SESBANIA VIRGATA STIMULATES THE OCCURRENCE OF ITS MICROSYMBIONT IN SOILS BUT DOES NOT INHIBIT MICROSYMBIONTS OF OTHER SPECIES

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ABSTRACT: The legume species *Sesbania virgata* establishes a specific and efficient symbiosis with *Azorhizobium doebereinerae*. Previous studies have shown that *A. doebereinerae* occurrence correlates to the presence of *S. virgata*. This work aimed to evaluate the occurrence of *A. doebereinerae* and of other nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB) in soil samples collected adjacent to and 10 m away from the stems of five *S. virgata* plants in pasture areas. Symbiotic characteristics of isolates from these NFLNB populations were also studied. *S. virgata* and the four promiscuous legume species *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Phaseolus vulgaris* and *Vigna unguiculata* were inoculated with soil samples to trap *A. doebereinerae* and other NFLNB. NFLNB capable of inducing nodulation in at least one of these legumes were found in all samples. *M. atropurpureum* was the most promiscuous species, as it trapped the highest number of NFLNB cultural types from soil suspensions. The other species were less promiscuous in the following order: *V. unguiculata*, *P. vulgaris*, and *L. leucocephala*. Isolates of the promiscuous legumes were classified into seven cultural groups. One of these groups, isolated from all promiscuous species, showed fast-growth alkali-reaction in culture medium (like *Azorhizobium*); it was identified as *Cupriavidus*. This is the first report of symbiosis of *Cupriavidus* with Papilionoideae species. The symbiotic efficiency of promiscuous hosts with NFLNB varied, but it was always less than that of controls with mineral nitrogen or an inoculant strain. *S. virgata* was efficiently nodulated only by *A. doebereinerae*, which occurred mainly in samples collected close to the plant stem, corroborating a high stimulus by its host species. A high diversity of NFLNB occurs as saprophytes close to the *S. virgata* root system.
Key words: *Azorhizobium*, *Cupriavidus*, Leguminosae, nodulation, nitrogen-fixing species

SESBANIA VIRGATA ESTIMULA A OCORRÊNCIA DE SEU MICROSSIMBIONTE NOS SOLOS, MAS NÃO INIBE OS MICROSSIMBIONTES DE OUTRAS ESPÉCIES

RESUMO: A espécie de leguminosa *Sesbania virgata* estabelece uma simbiose especifica e eficiente com *Azorhizobium doebereinerae*. Estudos prévios indicam que a ocorrência de *A. doebereinerae* esta relacionada à presença de *S. virgata*. Avaliou-se a ocorrência de *A. doebereinerae* e de outras Bactérias Fixadoras de Nitrogênio Nodulíferas em Leguminosas (BFNNL) em amostras de solos coletadas próximo e a 10 metros do caule de cinco plantas de *S. virgata* em áreas de pastagem. As características simbióticas de isolados das populações dessas BFNNL foram também estudadas. Para captura de *A. doebereinerae* e de outras BFNNL, essas amostras de solos foram inoculadas em *S. virgata* e nas leguminosas promíscuas *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Phaseolus vulgaris* e *Vigna unguiculata*. Todas as amostras de solos apresentaram BFNNL capazes de nodular, pelo menos, uma espécie. *M. atropurpureum* foi a espécie mais promíscua capturando um grande número de tipos culturais de BFNNL das suspensões de solo. As outras espécies foram menos promíscuas na seguinte ordem: *V. unguiculata*, *P. vulgaris*, e *L. leucocephala*. Os isolados das espécies hospedeiras consideradas promíscuas foram agrupados em sete tipos culturais. Um desses grupos, isolado de todas as espécies promiscuas, apresentou crescimento rápido e reação alcalina em meio de cultura (como *Azorhizobium*) e foi identificado como *Cupriavidus*. Este é o primeiro relato da simbiose

de *Cupriavidus* com espécies de Papilionoideae. A simbiose de BFNNL com as demais espécies teve eficiência variável, mas foi sempre menor que a da adubação nitrogenada e da estirpe recomendada como inoculante. *S. virgata* formou simbiose somente com *A. doebereinerae*, a qual foi mais freqüente nas amostras de solos coletadas próximo ao caule da planta, corroborando alto estímulo deste microsimbionte por sua espécie hospedeira. Uma alta diversidade de BFNNL ocorre saprofiticamente próximo ao sistema radicular de *S. virgata*.
Palavras-chave: *Azorhizobium*, *Cupriavidus*, Leguminosae, nodulação, espécies fixadoras de nitrogênio

INTRODUCTION

The genus *Sesbania* belongs to the Leguminosae family, subfamily Papilionoideae. It comprises about 70 species distributed mainly in the African and American continents. The plants of this genus can form symbiosis with nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB), which favor development in depleted and low natural fertility soils (Allen & Allen, 1981).

