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Specificity of the Legume *Sesbania virgata* (Caz.) Pers. and its Nodule Isolates *Azorhizobium johannae* with other Legume Hosts and Rhizobia. I.

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Abstract

Symbiotic properties of rhizobia are important characteristics both to describe new genera and species of these bacteria and to select inoculant strains. To verify the specificity of *Azorhizobium johannae* strains and *Sesbania virgata*, with currently known rhizobia species and their original hosts, three greenhouse experiments were carried out in Leonard jars. In the first experiment, seven *A. johannae* strains (BR 5401, UFLA 01-602, UFLA 01-54B, BR 5414, BR 5416, BR 5420, BR 5426) were tested separately, with ten legume species. These strains nodulated efficiently only *Sesbania virgata*. Although nodulation did occur with *Sesbania rostrata*, *Phaseolus vulgaris* and *Macroptilium atropurpureum*, it was inefficient. Nodulation was not observed with *Acacia mangium*, *Glycine max*, *Leucaena leucocephala*, *Lupinus* sp., *Medicago sativa* or *Vigna unguiculata*. In the second experiment, eight type or reference strains of known rhizobia species (ORS 571^T, USDA 205^T, NZP 5549^T, CIAT 899^T, UFLA 04-74B, NZP 2213^T, CFN 42^T and BR 2001) were tested with *S. virgata*. Only the strains ORS 571^T (*Azorhizobium caulinodans*) and BR 5401 (*A. johannae*) were able to nodulate *S. virgata*. In the last experiment seven strains of *A. johannae* (UFLA 01-54B, UFLA 01-602, BR 5401, BR 5414, BR 5416, BR 5420,

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BR 5426) were tested for ability to nodulate stems of *S. rostrata*. Only strain ORS 571^T, originally isolated from *S. rostrata*, was able to nodulate this species. The specificity of *A. johannae* and *S. virgata* symbiosis supports the proposition that *S. virgata* isolates as a new species of *Azorhizobium*.

Keywords: Wood legume species, Leguminosae, biological nitrogen fixation

1. Introduction

Among N_2-fixing systems, the legume rhizobia symbiosis is the most important because of its widespread occurrence, mainly with important economic plant species, and due to the efficiency of the process in relation to the other systems. Rhizobia are Gram-negative bacteria that are able to form differentiated structures called nodules, most commonly found on roots of legumes, and less frequently on stems. Although the ability to establish symbiosis with rhizobia has been known for only 25% of legumes, it is estimated that 88% of around 20,000 species of Leguminosae are able to establish symbioses. Knowledge about general characteristics of symbiotic bacteria is still sparse and focused largely on the grain legumes.

A significant number of the Leguminosae species are tropical trees, which have been intensively investigated in Brazil for rhizobia symbiosis over the last 20 years (Magalhães et al., 1982; Faria et al., 1989; Moreira et al., 1992, 1993, 1998). From these surveys many tropical species were shown to establish rhizobia symbiosis, while few did not. A high diversity was also demonstrated in the microsymbionts that belong to all known genera.

Sesbania virgata (Caz.) Pers. (syn. *S. marginata* Benthan) is a fast growing shrub about 2–4 m high, well adapted to flooded conditions that, produces a lot of seeds. Because of these characteristics, it has been used for revegetation of riparian forests, soil erosion control and rehabilitation of degraded areas, firewood and charcoal production. A beverage similar to coffee can be prepared with the seeds. It occurs in central, southeast and south Brazil, Argentina, Uruguay and Paraguay (Pott and Pott, 1994).

S. virgata (syn. *S. marginata*) microsymbionts Sm1 (BR 5401) and Sm5 (BR 5404) (Campêlo, 1976) were compared to 169 strains isolated from other forest species and to known rhizobia species by SDS-PAGE of total proteins (Moreira et al., 1993). These strains formed a separate group without significant similarity to the other strains. Their cultural characteristics on YMA were fast to intermediate growth, alkaline reaction and little gum production, similar to those of *Azorhizobium caulinodans*. Additional strains were isolated from *S. virgata* growing at diverse sites in Brazil with similar cultural characteristics

(Barberi et al., 1998; Faria, unpublished). Partial 16 S rRNA sequence of BR 5401 revealed that it was most similar to *Azorhizobium caulinodans* ORS 571 (Moreira et al., 1998). The highest affinity of strains isolated from *S. virgata* and significant differences with them and ORS 571^T led to the proposition of a new species *Azorhizobium johannense* (Moreira et al., 2000). Subsequently, it was suggested that *A. johannae* is a more appropriated epithet (P.W. Young, personal communication).

