhizobia also colonize the tissue of grasses. According to the authors, the main form of rhizobia invasion in grass roots is through breaks in the skin and fissures created during the emergence of lateral roots. This infectious process is independent of gene-nod, that is, it is not related to the symbiotic process of nodulation and does not involve the formation of cord infection either, being confined to the intercellular space (Reddy *et al.*, 1997). By using labeled bacteria with the Gus gene, Osório Filho (2009) proved not only the infection of root tissue but also of rice leaves by rhizobia. These bacteria can move through the xylem to the shoot (Yanni *et al.*, 1997).

Several studies have reported an increase in the yield of grass species due to inoculation with rhizobia, even if these bacteria are not able to fix N in association with grasses (Yanni *et al.*, 2001; Mishra *et al.*, 2006; Hahn, 2013; Machado *et al.*, 2013; Osório Filho *et al.*, 2014). That way, besides inserting the atmospheric N in the soil-plant system by symbiotic fixation, the symbiotic rhizobia of native legumes and adapted to soil and climatic conditions of RS and SC can directly stimulate cultivated grasses that may be offered to animals in intercropping/succession to these legumes.

The influence on the yield of grasses is given as a result of the production of fitoestimulating substances,

such as hormones of the auxin groups (Biswas *et al.*, 2000), cytokinins (Persello-Cartieaux *et al.*, 2003), gibberellins (Yanni *et al.*, 2001), ABA (Dangar and Basu, 1991), or indirectly, by the residual nitrogen in root exudates and legume tissues when decomposed.

The search for biological associations that can benefit development of plants of economic interest and reduce the usage or enhance the use of mineral fertilizers can make the exploitation of pastoral systems more sustainable, economically and ecologically. The exploitation of pastoral systems in a more sustainable way, with less use of mineral inputs without damage to yields can be obtained by using nitrogen-fixing rhizobia, which action can promote growth of cultivated grasses.

Therefore, it is of great importance the prospection of rhizobia which are already adapted to the soil and climatic conditions of RS and SC states and which remain in native plants rhizosphere in efficient symbiotic associations. Thus, the study of these symbiotic rhizobia in native forage legumes is necessary, when interacting with forage grasses that are important to livestock of RS and SC states, and that can be grown in association with these native legumes.

The aim of this study was to evaluate the effect of rhizobia isolated from adesmia native legumes (*Adesmia latifolia*) and serradella (*Ornithopus micrantus*) on the yield of four grasses of economic importance in the states of RS and SC: sudan grass (*Shorghum sudanense* L. cv BRS. Estribo), millet (*Pennisetum glaucum* (L.) R. Brown cv. BRS1503), aries (*Panicum maximum* cv. Aries) and sorghum Moench cv. BRS810), as well as compare the rhizobia studied and group them by genotypic similarity.

Materials and Methods

Rhizobia Cultures Studied

Serradella rhizobia UFRGS Om57, UFRGS Om59 and UFRGS Om148 were studied for their ability to promote plant growth. Those rhizobia were obtained from the collection of cultures of UFRGS Soil Microbiology Laboratory, the adesmia isolated EEL46210, donated by Epagri of Lages-SC and the SEMIA 929 and SEMIA 6437 strains, released by the Ministry of Agriculture, Livestock and Supply (MAPA) for the production of inoculants for serradella and adesmia, kindly provided by the collection of SEMIA strains of FEPAGRO.

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

Four experiments were performed with rhizobia inoculation in plants of forage grasses: sudan grass (*Shorghum sudanense* L. cv. BRS Estribo), millet (*Pennisetum glaucum* (L.) R. Brown cv. BRS1503), aries (*Panicum maximum* cv. Aries) and sorghum (Sorghum (*Sorghum bicolor* (L.) Moench cv. BRS810).