Sesbania virgata, native to South America, is found in the South, Southeast, and Midwest regions of Brazil, as well as in Paraguay, Argentina, and Uruguay (Pott & Pott, 1994). It is a fast-growing pioneer species that flowers and fruits several times a year and tolerates flooding; these properties allow it to be used in the recovery of degraded areas and gallery forest reforestation. This species has pharmacological properties and the treatment of mice with the lyophilized juice of the leaves reduced the reaction to painful stimulation and inflammatory edema (Braggio et al., 2002). The transference of biologically fixed nitrogen from *S. virgata* plants inoculated with rhizobia and mycorrhiza to *Eucalyptus grandis* plants indicate that intercropping can be beneficial to this latter species (Rodrigues et al., 2003).

Multiple strains of *Azorhizobium doebereinerae* isolated only from *S. virgata* showed similar total protein profiles and cultural characteristics: they were fast growers with alkaline reaction, and low production of gum in culture medium 79 (Fred & Waksman, 1928), distinguishing them from other NFLNB easily (Moreira et al., 2006). Strains of *A. doebereinerae* (syn. *A. johannae*) were found to have similar symbiotic behavior in monoxenic conditions, capable of nodulating few hosts and forming efficient symbiosis with only *S. virgata* (Gonçalves & Moreira, 2004). The presence of *A. doebereinerae* in several ecosystems in the south of Minas Gerais State was found to be related to the occurrence of *S. virgata*, as this microsymbiont was found only in soil samples collected close to the radicular system of *S. virgata* (Florentino & Moreira, 2009).

The occurrence of *A. doebereinerae* and of other nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB) was evaluated in soil samples collected adjacent to and 10 m away from the stems of five *S. virgata* plants in pasture areas. The symbiotic characteristics of isolates from these NFLNB populations were also studied to verify whether *S. virgata* favors the occurrence of *A. doebereinerae* by inhibiting the other NFLNB in natural conditions.

MATERIAL AND METHODS

Soil sampling in areas containing *Sesbania virgata* plants

Soil samples near five *S. virgata* plants were collected in February 2006 from distinct pasture areas in the state of Minas Gerais, Brazil: three located in Nepomuceno (21º14' S and 45º13' W) and two in Ribeirão Vermelho (21º13' S and 45º02' W). Two composite soil samples were taken from each plant, one close to the stem of each plant and another 10 m away from it, totaling ten composite samples. Samples were taken at the depth of 0-20 cm. Each composite sample was made up of four simple samples. During sampling, the litter was removed and the tools were flame-sterilized before each compound sampling to prevent contamination. The samples were kept in plastic bags and preserved in a cold chamber (4ºC) until inoculation of the soil suspensions into the legume species.

NFLNB trapping with trap plant species

Five experiments were carried out under greenhouse conditions. In each experiment, ten soil samples were inoculated into *S. virgata*, *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Phaseolus vulgaris*, and *Vigna unguiculata*. Except for *S. virgata*, these species are promiscuous plant species capable of symbiosis with more than one NFLNB species.

Each experiment also included inoculations with the type strains of the two described species of the *Azorhizobium* genus: *A. caulinodans* strain ORS 571^T, which was isolated from *S. rostrata* (Dreyfus et al., 1988); and *A. doebereinerae* strain BR 5401^T (Moreira et al., 2006), which is also recommended by MAPA as an efficient symbiont of *S. virgata* by Faria & Melo (1998). Additionally, there were two control treatments without inoculation for each experiment (species): one included mineral nitrogen and the second did not. Therefore, in total, there were 14 treatments for each plant species investigated.

We also inoculated each promiscuous host species with its recommended inoculating strain: BR 827 (for *L. leucocephala*), CIAT 899^T (for *P. vulgaris*), and UFLA 03-84 (for *V. unguiculata*). These tests were used as positive controls to test if the experimental conditions favored nodulation and nitrogen fixation. For *M. atropurpureum*, we used strain $St_2$12, isolated from Amazon soils, whose symbiotic efficacy has been demonstrated in previous experiments in our laboratory.

Samples of 50 g of soil suspended in 50 mL sterilized saline solution (0.55% NaCl) were agitated at 120 rpm for 30 min and then inoculated into the host species. Next, 1 mL of each suspension was inoculated in the host species and identified as follows: A0, A10, B0, B10, C0, C10, D0, D10, E0, and E10. A0, A10, B0, and B10 which refer to soil samples collected at the stem (A0 and B0) and 10 m away (A10 and B10) from *S. virgata* plants found in the two pasture areas close to Ribeirão Vermelho. C0, C10, D0, D10, E0, and E10 indicate the soil samples collected at the stem (C0, D0 and E0) and 10 m away (C10, D10, and E10) from *S. virgata* plants found in the three pasture areas close to Nepomuceno Town.

For inoculation, NFLNB recommended strains were grown in liquid culture medium 79 (Fred & Waksman, 1928), also known as YMB (Vincent, 1970), containing bromothymol blue, pH 6.8, under agitation at 28ºC. The fast-growing strains, BR 5401^T, ORS 571^T, BR 827, $St_2$12, and CIAT 899^T, were cultured for three days and the slow-growing strain UFLA 03-84 was cultured under the same conditions for five days.