Graham et al. (1991) proposed minimal standards for the proposition of new rhizobia species. Among morphologic, genetic, physiological and biochemical traits, it was also recommended that the symbiotic characteristics should be considered. However, recent proposals of new rhizobia species emphasized genetic characteristics and the symbiotic characteristics have not been reported in detail. These traits are important not only for laboratories with limited resources but also from the practical point of view of selecting and recommending strains for inoculant production.

The objective of this work was to verify the symbiotic relationships of *Azorhizobium johannae* and *Sesbania virgata* with other important partners, i.e., type or reference strains of known rhizobia species and the original hosts of these strains.

2. Material and Methods

Three experiments were carried out, using completely randomized designs, in Leonard jars (Vincent, 1970) at the Soil Science Department greenhouse of the Federal University of Lavras, Minas Gerais, Brazil.

Leonard jars: The upper part of the jars contained, a 1:1 mixture of sand and vermiculite. The lower part was filled with 300 ml of Jensen's nutrient solution with the following composition in g l^{-1} distilled water: K_2HPO_4 0.2; $MgSO_4.7H_2O$ 0.2; NaCl 0.2; $CaHPO_4$ 1.0; $FeCl_3.6H_2O$ 0.1; H_3BO_3 0.00286; $MnSO_4.4H_2O$ 0.00203; $ZnSO_4.7H_2O$; 0.0022; $CuSO_4.5H_20$ 0.00008; $Na_2MoO_4H_2O$ 0.00009; $CoCl_2$ 0.00003; pH 6.7 (Vincent, 1970). Jars with substrate and nutrient solution were autoclaved for two hours at 1.5 kg/cm^2 and 127°C.

Preparation and planting of seeds: For some species, seed dormancy was broken by a treatment with concentrated H_2SO_4 followed by successive washings with sterile water. Time of exposure to H_2SO_4 varied according to the species: 30 minutes for *S. virgata*, *S. rostrata* and *L. leucocephala*, 25 minutes for *A. mangium*, and 4 minutes for *M. atropurpureum*. All species seeds were superficially disinfected with ethanol 95% for 5 minutes, sodium hypochlorite for 2 minutes, and washed thoroughly with sterile water.

Seeds were planted 1 cm below the surface of the growth substrate,

inoculated with rhizobia strains and covered with a sterile mixture of sand, benzene and paraffin (5:1:0.015 m/m/m) to avoid contamination. Six seeds were planted per jar. Five to nine days after germination, the plants were reduced to two plants.

Inocula preparation: All rhizobia strains were streaked separately on YMA (Vincent, 1970), and isolated colonies were transferred to semi-solid (1.75 g agar l^{-1}) YM medium. When cultures reached exponential phase, i.e., after three to four days for fast growers, five to six days for intermediate growers and seven to eight days for slow growers, 1 ml of these cultures were inoculated per plant. Three replicates were performed for each strain and treatment means were compared by Duncan's test at 5% probability.

Azorhizobium johannae strains tested were isolated from plants growing in the Municipalities of Seropédica/RJ (BR 5401), Campo Grande/RJ (BR 5426), Volta Redonda/RJ (BR 5420); Itaguaí/RJ (BR 5416), Paracambi/RJ (BR 5414), Lavras/MG (UFLA01-602) and Itutinga/MG (UFLA 01-54B).

In the first experiment, carried out in March 1998, nodulation capability and efficiency of seven *A. johannae* strains (UFLA 01-602; UFLA 01-54B; BR 5401; BR 5414; BR 5416; BR 5420; BR 5426) were tested with 10 leguminous species, all original hosts of known rhizobia species. These strains were also compared to three control treatments: 1) recommended rhizobium strain as inoculant to each plant species; 2) 280 mg l^{-1} NH_4NO_3; 3) absolute control: without mineral nitrogen or rhizobia. Plant species tested, and their recommended inoculant strains, were: *Sesbania virgata* and BR 5401^T (*Azorhizobium johannae*), *Sesbania rostrata* and ORS 571^T (*Azorhizobium caulinodans*), *Macroptilium atropurpureum* and BR 1010 (*Bradyrhizobium* sp.), *Leucaena leucocephala* and BR 827 (*Sinorhizobium* sp.), *Vigna unguiculata* and BR 2001 (*Bradyrhizobium* sp.), *Glycine max* and BR 29 (*Bradyrhizobium elkanii*), *Medicago sativa* and UFLA04-74B (*Sinorhizobium* sp.), *Phaseolus vulgaris* and CIAT 899^T (*Rhizobium tropici*), *Lupinus albus* and BR 29 (*Bradyrhizobium elkanii*), *Acacia mangium* and BR 3617 (*Bradyrhizobium* sp.). Sixty five days after sowing, plants were analyzed for shoot and nodule dry weight, nodule number, and morphology.