The experiments were conducted in Nova Alvorada-RS, which is located in the mid-northeastern Rio Grande do Sul (RS), with latitude 28º40'39" south and longitude 52º09'59" west. The altitude is 427 meters and the climate, humid subtropical Climate (Köppen-Geiger climate classification: Cfa). The experiments were conducted in protected and controlled lighting environment, with 12 hours of artificial light, and the light bulbs turned on at 7:30 AM, and turned off at 7:30 PM.

Inert substrate composed of expanded vermiculite and sand mixture was used, in the ratio 2:1 (v/v), arranged in 400 mL plastic cups. The sand was previously washed in running water, in order to exclude any colloidal particles still present therein. After mixing homogeneously the substrate, it was placed in cotton bags and sterilized in an autoclave at a temperature of 120°C and 1 atm for a period of 90 minutes. After sterilization and cooling of the substrate, it was deposited in plastic cups, where the seeds were sown.

The seeds of the four studied species were sterilized by successive immersions in alcohol (70%) for 30 sec, followed by sodium hypochlorite (2.5%) for 30 sec and seven consecutive washes with sterile distilled water by autoclaving at 120°C for 15 min. Then, the seeds were placed between sheets of sterile paper towel, forming moistened rolls with sterile distilled water, covered with tin foil paper and placed in the greenhouse for germination at 28°C for 48 h. After germination, six plants which rootlets had length of 1 to 3 mm were placed in each pot using forceps and flamed glass rod. Seven days after transplantation, the lopping was performed for all experimental units, remaining two plants per pot.

The preparation of inocula of rhizobia was carried out in erlenmeyers of 250 mL, containing 80 mL of culture medium liquid Mannitol Yeast (Vincent, 1970) placed to incubate in an orbital shaker at 28°C, with constant shaking at 120 rpm for seven days. After the incubation period, the cultures broth showed minimal cellular concentration of 1.10^8 colony forming units (cfu) per mL, quantified in a Neubauer chamber (Moura *et al.*, 1987). 2 mL of the culture broth were applied in the inoculated treatments at seven days after sowing, using sterile glass pipette. In each experimental unit of the non-inoculated controls, it was administered the same volume of sterile liquid LM medium of culture (Vincent, 1970).

Nitrogen (N) was applied at seven days after sowing in all experimental units using a solution of ammonium nitrate (NH_4NO_3), with the concentration of 5,7 $g.L^{-1}$. Due to the fact that there is not symbiotic fixation of N by rhizobia in grasses, all inoculated treatments received application of N. Thus, in inoculated treatments and in the NI50 Control 5 mL were applied, whereas in the NI100 10 mL of solution of NH_4NO_3 were applied. To meet the need of the other nutrients which are essential to the growth and development of plants it was used nutrient solution Sarruge (1975), diluted to 25%, of which were applied weekly 50 mL to each of the experimental units, over the first 15 days of the

experiment. Subsequently, the applications were carried out with 4-day intervals until the time of harvest, 45 days after sowing.

Accordingly, each of the four experiments was composed of eight treatments: six inoculated treatments, all with N rate equivalent to 50 kg $N.ha^{-1}$; two control treatments without inoculation, one with N rate equivalent to 50 kg $N.ha^{-1}$ (NI50); and the other with N rate equivalent to 100 kg $N.ha^{-1}$ (NI100). Each treatment consisted of three replications. The study composed of four experiments was designed entirely by chance. At 15 and 30 days after emergence, plant heights were measured with the aid of a graduated scale. At the end of 45 days, the plants were collected, separating the shoot of root system. The roots were washed in running water to remove the particles of sand and vermiculite. After that, roots and shoots were stored in a greenhouse at 65°C for drying to constant weight. The shoot dry mass parameters, root dry mass, relative efficiency index (RER%), adapted from Brockwell (1966) by Machado *et al.* (2013) and plants height were evaluated.