S. virgata, *M. atropurpureum*, *P. vulgaris*, and *V. unguiculata* plants were cultured in 600-mL recycled dark glass flasks with quarter-strength 500 mL of Jensen nutritive solution without nitrogen (1 g L^{-1} $CaHPO_4$, 0.2 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} NaCl, 0.1 g L^{-1} $FeCl_3$, 2.86 mg L^{-1} H_3BO_3, 2.03 mg L^{-1} $MnSO_4.4H_2O$, 0.22 mg L^{-1} $ZnSO_4.7H_2O$, 0.08 mg L^{-1} $CuSO_4.5H_2O$, and 0.09 mg L^{-1} $Na_2MoO_4.H_2O$) (Jensen, 1942) with pH adjusted to 6.8. Each flask contained a rectangular filter paper sheet (3 × 25 cm, length equal to the glass height) that served as a support and to conduct the solution to the roots, especially when they were small. Flasks were sealed with adhesive tape to prevent contamination. Caps were bored for the introduction of the radicula and the respective inoculants (strain culture or soil suspension) or the mineral nitrogen solution.

Before planting, the flasks with Jensen's solution were autoclaved for 40 min at 1.5 kg cm^{-2} and 127ºC. It was not necessary to refill the solution during the experiment.

In the experiment with *L. leucocephala*, plants were grown in Leonard jars with sand and vermiculite, since this species was known to develop poorly well in Jensen's solution. The top part of the Leonard jars was filled with 1:1 (v:v) sand and vermiculite, while the bottom part was filled with quarter-strength Jensen's solution, without diluted nitrogen. Jars were first autoclaved as previously described for the flasks. After planting, the jar top soil was covered with a thin layer of 5:1:0.015 (v:v:v) sterilized sand:benzene: paraffin to prevent contamination. The jar was refilled periodically with sterilized Jensen's solution without nitrogen as previously described.

Seed dormancy of *S. virgata*, *L. leucocephala*, and *M. atropurpureum* was interrupted with 98.08% sulfuric acid for 40, 35, and 50 min, respectively. *P. vulgaris* and *V. unguiculata* seeds were disinfected superficially with alcohol and sodium hypochlorite (2%) for 30 and 120 s, respectively. After scarification and disinfection, the seeds were washed with sterilized water, placed in Petri dishes with sterilized cotton and filter paper, and wetted with water for 24 h. During planting, the teguments of the germinated seeds of the species cultivated in the flasks, *S. virgata*, *M. atropurpureum*, *P. vulgaris*, and *V. unguiculata*, were removed. Seeds of these species were placed in the flasks and immediately inoculated with either 1 mL of soil suspension or the NFLNB recommended strain. Only one plant was cultivated in each flask.

For *L. leucocephala*, four seeds were planted per jar, which were inoculated with either 1 mL of soil suspension or NFLNB each at plantation. After germination, the plants were thinned out to two plants per jar.

S. virgata, *L. leucocephala*, and *M. atropurpureum* were grown for 40 days and *P. vulgaris* and *V. unguiculata* for 30 days. All plants were evaluated for shoot dry matter weight (SDMW), nodule dry matter weight (NDMW), and number of nodules (NN).

The statistical design was completely randomized (CRD) with four repetitions. SDMW, NDMW, and NN were statistically analyzed by Scott-Knott test at 5%.

Characterization of the cultures of nodule isolates from legume species

For the isolation of the NFLNB, four nodules were randomly sampled per plant, for all of the five nodulating legume species. Nodules were disinfected with alcohol and hydrogen peroxide (H_2O_2) for 30 and 60 s, respectively, and washed with sterilized water six times successively, before maceration in the culture medium 79.

Isolates were characterized in culture medium 79, pH 6.8 at 28ºC, and the following variables were analyzed: time for the appearance of the isolates' colony, change in the pH of the culture medium, and the amount of gum produced. The nodule isolates obtained

from the five species were labeled with the initials of each origin host, *S. virgata* (Sv), *L. leucocephala* (Ll), *M. atropurpureum* (Ma), *P. vulgaris* (Pv), and *V. unguiculata* (Vu) followed by A0, A10, B0, B10, C0, C10, D0, D10, E0, and E10, indicating the soil sampling distance from *S. virgata* (close to or 10 m away from the stem), and a Roman number (I, II, III, or IV) identifying the isolate origin nodule. Further, they received codes of UFLA's bacterial collection.

Identification of the fast-growing alkali producing isolates

The cultural characterization of the 342 isolates obtained from all of the promiscuous legume species showed that 84 of these were fast-growers with alkaline reaction in the culture medium, similar to *A. doebereinerae*. However, these isolates had gum production at slightly higher levels than that of *A. doebereinerae*. To determine if these isolates belonged to the *A. doebereinerae* species, 12 were selected for evaluation of symbiosis characteristics by inoculation in *S. virgata*. Homologous isolates (7) were also tested in this species. Therefore, we tested seven isolates of *S. virgata*, four isolates of *L. leucocephala*, three isolates of *M. atropurpureum*, four isolates of *P. vulgaris*, and one isolate of *V. unguiculata*. Previously adopted procedures were followed in plant cultivation, seed disinfection, inoculation, and the use of control treatments.

The total protein profiles of seven isolates were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gene sequencing of 16S rDNA. One of these isolates was obtained from a *S. virgata* nodule. Inoculation in *S. virgata* and the total protein profile were used because, according to previous studies (Gonçalves & Moreira, 2004; Moreira et al., 2006; Florentino & Moreira, 2009), the protein profiles and the symbiosis efficiency of all *A. doebereinerae* isolates were highly similar to each other and to those of strain BR 5401^T.

