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BASIC AREAS
Article

New strains of *Bradyrhizobium* increase nitrogen accumulation in velvet bean biomass

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ABSTRACT: The use of *Stizolobium aterrimum* (velvet bean) as green manure has been encouraged in agriculture as a source of nitrogen-rich phytomass for incorporation into the soil to enhance subsequent crop yield. The nitrogen-fixing capacity of this legume can be optimized through symbiosis with selected strains of rhizobia. The aim of this study was to evaluate the effectiveness of new strains of *Bradyrhizobium* in promoting the growth of velvet bean plants, both in potted soil and in the field. The study involved three experiments (two in pots and one in the field) with four replications of each in an Oxisol. An additional experiment was carried out to estimate the native rhizobial community in the experimental soil. The efficiency of the strains was estimated by compared plant growth with them to plant growth in two non-inoculated controls (one without mineral N and the other with mineral N) and plant growth with the strain BR2811, which is approved as an inoculant for velvet bean plants. Evaluations of velvet bean were performed at 60 and 100 days after emergence in non-sterile soil pots, and at 170 days after sowing (at flowering) in the field. Native rhizobia are efficient in symbiosis with velvet bean, as they were similar to BR2811 and to the control with mineral N, but they had lower biological nitrogen fixation efficiency than the new strains tested–UFLA05-18 and UFLA05-20. The new strains increased velvet bean yield and accumulation of N in velvet bean phytomass and, therefore, have potential for being used as inoculants for this species.

Key words: *Stizolobium aterrimum*, biological nitrogen fixation, rhizobia, strain selection.

INTRODUCTION

Velvet bean (*Stizolobium aterrimum*) Piper & Tracy [syn *Mucuna pruriens* var. *utilis* (Wall. ex Wight) Baker ex Burck], also considered the queen of legumes, is a species of the Leguminosae family, originating in Africa, with a cycle that can vary from 140 to 180 days (Wutke et al. 2014). This annual or biennial legume requires a warm climate and tolerates low fertility. Furthermore, it is of great importance as a green manure (Borkert et al. 2003, Andrade Neto et al. 2010, Queiroz et al. 2010, Ambrosano et al. 2011, Matos et al. 2011, Tabaldi et al. 2012, Ambrosano et al. 2013) due to its biomass yield of up to 9 $t \cdot ha^{-1}$ and nitrogen fixation of up to 157 kg $N \cdot ha^{-1} year^{-1}$ (Wutke et al. 2014, Santos et al. 2017).

The use of legumes efficient in biological nitrogen fixation (BNF) as green manure can naturally improve soil fertility, reduce dependence on chemical fertilizers, and promote more sustainable agriculture. Given such advantages, the use of velvet bean under tropical conditions has been encouraged (Andrade Neto et al. 2010, Queiroz et al. 2010, Ambrosano et al. 2011, Ambrosano et al. 2013, Zaccheo et al. 2016) as beneficial to subsequent crops, such as maize (Pache Filho et al. 2014, Barros et al. 2020).

Due to the importance of BNF in enhancing nitrogen input into the soil-plant system of velvet bean, the process of selecting rhizobia strains efficient in fixing N_2 becomes essential. This is because native populations of soil rhizobia are

not always able to establish an efficient mutualistic symbiosis with the cultivated host. This particularly applies (although not always) to exotic/non-native introduced legumes. So far, BR2811 (SEMIA6158), isolated from *Crotalaria spectabilis*, belonging to *Bradyrhizobium elkanii*, is the only strain approved as an inoculant for velvet bean by the Brazilian Ministry of Agriculture (MAPA) (Brasil 2011). However, research results have indicated the nitrogen-fixing potential of other strains for this legume as well (Rangel et al. 2017, Costa et al. 2020). This includes strains UFLA05-18, UFLA05-19, and UFLA05-20 isolated from velvet bean growing in an arsenic-contaminated gold mine, whose efficiency surpassed that of the BR2811 strain and mineral N in experimentation in Leonard pots containing sterilized substrate and nutrient solution (Rangel et al. 2017). Considering these results, it is important to validate these strains in pots with soil and under field conditions to confirm their recommendation as inoculants for velvet bean.

Therefore, the objective of this study was to evaluate the symbiotic efficiency of *Bradyrhizobium* strains in velvet bean cultivated in an Oxisol, in pots with soil and in the field.