In the second experiment, carried out in September 1999, nodulation capability and efficiency of type and reference strains of known rhizobia species were assayed with *Sesbania virgata*. Treatments were the strains: ORS 571^T (*A. caulinodans*), NZP 5549^T (*B. japonicum*), BR 2001 (*Bradyrhizobium* sp.), USDA 205^T (*Sinorhizobium fredii*), CIAT 899^T (*Rhizobium tropici*), CNF42^T (*R. etli*), UFLA 04-74B (*Sinorhizobium* sp.), NZP 2213^T (*Mesorhizobium loti*), BR 5401^T (*A. johannae*), besides two controls without inoculation: with and without mineral nitrogen supplied as in the first experiment. Plants were harvested 70 days after planting and analyzed for shoot and nodule dry weight, nodule number, and morphology.

In the third experiment, also carried out in September 1999, seven *Azorhizobium johannae* strains (UFLA 01-602; UFLA01-54B; BR 5401; BR 5414; BR 5416; BR 5420; BR 5426) were tested for stem nodulation capability with *Sesbania rostrata*. Plants stem-inoculated with ORS 571^T were used as controls. These plants were cultivated in a separate greenhouse to avoid contamination of the tested treatments. Plants were analyzed for the presence of stem nodules 60 days after planting.

3. Results and Discussion

In all experiments, no nodules were formed on plants in control treatments with or without nitrogen, thus indicating the absence of contamination (Tables 1 and 3). In all treatments, isolation of bacteria from selected nodules followed by confirmation of the original inoculant phenotype was performed.

In the first experiment, *Azorhizobium johannae* strains were found to nodulate only *S. virgata* efficiently. They also nodulated *Sesbania rostrata*, *Phaseolus vulgaris* and *Macroptilium atropurpureum*, but with considerably lower inefficiency (Tables 1 and 2). Nodulation of *S. rostrata* by *A. johannae* may be due to the similarity with their original host. Nodulation of the other hosts confirm their well known promiscuity. Nodules in *S. virgata* were the indeterminate type, red inside, indicating the presence of leghaemoglobin. In other nodulated hosts they were of the determined type (spherical), weakly red or white inside indicating low efficiency. Only recommended strains induced dry shoot matter weight in some species equal to that of the mineral nitrogen treatment (*M. atropurpureum*, *P. vulgaris*, *S. rostrata*, *S. virgata*). In the other species dry shoot matter weight was below that of mineral nitrogen treatment, but it was still considered an efficient symbiosis (Table 2). Among all symbioses tested that of *A. caulinodans* ORS 571 and *S. rostrata* had the highest efficiency in nitrogen fixation (132% shoot dry matter in relation to the control receiving mineral nitrogen). No statistical differences were detected among *A. johannae* strain BR 5401, recommended as inoculant for *S. virgata* (Faria and Guedes, 1999), and the other strains isolated from this species, regarding their efficiency.

In the second experiment only BR 5401 nodulated *S. virgata* efficiently (Table 3). BR 5401 induced weight similar to that of mineral nitrogen treatment confirming the results of the first experiment. *S. virgata* was also nodulated by ORS 571 (*A. caulinodans*), however with a low efficiency. In other experiments carried out in plastic pouches or agar slants with sterile nutrient solution, ORS 571 was not able to complete nodule formation in *S. virgata*, although pseudonodules were observed. When tested for nodulation, in 12 other *Sesbania* spp., *A. caulinodans* produced only ineffective nodules (Boivin et al., 1997).

Table 1. Nodule number and weight (between brackets) in ten plant species inoculated with different *Azorhizobium johannae* strains and with their respective strains recommended as inoculants. Experiment in Leonard jars with nutrient solution. Mean of three replicates*.