Analysis of total proteins

The cultivation conditions of the six isolates of the promiscuous species, one isolate of *S. virgata*, and two type strains were rigorously standardized. They were cultivated in culture medium 79 and successively cultivated twice in solid TY medium. The isolated colonies were inoculated into 50 mL of liquid TY culture medium and cultivated for three days under constant agitation at 120 rpm at 28ºC. Next, the medium of each culture was centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatant was discarded and the pellet was resuspended in NaPBS buffer for cells washing. The pellet, 70 mg, was transferred to 1.5 mL microtubes containing 0.9 mL of sample buffer (TTA) and 0.1 mL of 20% SDS for solubilization of the proteins. This mixture was heated at 95ºC for 10 min and then centrifuged at 12,000 rpm for 10 min at 4ºC. Thirty microliters of the supernatant was subject to electrophoresis in polyacrylamide gel (PAGE). The Laemmli method (1970) was used with modifications as described by Jackman (1985) and Moreira et al. (1993). Electrophoresis was carried out using a discontinuous gel system with 12% polyacrylamide as the separation gel and 5% as the stacking gel.

Amplification and gene sequencing of 16S ribosomal DNA

The seven isolates used for protein profiles characterization were grown in culture medium 79. Isolated colonies were removed, placed in 1.5-mL microtubes with 1 mL sterile ultrapure water, and heated to 95ºC for 10 min. A 10-μL aliquot was used in polymerase chain reaction (PCR) with a final volume of 50 μL per reaction. The final concentrations of the reagents, per reaction, were 0.2 μM of each primer 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991), 2.5 mM of magnesium chloride, PCR buffer at 1x, 0.2 μM of each dNTP, and 0.02 U Taq DNA polymerase (Taq DNA polymerase, Invitrogen). The amplification reaction was carried out in an Eppendorf Mastercycle® thermocycler under the following conditions: initial denaturation at 94ºC for 5 min, 30 cycles of denaturation (94ºC for 40 s), annealing (55ºC for 40 s), extension (72ºC for 1.5 min), and one final extension at 72ºC for 7 min. An aliquot of each PCR reaction (20 μL) was analyzed using a 1% (w/v) agarose gel with TAE buffer and ethidium bromide staining (5 μg mL^{-1}). PCR products were purified from successful PCR reactions via ethanol precipitation and partial DNA sequencing (with the 27F primer) was performed with a 3730xl sequencer.

Sequences were evaluated by Phred of 20 and then compared with GenBank sequences using the Basic Local Alignment Search Tool (NCBI).

RESULTS AND DISCUSSION

Symbiotic efficiency of *A. doebereinerae* and other NFLNB populations occurring in soils collected close to the stem and 10 m away from *Sesbania virgata*

The absence of nodules in the controls (with and without mineral nitrogen) in any of the five experiments demonstrated the absence of contamination. *S. virgata* and *L. leucocephala* nodules had indeterminate growth, while *M. atropurpureum*, *P. vulgaris*, and *V. unguiculata* had determinate growth.

Strain *A. doebereinerae*, BR 5401^T, nodulated *S.virgata* and all legume species that were considered promiscuous under axenic conditions, while strain ORS 571^T nodulated *L. leucocephala*, *P. vulgaris*, and *V. unguiculata* (Table 1). With the exception of *L. leucocephala*, all promiscuous plants inoculated with these two strains had small nodules and a whitish color, indicating the absence of leghemoglobin. The shoot dry matter (SDMW) of these plants was similar to those of non-inoculated and non-fertilized plants (Table 2). These data corroborate the nodulation of *M. atropurpureum*, *P. vulgaris*, and *S. virgata* by strains BR 5401^T and ORS 571^T previously reported by other authors (Goncalves & Moreira, 2004).

Sesbania virgata

The control plants inoculated with strain BR 5401^T had a large number of nodules (NN), corroborating the high affinity and symbiotic efficiency of this strain when inoculated in *S. virgata*. The control plants inoculated with strain *A. caulinodans*, ORS 571^T, had pseudonodules, as previously reported by Gonçalves & Moreira (2004). Only one (E0) of the five samples collected close to the stem of *S. virgata* did not have NFLNB. The SDMW of treatments A0, B0, C0, and D0 were similar to each other, and in the case of C0, even higher than that of the treatment inoculated with strain BR 5401^T. The NN of all the treatments inoculated with these soil samples was higher than that of

Table 1 - Number of nodules (NN) per plant and nodule dry matter weight (NDMW-results in parentheses) of legume species inoculated with different treatments: soil suspensions of samples collected close to the stem (A0, B0, C0, D0, E0) and 10 m away (A10, B10, C10, D10, E10) from *S. virgata;* the type strains of the two species of genus *Azorhizobium* and one highly efficient strain for each species.