The ability of the rhizobia to promote accumulation of total dry mass in the inoculated plants compared to the control treatments was assessed by calculating the relative efficiency index RER (%), according to the following formula:

$$RER\ (\%) = \frac{(\text{Inoculated MS} - \text{NI 50 Control MS})}{(\text{NI 100 Control MS} - \text{NI 50 Control MS})} * 100$$

Which: RER (%) = Relative Efficiency Ratio;

Inoculated MS = total dry mass of the inoculated treatment, with dose equivalent to 50 kg N ha^{-1};

NI 50 Control MS = total dry mass of the NI 50 Control treatment, uninoculated and with addition of nitrogen equivalent to 50 kg N ha^{-1};

NI 100 Control MS = total dry mass of the NI 100 Control treatment, uninoculated, with the addition of nitrogen equivalent to 100 kg N ha^{-1}.

Sequencing of the 16S rRNA Region

The sequencing of the 16 S region of the ribosomal DNA of the strains currently released by MAPA (Ministry of Agriculture, Livestock and Supply) for the production of serradella inoculants (SEMIA905 and SEMIA929) and adesmia (SEMIA6437) and also of isolated rhizobia UFRGS Om57; UFRGS Om59; UFRGS Om148 and EEL46.210 was carried out. The extraction of genomic DNA from rhizobia was performed starting from cells grown in tubes containing culture medium LM (Vincent, 1970) and incubated at 28°C for 48 h under constant agitation at 120 rpm.

The sequencing of the samples was performed using the automated sequencer AB 3500 Genetic Analyzer armed with capillaries of 50 cm and POP7 polymer (Applied Biosystems). The DNA patterns were marked using 2.5 pmol of primers 27F (BacPaeF): 5'AGA GTT TGA TCC TGG CTC AG 3' and 1525R (Bac1542R): 5'AGA AAG GAG GTG ATC CAG CC 3' and 0.5 µL of the reagent Big Dye Terminator v3.1 Cycle Sequencing Standart (Applied Biosystems) in a final volume of 10 µL. The labeling reactions were performed in an LGC XP Cycler thermocycler with an initial denaturation step at 96°C for 3 min followed by 25 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min. Once labeled, the samples were purified by precipitation with isopropanol at 75% and washing with etanol at 60%. The precipitated products were diluted in 10 µL of formamida Hi-Fi (Applied Biosystems), denatured at 95°C for 5 min, chilled on ice for 5 min and electro injected in automated sequencer.

The sequencing data were collected using the Data Collection 2 program (Applied Biosystems). Nucleotide sequences obtained were analyzed using the algorithm Megablast (Tamura *et al.,* 2007) and the sequences were compared and grouped by similarity based on the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) by NTSYS 2.0 software (Rohlf, 1998).

Results

Effect of Rhizobia Inoculation on the Growth of Forage Grasses Plants

In inoculated Sudan grass plant, it was noted that five rhizobia (UFGRS Om57, UFRGS Om148, EEL46.210, SEMIA929 and SEMIA6437) increased the dry root mass, being equivalent to the NI100 Control treatment (Table 1). Inoculation also increased plant height. Three isolated stimulated plant height at 15 days (SEMIA 929, EEL46210 and UFRGS Om57) and four isolated did the same at 30 days after emergence (SEMIA 6437, UFRGS Om57, UFRGS Om59 and UFRGS Om148). Despite the stimulation of root dry mass and plant height, in the inoculated plants it was observed dry mass of shoot inferior to the control treatments (Table 1). The Relative Efficiency Ratio (RER%) obtained with the inoculations in sudan grass reveals positive effect on the total dry mass of plants, observed in the treatments SEMIA 929, SEMIA 6437, EEL46210, UFRGS Om57 and UFRGS Om148 compared to control treatments (Fig. 1).