MATERIALS AND METHODS

Three trials were carried out to determine the symbiotic efficiency of strains of the *Bradyrhizobium* genus on velvet bean cv. comum in a Oxisol (two in pots and one in the field) from September 2017 to June 2018. A prior experiment was carried out in recycled bottles with nutrient solution to determine the number of native rhizobial populations in the Oxisol that were able to nodulate velvet bean cv. comum.

The soil in the pots of the experiments conducted in a greenhouse was collected from the same area where the field experiment was set up. The chemical and physical characteristics of the soil are shown in Table 1. The land use sequence of the experimental area was as follows: cereal crop cultivation, fallow, common bean cultivation without technology or inoculation, compaction, conventional soil tillage to implement this experiment.

Table 1. Chemical and physical characteristics and textural class of soil samples collected at a depth of 0 to 20 cm, before implementing the experiments in pots with soil and in the field.

Characteristic*	Unit	Oxisol	Interpretation**
pH H_2O (1:2.5)	-	6.0	M
P (Mehlich I)	$mg \cdot dm^{-3}$	28.51	VG
K (Mehlich I)	$mg \cdot dm^{-3}$	136.85	VG
Ca	$cmol_c \cdot dm^{-3}$	2.38	M
Mg	$cmol_c \cdot dm^{-3}$	0.92	G
Al	$cmol_c \cdot dm^{-3}$	0.06	VL
H + Al	$cmol_c \cdot dm^{-3}$	2.45	L
SB	$cmol_c \cdot dm^{-3}$	3.65	G
T	$cmol_c \cdot dm^{-3}$	6.10	M
t	$cmol_c \cdot dm^{-3}$	3.71	M
m	%	1.62	VL
V	%	59.82	M
Organic matter	$dag \cdot kg^{-1}$	1.88	M
Sand	$g \cdot kg^{-1}$	520	CS
Silt	$g \cdot kg^{-1}$	100	CS
Clay	$g \cdot kg^{-1}$	380	CS
Texture Class	Clayey	Clayey	
Geographic coordinates	21°14'45" S, 44°59'59" W		
Altitude	920 m		
Climate (Köppen)	Cwa (humid subtropical)		

*pH in water (v/v 1:2.5); P and K: Mehlich-1 extractant; Ca, Mg, Al: 1 $mol \cdot L^{-1}$ KCl extractant; H + Al: potential acidity, extracted by 0.5 $mol \cdot L^{-1}$ calcium acetate at pH 7; SB: sum of bases; T: cation exchange capacity at pH 7; t: cation exchange capacity; m: aluminum saturation; V: base saturation; organic matter, Walkley Black method; sand, silt, and clay: pipette method; **M: medium; VG: very good; G: good; CS: clayey soil; L: low, VL: very low. Source: Alvarez et al. (1999).

The three trials were conducted simultaneously. In the two greenhouse trials, strains isolated from velvet bean (UFLA05-18, UFLA05-19, UFLA05-20) and the strain BR2811 (SEMIA6158) were tested. In the field trial, these strains were used as well as the strains INPA104 A, UFLA04-212, and UFLA03-144 from the *Bradyrhizobium* genus, which had shown efficiency in previous studies in velvet bean (*Stizolobium aterrimum*), cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*), and/or fava (*Phaseolus lunatus*) (Table 2). Inoculants for the pot experiments were prepared in liquid 79 medium (Fred and Waksman 1928) at 28°C, with shaking at 110 rpm for 96 hours. For the field experiment, the inoculum consisted of a culture mixture of liquid 79 medium and autoclaved peat at a 2:3 (v:w) ratio. The quality of the inoculants was monitored by counting the number of colony forming units (CFU) on solid 79 culture medium, respecting the minimum legal requirement of 10^9 CFU per mL or g of inoculant.

Table 2. Strains used in experiments in pots with non-sterile soil and in the field.