Treatment	Legume species				
	Sesbania virgata	*Leucaena leucocephala*	*Macroptilum atropurpureum*	*Phaseolus vulgaris*	*Vigna unguiculata*
A0	152 a[1] (107.00 c)[2]	14 d (60.00 b)	4 f (9.00 d)	95 b (31.25 c)	0 f (0.00 f)
A10	53 c (43.75 d)	0 e (0.00 e)	3 f (6.75 d)	78 c (37.75 c)	0 f (0.00 f)
B0	136 a (129.50 b)	20 c (40.00 c)	23 c (19.75 c)	69 c (50.75 b)	45 a (70.00 a)
B10	0 c (0.00 e)	0 e (0.00 e)	20 c (17.50 c)	39 d (16.75 d)	17 c (10.00 e)
C0	143 a (131.00 b)	0 e (0.00 e)	6 f (9.25 d)	151 a (38.25 c)	35 b (70.00 a)
C10	0 c (0.00 e)	0 e (0.00 e)	30 b (24.25 b)	34 d (3.50 e)	18 c (20.00 d)
D0	140 a (107.75 c)	0 e (0.00 e)	7 e (7.50 d)	37 d (12.50 d)	3 e (10.00 e)
D10	0 c (0.00 e)	13 d (20.00 d)	5 f (5.00 d)	0 e (0.00 e)	6 d (30.00 c)
E0	0 c (0.00 e)	0 e (0.00 e)	5 f (9.25 d)	0 e (0.00 e)	0 f (0.00 f)
E10	0 c (0.00 e)	33 b (90.00 a)	50 a (18.00 c)	94 b (64.50 a)	40 a (40.00 b)
BR 5401^T	105 b (155.00 a)	76 a (20.00 d)	11 d (3.50 e)	105 b (16.25 d)	32 b (10.00 c)
ORS 571^T	0 c (0.00 e)	22 c (20.00 d)	0 g (0.00 e)	81 c (18.75 d)	30 b (10.00 e)
Highly efficient Strain	-[3]	0 d (0.00 e) (BR 827)	30 b (34.75 a) (St212)	86 c (61.50 a) (CIAT899T)	40 a (70.00 a) (UFLA03-84)
Control[4]	0 c (0.00 e)	0 e (0.00 e)	0 g(0.00 e)	0 e (0.00 e)	0 f (0.00 f)
Mineral N[5]	0 c (0.00 e)	0 e(0.00 e)	0 g (0.00 e)	0 e (0.00 e)	0 f (0.00 f)
CV (%)	18.57 (10.50)	15.42 (38.95)	33.27 (32.86)	19.98 (24.25)	21.44 (20.76)

[1]Values followed by different letters in the same column are different (Scott-Knott test, $p < 0.05$); NDMW and NN data were transformed to square root (x + 1). [2]NDMW results in parentheses – mg plant^{-1}; [3]BR 5401^T for *S. virgata*; [4]Without mineral N and inoculation; [5]Mineral N(28 mg kg^{-1}).

Table 2 - Shoot dry matter weight of legume species inoculated with different treatments: soil suspensions of samples collected close to the stem (A0, B0, C0, D0, E0) and 10 m away (A10, B10, C10, D10, E10) from *S. virgata;* the type strains of the two species of genus *Azorhizobium* and one highly efficient strain for each species.

Treatment	Legume species				
	Sesbania virgata	*Leucaena leucocephala*	*Macroptilium atropurpureum*	*Phaseolus vulgaris*	*Vigna unguiculata*
	g per plant				
A0	1.03 b[1]	0.25 d	0.06 e	0.38 d	0.10 f
A10	0.70 c	0.21 e	0.04 e	0.41 c	0.17 e
B0	1.07 b	0.23 d	0.08 d	0.41 c	0.47 b
B10	0.10 d	0.10 f	0.09 d	0.36 d	0.16 e
C0	1.16 a	0.22 e	0.02 f	0.49 c	0.20 d
C10	0.10 d	0.20 e	0.10 d	0.41 c	0.23 d
D0	1.01 b	0.22 e	0.04 e	0.34 d	0.15 e
D10	0.09 d	0.21 e	0.03 f	0.36 d	0.19 e
E0	0.11 d	0.20 e	0.04 e	0.32 e	0.15 e
E10	0.10 d	0.25 d	0.14 c	0.35 d	0.33 c
BR 5401^T	1.08 b	0.29 c	0.02 f	0.25 e	0.08 f
ORS 571^T	0.11 d	0.35 b	0.02 f	0.25 e	0.07 f
Inoculant strain	-[2]	0.10 f (BR 827)	0.18 b (St212)	0.58 b (CIAT 899T)	0.30 c (UFLA 03 84)
Control[3]	0.08 d	0.11 f	0.02 f	0.23 e	0.09 f
Mineral N (28 mg kg^{-1})	1.05 b	0.63 a	0.21 a	0.84 a	0.72 a
CV (%)	9.60	8.20	18.48	13.08	18.05

[1]Values followed by different letters in the same column are different (Scott-Knott test, $p < 0.05$); [2]BR 5401^T for *S. virgata*; [3]Without mineral N and inoculation

the treatment inoculated with strain BR 5401^T. However, the treatment inoculated with this strain had a NDMW higher than those of the treatments inoculated with these soil samples (Table 1).