The SEMIA 929 strain stimulated millet plants (Table 1). The millet inoculated with the SEMIA 929 strain had an increase of dry mass in the shoot, being superior to the NI50 Control treatment and to all other inoculated treatments. Fig. 1 shows RER% of SEMIA 929 exceeding 150%, which indicates that in addition to fixing atmospheric N in serradella, SEMIA 929 stimulates the yield of millet through other mechanisms. With the inoculation of SEMIA 929 strain in millet there was an increase of root dry mass and plants height at 15 days after emergence. All these results provide strong

Table 1: Plant shoot and root dry mass production after cultivation by a period of 45 days and plant height at 15, 30 and 45 days after seeding of Sudan grass (*S. sudanense*), millet (*P. glaucum*), aries grass (*P. maximum*) and sorghum (*Sorghum bicolor*) inoculated with rhizobia

Plants	Cont. NI100	SEMIA929	SEMIA6437	EEL46210	UFRGS Om57	UFRGS Om59	UFRGS Om148	Cont. NI50	CV(%)
	Shoot dry mass (mg)								
S. Sudanense	31 a	11 c	10 c	14 c	14 c	12 c	15 c	21 b	21,5
P. glaucum	19 a	14 b	9 c	6 c	11 c	6 c	10 c	10 c	32,5
P. maximum	5$^{n.s.}$	3	3	3	2	5	3	5	61,8
S. bicolor	41 a	35 b	23 b	25 b	48 a	26 b	31 b	32 b	32,4
	Root dry mass (mg)								
S. Sudanense	96 a	100 a	100 a	92 a	67 a	27 b	71 a	36 b	43,9
P. glaucum	186 a	245 a	57 b	60 b	104 b	79 b	100 b	100 b	44,1
P. maximum	.	.	.	.	.	.	.	.	.
S. bicolor	112 a	68 b	45 b	141 a	147 a	64 b	79 b	72 b	53,3
	Plant height at 15 days after seeding (cm)								
S. Sudanense	19,2 a	19,6 a	15,5 b	17,4 a	16,9 a	14,7 b	13,1 b	14,3 b	16,1
P. glaucum	9,6 a	11,5 a	10,1 a	9,8 a	9,5 a	7,6 b	11,5 a	7,7 b	18,9
P. maximum	5,7$^{n.s.}$	4,6	5,2	4,8	4,4	4,3	4,9	3,6	15,7
S. bicolor	27,5 a	23,6 b	23,4 b	23,9 b	23,3 b	23,3 b	24,3 b	22,8 b	8,1
	Plant height at 30 days after seeding (cm)								
S. Sudanense	23,1 a	19,5 b	22,5 a	20,1 b	22,0 a	21,3 a	22,5 a	19,8 b	8,5
P. glaucum	12,2$^{n.s.}$	14,1	14,0	14,00	14,9	12,4	14,2	10,3	28,1
P. maximum	7,4 a	5,9 b	6,7 a	6,1 b	5,4 b	5,4 b	6,1 b	4,8 b	15,5
S. bicolor	33,7 a	28,4 b	28,0 b	28,2 b	28,1 b	28,8 b	28,7 b	27,8 b	6,7
	Plant height at 45 days after seeding (cm)								
S. Sudanense	26,5$^{n.s.}$	24,4	23,3	22,2	22,9	21,2	23,2	25,2	12,7
P. glaucum	16,6$^{n.s.}$	15,9	14,8	14,4	13,2	11,7	17,2	12,1	19,5
P. maximum	7,7 a	6,3 b	6,7 a	6,0 b	5,2 b	5,5 b	6,0 b	6,8 a	12,0
S. bicolor	34,1 a	28,1 b	28,8 b	28,8 b	29,5 b	28,1 b	29,3 b	30,2 b	6,2

Values are the average of three replicates with two plants per replication. Control NI100: non-inoculated control treatment with the addition of 100 kg of N ha^{-1}; Controle NI50: non-inoculated control treatment with the addition of 50 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 (15%); n.s.: no significant differences

evidence of the positive and specific interaction between SEMIA 929 and millet cv. BRS1503.

The results obtained with rhizobia inoculation in aries grass are presented in Table 1. Because of the little dry mass production of shoots and the variation between repetitions within the treatments, it was not possible to identify differences in the level of significance of 15%. It was also impossible to measure root dry mass, due to the values being very small and undetectable in this study. Because of the equality of the values of dry mass of shoot of NI100 Control and NI50Control treatments, it was impossible to determine the RER% of inoculations in aries grass.