Strains	Nodule number no. plant^{-1} (nodule weight g plant^{-1})									
	A. mangium	*G. max*	*L. leuco.*	*Lupinus* sp.	*M. atropurp.*	*M. sativa*	*P. vulgaris*	*S. rostr.*	*S. virgata*	*V. unguic.*
A. caulinodans										
ORS 571**	0	0	0	0	10C (4.2C)	0	28.5C (6.3C)	30A (33.9A)	13.5AB (25.2B)	0
A. johannae										
BR 5401**	0	0	0	0	12BC (5.3BC)	0	170.5BC (40.9BC)	8.5B (9.1B)	20A (42.7A)	0
BR 5420	0	0	0	0	16B (7.4BC)	0	164.5BC (37.9BC)	6.5BC (7.7B)	15.5AB (31.4B)	0
UFLA 01-602	0	0	0	0	13.5BC (6.4BC)	0	100.5BC (23.6BC)	5BC (6.1B)	19AB (35.4B)	0
UFLA 01-54B	0	0	0	0	15BC (7.5BC)	0	55BC (12.2BC)	7.5BC (5.4B)	17AB (35.1B)	0
BR 5416	0	0	0	0	12.5C (4.9BC)	0	94.5BC (15.3BC)	4.5BC (4.8B)	15AB (37.6B)	0
BR 5414	0	0	0	0	12.5BC (5.4BC)	0	220AB (60.6AB)	5.5BC (4.4B)	18AB (36.9B)	0
BR 5426	0	0	0	0	15.5BC (6.8BC)	0	170.6BC (40.2BC)	6BC (6.4B)	13AB (27.6B)	0
Inoculant**	12.5A BR 3617	95.2A BR 29	14A BR 827	21.8A BR 29	21.5A (9.2A) BR 1010	75 A(217A) UFLA 474B	355A(85.1A) CIAT 899	–	–	29.2A(7.9A) BR 2001

*Means with the same letter in columns do not differ by Duncan's test at 5%.**Strain recommended as inoculant. ***No mineral nitrogen and no inoculation.

Table 2. Dry shoot matter in ten plant species inoculated with different *Azorhizobium johannae* strains and their respective strains recommended as inoculants. Experiment in Leonard jars with nutrient solution. Mean of three replicates*.

Strains	Shoot dry matter (g plant^{-1})									
	A. mangium	*G. max*	*L. leuco.*	*Lupinus* sp.	*M. atropurp.*	*M. sativa*	*P. vulgaris*	*S. rostr.*	*S. virgata*	*V. unguic.*
A. caulinodans										
ORS 571**	0.06C	0.47C	0.16C	0.69E	0.47B	0.11D	0.36B	6.2A	1.20BCD	0.25C
A. johannae										
BR 5401**	0.07C	0.59C	0.15C	1.11C	0.67B	0.20C	0.64B	0.49C	2.25A	0.33C
BR 5420	0.07C	0.56C	0.20C	1.04C	0.35B	0.12D	0.67B	0.06C	2.07AB	0.22CD
UFLA 01-602	0.08C	0.45C	0.15C	0.87D	0.70B	0.12D	1.12AB	0.06C	1.75ABC	0.22CD
UFLA 01-54B	0.06C	0.53C	0.17C	0.72E	0.42B	0.11D	0.57B	0.45C	1.69ABC	0.20CD
BR 5416	0.07C	0.55C	0.18C	1.05C	0.86B	0.18C	0.45B	0.06C	1.76ABC	0.29C
BR 5414	0.06C	0.45C	0.16C	0.61D	0.62B	0.17C	1.24AB	0.05C	1.49ABC	0.19CD
BR 5426	0.06C	0.43C	0.15C	1.03C	0.93B	0.11D	1.19AB	0.45C	1.75ABC	0.22CD
Inoculant**	0.7B BR 3617	3.32B BR 29	1.84B BR 827	1.27B BR 29	1.88A BR 1010	0.60B BR 474B	1.80A CIAT 899	–	–	2.03B BR 2001
N (260 mg l^{-1})	0.85A	4.01A	2.10A	1.54A	2.33A	0.73A	2.15A	4.7B	2.4A	2.88A
Control***	0.06C	0.40C	0.13C	0.55F	0.25B	0.12D	0.30B	0.04C	0.47D	0.08D

*Means with the same letter in columns do not differ by Duncan's test at 5%. **Strain recommended as inoculant. ***No mineral nitrogen and no inoculation.

Table 3. Nodule numbers and weight and dry shoot weight of *S. virgata* inoculated with its homologous strains and type strains of known rhizobia species. Experiment in Leonard jars with nutrient solution. Mean of three replicates*.

Strains	*Sesbania virgata* Nodule number (no. plant $^{-1}$*)	Nodule weight (g plant $^{-1}$*)	Dry shoot weight (g plant^{-1}*)
BR 5401^{T}** *A. johannae*	91.9 A	0.55 A	2.85 A
ORS 571^{T} *A. caulinodans*	55.5 B	0.25 B	1.66 B
NZP 5549^{T} *B. japonicum*	0	0	0.20 D
BR 2001 *Bradyrhizobium* sp.	0	0	0.20 D
USDA 205^{T} *S. fredii*	0	0	0.19 D
CIAT 899^{T} *R. tropici*	0	0	0.42 CD
CFN 42^{T} *R. etli*	0	0	0.24 CD
UFLA 04-74 B *Sinorhizobium* sp.	0	0	0.18 D
NZP 2213^{T} *M. loti*	0	0	0.33 CD
Mineral N (280 ppm)	0	0	3.37 A
Control***	0	0	0.18 D

*Means with the same letter in columns do not differ by Duncan's test at 5 %.**^{T} = Type strains. *** No mineral nitrogen and no inoculation.