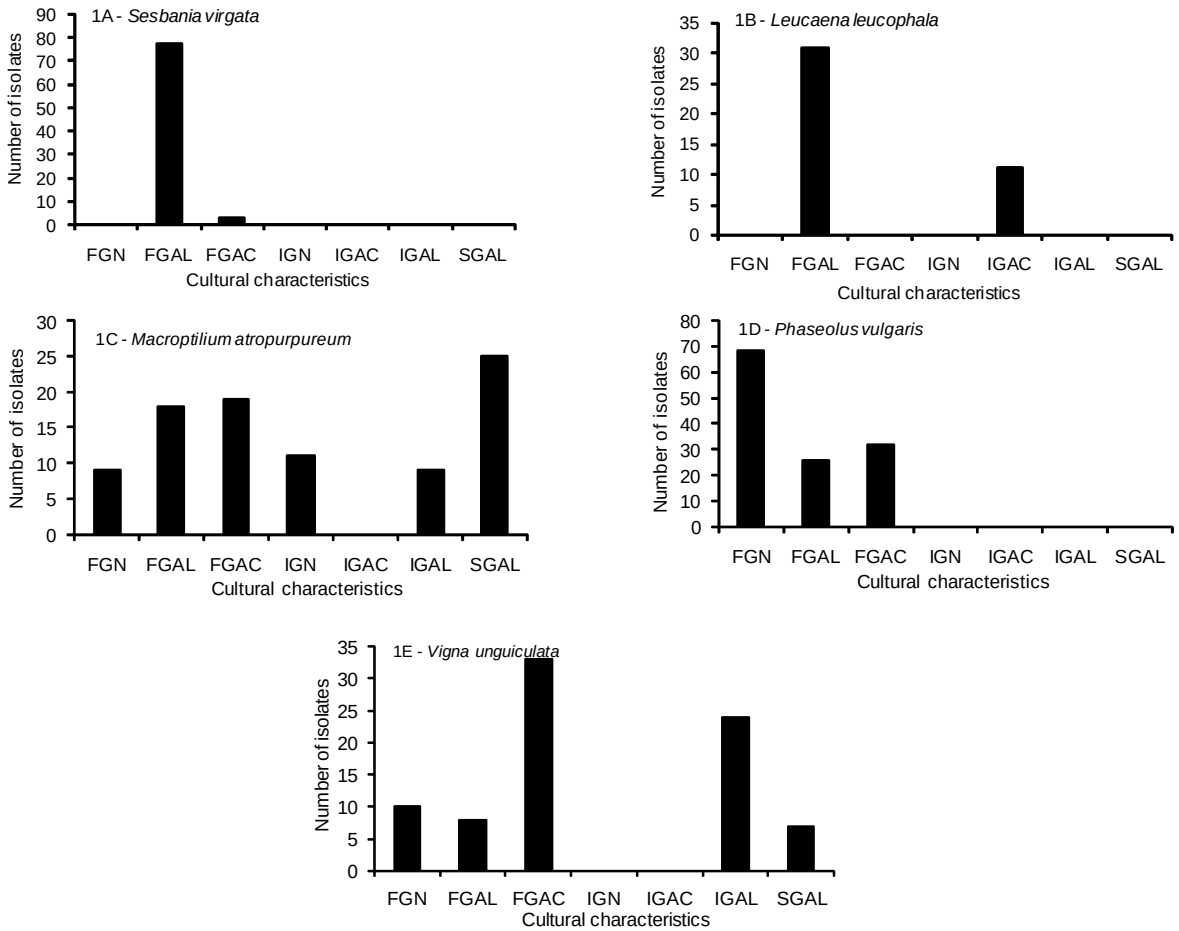
Only one of the samples collected at 10 m away from the stem of *S. virgata* (A10) had NFLNB capable of nodulating *S. virgata*. However, it had SDMW, NDMW, and NN lower than those of the treatments inoculated with soils collected close to the stem of *S. virgata* and with strain BR 5401^T (Tables 1 and 2). The density of the NFLNB populations capable of nodulating *S. virgata* is low at this distance from the host. Florentino & Moreira (2009) have previously reported that the microsymbiont did not occur in soil samples collected 20 and 40 m away from *S. virgata*, and that the distance of influence of this species on its microsymbiont may be smaller than 10 m.

In general, nodulation in *S. virgata* is always related to high symbiosis effectiveness (Tables 1 and 2), which is in agreement with the results of Gonçalves & Moreira (2004) and Florentino & Moreira (2009). Another species belonging to the genus *Sesbania* (*S. sesban*) also establish symbiosis only with a limited range of microsymbionts in a small percentage of soil samples and its symbiosis was always highly efficient, indicating that these species (*S. virgata* and *S. sesban*) have similar symbiotic characteristics (Bala et al., 2003 a,b).

Of the 80 isolates of *S. virgata* that were obtained, 96% had cultural characteristics similar to those of *A. doebereinerae*, with fast growth, alkaline reaction in the culture medium, and scarce gum production (Figure 1.A). The remaining 4% of isolates were not able to induce nodules in *S. virgata*, and can thus be considered contaminations.