Yet in sorghum plants (cv. BRS 810), it was observed that the inoculation of rhizobia UFRGS Om57, serradella isolated, induced increase in shoot dry mass, being equivalent to that produced in non-inoculated plants and receiving addition of nitrogen equivalent to 100 kg of N ha^{-1} (NI 100 Control) and surpassing all other treatments (Table 1).

In Fig. 1 it is posible to check the relative efficiency (RER%) of rhizobia inoculated in sudan grass, millet and forage sorghum. With the exception of isolated UFRGS Om59, all rhizobia tested increased the total dry mass production (RER% > 0) in at least one of the studied species. It was observed positive Relative Efficiency Ratio (RER%) of strain SEMIA 929 and of the isolated UFRGS Om57, with inoculation in sudan grass, millet and forage sorghum. With these results it is demonstrated the ability of the rhizobia SEMIA 929 and UFRGS Om57 to promote the growth of the grasses studied, by means of plants growth promotion mechanisms, despite of not fixing atmospheric N when inoculated in grasses (Fig. 1).

Genotypic Comparasions

DNA sequences were obtained with a length of around 250 base pairs, which were compared to each other and grouped in a similarity dendrogram (Fig. 2). The dendrogram was assembled according to the similarity coefficient (SC), whereby identical sequences correspond to a SC equal to 1, while SC equals to 0 corresponds to the total dissimilarity between the sequences. It can be seen that the similarity between the sequences obtained ranged between 0.35 and 0.77 (Fig. 2).

Discussion

In the SEMIA6437, EEL46210, UFRGS Om57 and UFRGS Om148 treatments, it was observed an increase in millet plant height at 15 days after emergence. This stimulatory effect soon after millet emergence observed at 15 days, is due to the acceleration in the early growth and might induce greater competition for water, light and essential nutrients. This stimulatory effect is of great

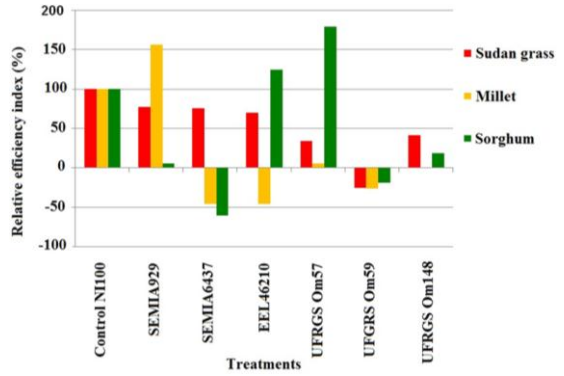

Fig. 1: Relative efficiency index (%) of rhizobia inoculation on dry mass increasing of plants of sudan grass, millet and sorghum

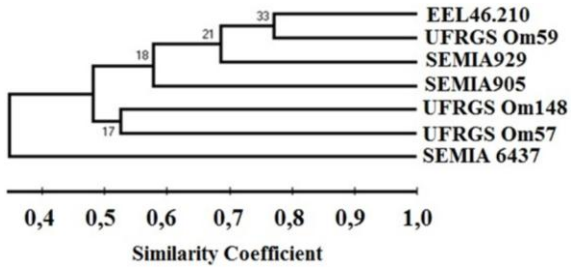

Fig. 2: Dendrogram of similarity of rhizobia based on fingerprint of bands of genomic DNA amplified. Grouping was obtained by Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

importance for increasing the competitiveness of the plants under study, under field conditions. Other authors also observed stimuli in the initial establishment phase of plants inoculated with rhizobia (Vargas *et al.*, 2009; Stroschein *et al.*, 2011; Tan *et al.*, 2014), which is important interaction on growth and early crop development.