Original code	Origin (host)	Symbiotic N_2 fixation efficiency in axenic conditions	Plant growth-promoting traits	Antibiotic resistance	As, Cd, Zn resistance[4]	Identification	References
BR2811 (SEMIA6158)[1,2,3]	*Crotalaria spectabilis*	*Crotalaria spectabilis and Stizolobium aterrimum*	-	OXA, VAN		*Bradyrhizobium elkanii*	Menna et al. (2006), Rangel et al. (2017), Costa et al. (2020)
UFLA05-18[2,3]	*Stizolobium aterrimum*	*Stizolobium aterrimum*	-	AMP, CEFT, CFD, OXA, VAN	Cd	*Bradyrhizobium sp.*	Rangel et al. (2017)
UFLA05-19[2,3]	*Stizolobium aterrimum*	*Stizolobium aterrimum*	IAA, SID	-		*Bradyrhizobium elkani*	Rangel et al. (2017)
UFLA05-20[2,3]	*Stizolobium aterrimum*	*Stizolobium aterrimum*	IAA, SID, $Ca_3(PO_4)_2$ solubilization	CEFT, CFD, VAN		*Bradyrhizobium sp.*	Rangel et al. (2017)
INPA104 A[3]	*Calliandra surinamensis*	*Macroptilium atropurpureum Phaseolus lunatus Stizolobium aterrimum*		AMP, CIP, CLO, CRX, DOX, ERI, GEN, NEO, PEN		*Bradyrhizobium sp.*	Moreira et al. (1993), Guimarães et al. (2015), Costa et al. (2017, 2020)
UFLA04-212[3]	*Macroptilium atropurpureum*	*Macroptilium atropurpureum*		GEN, NEO	-	*Bradyrhizobium brasiliense*	Florentino et al. (2009), Costa et al. (2017), Costa et al. (2020)
UFLA03-144[3]	*Vigna unguiculata*	*Vigna unguiculata Phaseolus lunatus Stizolobium aterrimum*				*Bradyrhizobium viridifuturi*	Guimarães et al. (2012), Costa et al. (2017, 2019)

[1]Strain that was approved by the Brazilian Ministry of Agriculture as an inoculant for *S. aterrimum*; [2]strains used in experiment in pots with non-sterile soil; [3]strains used in the field; IAA: indole-3-acetic acid production; SID: siderophore production. Based on the $Ca_3(PO_4)_2$ solubilization index; AMP: Ampicillin; CFD: Cefadroxil; CEFT: Ceftriaxone; CIP: Ciprofloxacin; CLO: Chloramphenicol; CRX: Cefuroxime; DOX: Doxycycline; ERI: Erythromycin; OXA: Oxacillin; PEN: Penicillin; VAN: Vancomycin; GEN: Gentamycin; NEO: Neomycin; [4]Arsenio, cadmium, and zinc resistance.

Most probable number of soil rhizobia

The most probable number (MPN) method (Vincent 1970) was used to estimate the density of the soil native rhizobial community capable of nodulating velvet bean. The velvet bean cv. comum used in the pot and field experiments was used

as the bait plant. Before setting up the field experiment, soil samples were collected from five points in the experimental area at the 0–20 cm soil depth, composing a single sample that underwent serial decimal dilutions.

The experiment was conducted in a greenhouse at the Soil Science Department at the Universidade Federal de Lavras using a completely randomized design with three replications. Recycled dark amber longneck bottles with a 500-mL capacity were used, containing Hoagland and Arnon's (1950) sterile nutrient solution for plant growth. Before sowing, velvet bean seeds were scarified in 98% sulfuric acid for 15 minutes, washed six successive times in sterilized distilled water, and soaked for 20 minutes in the last wash water. Subsequently, the seeds were pre-germinated in a sterile Petri dish containing moistened cotton and then incubated for two days at the temperature of 28°C. During sowing, the pre-germinated seeds were inoculated with 1 mL of soil suspensions from serial dilutions ranging from 10^{-1} to 10^{-6} CFU. A positive control with the BR2811 strain was included, along with two negative controls without inoculation (one with 5.25 $g \cdot L^{-1}$ and another with 52.5 $g \cdot L^{-1}$ of mineral N).