Leucaena leucocephala

No nodules were detected in *L. leucocephala* plants inoculated with strain BR 827. In another study, the inoculation with this strain produced efficient nodules (Gonçalves & Moreira, 2004). In our study, some treatments produced nodules, demonstrating that the experimental conditions were adequate for the expression of symbiosis and that was likely some problem with the strain BR 827. Only four of the soil samples inoculated in *L. leucocephala*, A0, B0, D10, and E10, had NFLNB capable of symbiosis with this host. Comparing the treatments A0 and B0, both inoculated with soil samples collected close to the stem of *S. virgata*, B0 had the larger NN.



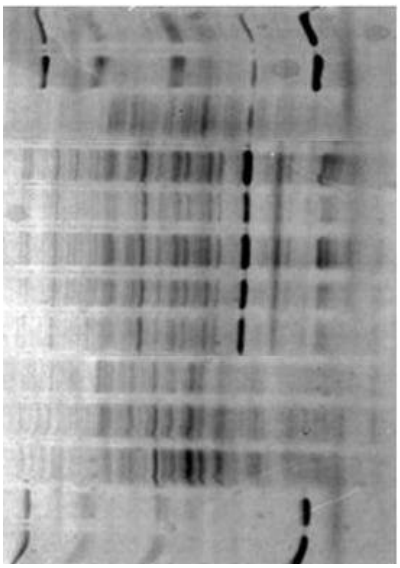
Figures 1 - A, B, C, D, E – Number of bacterial isolates in each of the cultural types in medium 79, listed by legume species. FGN – Fast growing without modifying the pH of the culture medium, FGAL – fast growing while alkalinizing the culture medium, FGAC – fast growing while acidifying the culture medium, IGN – Intermediate growing without modifying the pH of the culture medium, IGAC – Intermediate growing while acidifying the culture medium, IGAL – Intermediate growing while alkalinizing the culture medium, SGAL – slow growing while alkalinizing the culture medium.

Treatment E10 had SDMW and NN larger than those of treatment D10 (Tables 1 and 2). In comparison to the treatment with mineral nitrogen, the populations were not very efficient.

The 42 isolates of *L. leucocephala* that were obtained were arranged into two groups: the first, with 32 isolates, showed fast growth, alkaline reaction in culture medium, and gum production slightly larger than that of *A. doebereinerae*; the second, with ten isolates, showed intermediate growth, acidic reaction in culture medium 79, and mean gum production (Figure 1.B). *L. leucocephala* is considered a promiscuous species capable of forming symbiosis with either fast- or intermediate-growing NFLNB, that acidify or that do not modify the pH of the medium. These species were reported as belonging to the genera *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Moreira et al., 1998). In this study, most isolates had cultural characteristics distinct from those of the three genera cited above.

Macroptilium atropurpureum

All soil samples had NFLNB capable of symbiosis with *M. atropurpureum*. Among the treatments inoculated with soil samples collected close to the stem of *S. virgata*, the treatment inoculated with sample B0 had higher values of SDMW and NN, than treatments inoculated with the other soil samples. These values, however, were lower than the values of the treatment inoculated with strain $St_2$12 (Tables 1 and 2). E10 was the treatment inoculated with soil samples collected 10 m away from *S. virgata* that had the highest NDMW and NN values, with NN values higher than that of the treatment inoculated with strain $St_2$12. The 91 isolates of *M. atropurpureum* were grouped into six cultural types (Figure 1.C). The characteristics of fast growth, alkaline reaction in culture medium, and gum production slightly higher than that of *A. doebereinerae* were found only in 18 isolates. This large cultural diversity of nodule isolates of *M. atropurpureum* was also shown by



Total protein profile of isolates	Isolate-Identification by partial gene sequencing of 16S rDNA		Number of base pairs	Genbank accession-best hit	(%)*
PM					
	UFLA 04-121	*Cupriavidus* sp.	396	AB266612	98
	UFLA 02-128	*Cupriavidus* sp.	411	AB266612	98
	UFLA 02-129	*Cupriavidus* sp.	539	AB266612	98
	UFLA 01-657	*Cupriavidus* sp.	539	AB266612	98
	UFLA 01-658	*Cupriavidus* sp.	574	AB266612	98
	UFLA 01-659	*Cupriavidus* sp.	411	AB266612	98
	UFLA 01-656	*Azorhizobium* sp.	698	DQ664248	100
	BR 5401^T	*Azorhizobium doebereinerae*			
	ORS 571^T	*Azorhizobium caulinodans*			
PM					

Figure 2 - Total cell protein profiles and identification by partial gene sequencing of 16S rDNA (compared to Genbank) of isolates of *S virgata*, SvB0II2, *L. leucocephala*, LlB0III3, LlB0IV3 and LlE10I1, *P. vulgaris*, Pv0IV2 and Pv0III2 and *M. atropurpureum*, MaA0III2. *% similarity. Sequences determined in this study have been submitted to the public databases with accession numbers: GQ268819 (UFLA01-659); GQ268820 (UFLA02-129);GQ268821 (UFLA01-656);GQ268822 (UFLA02-128); GQ268823 (UFLA04-121);GQ268824 (UFLA01-658);GQ268825 (UFLA01-657).

Jesus et al. (2005)*;* however, they did not find fast-growing isolates with alkaline reaction.