Nodulation of *S. virgata* by *A. caulinodans* can be explained by the phylogenetic similarity of *A. johannae* with this species which may be extended to symbiotic genes. Allen and Allen (1981) reported inefficient nodulation of beans and cowpeas by 39 strains isolated from 6 *Sesbania* species without reciprocal nodulation, which agrees with the results found in this study.

In the third experiment, none of the *Azorhizobium johannae* strains nodulated stems of *S. rostrata*. This species was nodulated only by ORS 571. Pseudonodules were induced by *A. johannae* on the stems of *S. rostrata*. Rinaudo et al. (1991) found strains isolated from *S. rostrata* root nodules with quite different DNA:DNA homology, that could be proposed as new *Azorhizobium* spp. However, all of them were able to induce stem nodules in *S. rostrata*.

Specificity of *Sesbania* spp. has been pointed out before (Allen and Allen, 1981; Turk and Keyser, 1992; Bala and Giller, 2001). Microsymbionts of species belonging to this genus were identified as *Azorhizobium*, *Rhizobium*, *Mesorhizobium* or *Sinorhizobium* species (Dreyfus et al., 1988; Chen et al., 1991; Jarvis et al., 1997; De Lajudie et al., 1994; Boivin et al., 1997). In the case of *S. virgata* and *A. johannae* specificity seems to be more pronounced. Among 19 rhizobia strains only the homologous strain Sm1 (BR 5401) nodulated *S. virgata*, and this strain was also able to nodulate *Acacia molissima* and

Table 4. Specificity among type and reference strains of known rhizobia species and some leguminous species.

Strain/Host	Legume species									
	Mimosoideae						Papilionoideae			
	A. mangium	*L. leuco.*	*G. max*	*Lupinus* sp.	*M. atropurp.*	*M. sativa*	*P. vulgaris*	*S. rostr.*	*S. virgata*	*V. unguic.*
BR 3617 / *A. mangium*	E	–	–	–	–	–	–	–	NN	–
BR 29 / *G. max*	–	–	E	E	–	–	–	–	–	–
BR 827 / *L. leucocephala*	–	E	–	–	–	–	–	–	NN	–
BR 1010 / *M. atropurpureum*	–	–	–	–	E	–	–	–	NN	–
UFLA 04-74B / *M. sativa*	–	–	–	–	–	E	–	–	NN	–
CIAT 899 / *P. vulgaris*	–	–	–	–	–	–	E	–	NN	–
ORS 571 / *S. rostrata*	NN	NN	NN	NN	e	NN	e	E	e	NN
BR 5401 / *S. virgata*	NN	NN	NN	NN	e	NN	e	e	E	NN
BR 2001 / *C. juncea*	–	–	–	–	–	–	NN	–	NN	E

NN = no nodules; E = nodules, efficiency >70% (plant weight:strain/N-mineral); e = nodules, efficiency <70%; – not tested.

Erythrina speciosa, but failed to nodulate nine other tree species (Papilionoideae and Mimosoideae) on a cross inoculation study (Campêlo, 1976). Veasey et al. (1997) found *S. virgata* and *S. punicea* were the only ones, among 13 shrub and tree species including *S. exasperata*, *S. sesban* and *S. tetraptera*, that did not nodulate with the native rhizobia population of a red-yellow latossol. Moreira (1991) characterized 598 strains isolated from 49 woody genera from diverse natural ecosystems. Strains with cultural characteristics similar to *Azorhizobium* were only found among those isolated from *S. virgata*, which, in turn has been found naturally nodulated by *A. johannae* only. In this work, under axenic conditions *A. johannae* was found to nodulate siratro and beans (Table 4), suggesting that specificity is greater under natural soil conditions.

4. Conclusions

Azorhizobium johannae efficiently nodulates *S. virgata* only. While it is capable of nodulating other hosts such as *Macroptilium atropurpureum*, *Phaseolus vulgaris* and *S. rostrata*, the efficiency was substantially reduced compared to *S. virgata*. *A. johannae* was not able to form stem nodules with *S. rostrata*. Strains belonging to *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* do not nodulate *S. virgata*.

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