The Hoagland solution used in all the inoculated treatments and in the control with low mineral N concentration (5.25 $mg \cdot L^{-1}$)/without inoculation contained the following composition: 0.4 mL of 236.16 $g \cdot L^{-1}$ $CaN_2O_6 \cdot 4H_2O$, 0.1 mL of 115.03 $g \cdot L^{-1}$ $NH_4H_2PO_4$, 0.6 mL of 101.11 $g \cdot L^{-1}$ KNO_3, 2 mL of 246.9 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 3 mL of 87.13 $g \cdot L^{-1}$ K_2SO_4, 10 mL of 12.6 $g \cdot L^{-1}$ $CaH_4P_2O_8 \cdot H_2O$, 200 mL of 1.72 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, and 1 mL of 10 $g \cdot L^{-1}$ $FeCl_3$; as well as 1 mL of micronutrients (2.86 $mg \cdot L^{-1}$ H_3BO_3, 2.03 $mg \cdot L^{-1}$ $MnSO_4 \cdot 4H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, and 0.09 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot H_2O$). The Hoagland solution used in the control with high mineral N concentration (52.5 $mg \cdot L^{-1}$)/without inoculation contained 4 mL of 236.16 $g \cdot L^{-1}$ $CaN_2O_6 \cdot 4H_2O$, 1 mL of 115.03 $g \cdot L^{-1}$ $NH_4H_2PO_4$, 6 mL of 101.11 $g \cdot L^{-1}$ KNO_3, 2 mL of 246.9 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and 1 mL of 10 $g \cdot L^{-1}$ $FeCl_3$; as well as 1 mL of micronutrients.

This trial was conducted at an average temperature of 23.6°C and 73% relative humidity. Plants were harvested 30 days after sowing, and root nodulation was assessed by considering the presence or absence of nodules at each dilution. The density of the rhizobial population (cells $\cdot g^{-1}$ of soil) was estimated according to the McCrady table (Döbereiner et al. 1995).

Symbiotic efficiency of *Bradyrhizobium* strains on velvet bean

Assessment of symbiotic efficiency in pots with non-sterile soil

The two experiments were conducted in a greenhouse from October 2017 to February 2018. Velvet bean was grown in 3-dm^3 pots for 60 days after emergence (DAE) (Experiment 1) and in 5-dm^3 pots for 100 DAE (Experiment 2). The treatments were composed of the strains UFLA05-18, UFLA05-19, and UFLA05-20 in the velvet bean selection phase, as well as BR2811. Two non-inoculated controls (without and with mineral N) were also used. A randomized block design was used, with four replications, for each of the experiments (evaluation period at 60 and 100 DAE).

Fertilization in the pots (300, 200, 200, 50, 50, 0.8, 1.5, 2, 3, 0.10, and 4 $mg \cdot dm^{-3}$ of P, K, Ca, Mg, S, B, Cu, Fe, Mn, Mo, and Zn, respectively) followed the recommendations of Malavolta et al. (1989). The soluble sources used were monobasic calcium phosphate (monohydrate) $Ca(H_2PO_4)_2 \cdot H_2O$, potassium chloride KCl, heptahydrated magnesium sulfate $(MgSO_4) \cdot 7H_2O$, boric acid H_3BO_3, pentahydrated copper sulfate $(CuSO_4) \cdot 5H_2O$, ferric chloride $FeCl_3$, tetrahydrated manganese chloride $(MnCl_2) \cdot 4H_2O$, dihydrated sodium molybdate $(Na_2MoO_4) \cdot 2H_2O$, and heptahydrated zinc sulfate $(ZnSO_4) \cdot 7H_2O$. The control treatment without inoculation and with mineral N also received 300 mg NH_4NO_3 dm^{-3}, divided into three applications: at sowing and at 10 and 20 DAE.

Before sowing, the seeds were scarified in 98% sulfuric acid for 15 minutes, washed six successive times in sterilized distilled water, and soaked for 20 minutes in the sterilized distilled water in the last wash. Four seeds were sown per pot, and plants were thinned five DAE, leaving two seedlings per pot. In strain treatments, each seed received 1 mL of inoculant. The pots were irrigated daily to maintain soil moisture close to field capacity, equivalent to 60% of the total volume of occupied pores. The average minimum temperature recorded during the experiment was 19.4°C, and the average maximum was 31.5°C.