Phaseolus vulgaris

Only one of the soil samples collected close to the stem of *S. virgata*, E0, did not have NFLNB capable of inducing the formation of nodules in *P. vulgaris*. Among the treatments that had nodules, A0, B0, and C0 had NN values similar and even higher than that of the treatment inoculated with strain CIAT 899^T. However, the values of SDMW of the treatments inoculated with these soil samples were lower than those of the treatment inoculated with strain CIAT 899^T (Table 1 and 2*)* and had a variable efficiency.

Of the samples collected 10 m away from *S. virgata*, only D10 did not have NFLNB capable of nodulating *P. vulgaris*. The NN value of treatment E10 was higher than that of the treatment inoculated with strain CIAT 899^T, despite its SDMW lower than that for CIAT 899^T.

We obtained 126 isolates of nodules of *P. vulgaris* with fast growth and a large variation of modification of the pH of the culture medium. Twenty-six isolates had fast growth, alkaline reaction in culture medium, and slightly higher gum production, similar to those of *A. doebereinerae* (Figure 1.D). Our findings contradict a report showing isolates with characteristics distinct from those of the genus *Rhizobium* of nodules of *P. vulgaris* did not have fast growth and alkaline reaction (Melloni et al., 2006).

Vigna unguiculata

Samples B0, C0, and D0, collected close to the stem of *S. virgata*, had NFLNB inducing nodules in *V. unguiculata*. The values of NN, NDMW, and SDMW of B0 were similar or higher than those of the treatment inoculation with the strain recommended for this culture, UFLA 03-84. Four of the soil samples collected 10 m away from *S. virgata* had NFLNB inducing nodule formation in *V. unguiculata*. Treatment E10 had the highest SDMW, NN, and NDMW values and SDMW and NN were similar to those of the treatment inoculated with strain UFLA 03-84.

We obtained 82 isolates of nodules of *V. unguiculata*; 52 of the isolates had fast growth and eight had cultural characteristics similar to those of *A. doebereinerae*, with fast growth, alkaline reaction in the culture medium, and gum production slightly higher than this species (Figure 1.E). In this study, most *V. unguiculata* isolates had fast growth in culture medium. These data differ from the results reported by Melloni et al. (2006), who reported that most *V. unguiculata* isolates had characteristics similar to those of the genus *Bradyrhizobium*: slow growth, alkaline reaction in culture medium, and gum production in culture medium 79 from low to medium.

Identification of the fast-growing alkali producing isolates

Symbiotic characteristics of *S. virgata*

Nodules were found only in *S. virgata* inoculated with homologous isolates, i.e., isolates of the same species. The isolates of the four legume species, *L. leucocephala*, *M. atropurpureum*, *P. vulgaris*, and *V. unguiculata* that had cultural characteristics similar to those of *A. doebereinerae*, with fast growth, alkaline reaction in the culture medium, and gum production slightly higher than this species, were not capable of nodulating *S. virgata*.

Protein profiles and 16S rDNA gene sequencing

Figure 2 shows the total protein profile and the 16S rDNA sequences of one isolate from *S. virgata* [SvB0II2 (UFLA 01-656)], three from *L. leucocephala* [LlB0III3 (UFLA 01-659), LlB0IV3 (UFLA 01-658) and LlE10I1 (UFLA 01-657)], one from *M. atropurpureum* [MaA0III2 (UFLA 04-121)], and two from *P. vulgaris* [PvB0IV2 (UFLA 02-129) and PvB0III2 (UFLA 02-128)]. The protein profile of *S. virgata* isolate SvB0II2 (UFLA 01-656) was very similar to that of strain BR 5401^T from *A. doebereinerae*, which was confirmed by its 16S rDNA sequencing.

The isolates of *L. leucocephala*, *P. vulgaris*, and *M. atropurpureum* had protein profiles distinct from those of isolates of *S. virgata* and of strain BR 5401^T from *A. doebereinerae*. According to the 16S rDNA sequencing, these isolates belong to genus *Cupriavidus*. This genus belongs to β-Proteobacteria and it was isolated from *Mimosa* species by Chen et al (2001). The species was first described as *Ralstonia taiwanensis* sp. nov. and later, it was moved to the genus *Cupriavidus* (Vandamme & Coenye, 2004). The genus *Cupriavidus* was previously reported to establish symbiosis only with *Mimosa* spp. (Chen et al., 2001; Chen et al., 2005a,b; Barrett & Parker, 2006). The cultural characteristics of the isolates are not reported in any of these works describing the *Cupriavidus* genus. However, our data based on phenotypic and genetic analysis indicate that *Cupriavidus* is able to establish symbiosis with Papilionoideae species.

CONCLUSIONS

Sesbania virgata favors the occurrence of its microsymbiont: *Azorhizobium doebereinerae* near its root system. However, *S. virgata* does not inhibit the occurrence of a high diversity of another nitrogen-fixing Leguminosae nodulating bacteria, which occur as saprophytes, as they are not able to nodulate this species.

Although *A. doebereineare* is able to nodulate some promiscuous legume species under axenic conditions, it is not able to compete with another nitrogen fixing Leguminosae nodulating bacteria in the soil, as it was not trapped by these hosts when tested from soil samples, which corroborate the high specificity of its symbiosis with *S. virgata*.

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