When inoculated in sorghum (cv. BRS810) the isolated UFRGS Om57 caused an um important effect that, if observed in field experiments, may allow the cultivation of inoculated forage sorghum and with the addition of half of mineral nitrogen fertilization, without loss of the crop yield. The Relative Efficiency Ratio (RER%) of inoculation of the UFRGS Om57 in sorghum was 179%, the highest throughout the study. The sorghum inoculation with isolated UFRGS Om57 induced stimulation to plant roots, and after 45 days, the root dry mass of plants was superior to the NI50 control treatment and equivalent to NI100 Control. These results demonstrate the high efficiency of the UFRGS Om57 isolated to promote forage sorghum cv. BRS810 growth and the great potential of this interaction for further field exploration. The isolated EEL46210 also stimulated sorghum, having an RER (%) of 124%, whereas the RER (%) of the isolated UFRGS Os148 was 19%. With these results, it is possible to observe certain specificity between rhizobia and the studied grasses. In other words, certain rhizobia are better able to stimulate certain grasses, while others may be inert or detrimental to the grasses yield. Other specific reports between rhizobia and non-leguminous species were also observed by Baset Mia *et al.* (2012) and Osório Filho *et al.* (2014).

As for the possible plant growth promoting mechanisms, when it comes to interaction with grasses, an important mechanism by which rhizobia work is the synthesis of auxin (Dobbelaere *et al.*, 2003). Rhizobia synthesize indole acetic acid (IAA), a type of auxin, mainly via the route of pyruvic indol-3-acid (IPyaA), which according to Costacurta and Vanderleyden (1995) may be subject to stringent regulation by plants metabolites. The IAA is the most common and best characterized phyto-hormone (Hayat *et al.*, 2010). According to Patten and Glick (1996), 80% of the bacteria isolated of the rhizosphere are capable of producing IAA, which consists of a major plant hormone, it regulates many aspects of growth and development of plants, from the division, elongation and celular differentiation to root formation, apical dominance, tropism, flowering, fruit ripening and senescence (Baca and Elmerich, 2003).

There are other classes of regulators of plant growth produced by rhizobacteria that promote plant growth, which can in some way influence the growth and development of plants. They are the gibberellins, cytokinins, ethylene, and abscisic acid (Zahir *et al.*, 2004). These regulators are more difficult to quantify and are related to different effects on the physiology of plants: gibberellins are related to stem elongation (Davies, 1995); cytokinins are reported as inducers of cell division, root development and the formation of root hairs (Frankenberger and Arshad, 1995); and abscisic acid aids in growth in stressful environments by water deficiency (Frankenberger and Arshad, 1995).

As regards genotypic characterization, the isolated UFRGS Om59 and EEL46.210 presented the highest similarity with the comercial SEMIA929, around 0,68. The SEMIA 6437 strain recommended for adesmia, was the most dissimilar to the rest of the group, with SC equal to 0.35. With these results we can infer that isolated EEL 46.210, UFRGS Om57, UFRGS Om59 and UFRGS Om148 differ genotypically from each other, and also differ the strains SEMIA 905, SEMIA 929 and SEMIA 6437. Frizzo (2007) and Tonon (2008) determined the similarity coefficient of ribosomal DNA of isolated rhizobial of *Lotus cornicultus* and *Lotus* sp. through the UPGMA method. The similarities varied from 0,23 to 0,90 and 0,10 to 0,40, respectively.

Phenotypic differences presented in Fig. 2 show that the five most promising isolates of the present study, when it comes to the biological nitrogen fixation and promotion of plant growth, are organisms that have not been studied yet, other than those recommended in the composition of rhizobial inoculants recommended to serradella and adesmia. These results are very encouraging, because through them it is revealed that the isolates of this study

have not yet been explored in the composition of commercial inoculants.

Conclusion

Among the nitrogen-fixing rhizobia of this study obtained from native legumes adesmia and serradela, it´s possible to select rhizobia able to increase the production of exotic forage grasses. These results provide new perspectives for rhizobia selection programs for composition of commercial microbial inoculants and possibilities to reduce the N fertilizer applied on pastures.

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