Sampling occurred at 60 (Experiment 1) and 100 DAE (Experiment 2). For each evaluation period, both plants from each pot were harvested, including the four replicates (pots) of each treatment, for determination of number of nodules (NN),

nodule dry matter (NDM), indirect reading of chlorophyll (IRC), root dry matter (RDM), and shoot dry matter (SDM). Shoot nitrogen accumulation (SNA) was determined at the 60 DAE harvest by multiplying the nitrogen content [obtained by the semi-micro Kjeldahl method (Sarruge and Haag 1979)] by SDM. IRC was obtained through readings taken with the Minolta SPAD-502 device on the last fully developed leaf of each plant (15 readings per plant). The nodules, roots, and shoots were dried in a forced-air oven at 60°C until a constant weight to obtain NDM, RDM, and SDM.

Evaluation of the symbiotic efficiency of *Bradyrhizobium* strains in the field

The field experiment was conducted from November 2017 to April 2018 (Experiment 3). A randomized block experimental design was used, with four replications. The inoculated treatments were composed of the six strains in the selection phase (Table 2), in addition to the control inoculated with the strain BR2811 and the uninoculated controls without (W0N) and with (WN) 70 kg of N-urea·ha^{-1}, with half of this N applied at sowing and the other half as topdressing at 20 DAE.

Each plot (10.8 m^2) consisted of four rows, each 4.5-m long, spaced at 0.6-m intervals, and the area used for data collection corresponded to the central rows. Conventional soil tillage involved plowing, harrowing, and furrowing to demarcate rows. None of the plots received base fertilization, as it is not a common practice when growing cover crops in the region.

Seeds were sown manually immediately after seed inoculation, using a density of four seeds per linear meter (Wutke et al. 2014). Rainfall and temperature were recorded over the growing period and can be found in Silva, 2023a. Weeds were controlled through manual weeding and ants through ant baits, whenever necessary. There was no need for control of other pests or diseases.

At velvet bean flowering, 170 days after sowing (DAS), three plants were removed from each plot with the aid of a template measuring 1 m^2 (rows 2 and 3) to determine the NN, NDM, SDM, and SNA. To determine NN, plant roots were harvested with the aid of a shovel, covering the entire volume of soil exploited by the root system. The nodules were carefully detached from their roots, washed, and dried in a forced-air circulation oven. The other methodologies related to plant assessment were those described in the previous session.

Statistical analysis

Analysis of variance was performed on the data obtained after they were first tested for normality (Shapiro-Wilk), homoscedasticity of variances (Breusch-Pagan), and independence (Dubin-Watson) using the R software (R Development Core Team 2019). To meet the assumptions of the analysis of variance, the data for NDM from both harvests in the potted soil experiment and NN from the field experiment were transformed to $(y+0.5)^{0.5}$, and the means of NDM from the latter experiment were first log-transformed log (y). When a significant effect was observed by the F-test [$p < 0.05$ or $p < 0.10$ (Brasil 2011)], the means of the treatments being tested were contrasted with the means of the controls in each experiment using the Dunnett's test, at the same level of significance. The control treatments used for the Dunnett's test were a noninoculated control with mineral N (WN), a noninoculated control without mineral N (W0N), and the strain BR2811 (approved as an inoculant for velvet bean). Furthermore, comparisons were made among the control treatments.

The means of the experimental controls were also contrasted with each other, following the same criteria mentioned above.

RESULTS AND DISCUSSION

Evaluation of the density of the native rhizobial community capable of nodulating velvet bean

The communities of native rhizobia capable of nodulating velvet bean in the Oxisol were approximately 1.1×10^2 cells per gram of soil (determined in axenic conditions). The presence of nodules on plants in the uninoculated controls (W0N

and WN) at 60 and 100 DAE in both pot experiments also indicated the presence of these native rhizobial communities in soils (Tables 3, 4, 5, and 6). Indeed, diverse microbiome studies have shown that *Bradyrhizobium* is a ubiquitous genus throughout the world (Su et al. 2020, Stone et al. 2021). Although native rhizobia are less numerous (1.1×10^2 rhizobial cells per gram of soil) in soils compared to the concentrations of cells in the inoculants, and they have a smaller NN than the inoculated treatments, the native rhizobia also proved to be efficient in BNF in velvet bean. They were especially efficient at 100 DAE in Experiment 2 (Table 6), in which they did not differ from the BR2811 strain and the WN control in any of the variables analyzed. This efficiency is related to the capacity of this community to survive and establish symbiosis, which is likely associated with favorable soil fertility and organic matter conditions (Table 1). Native rhizobial populations, when exceeding 50 cells per gram of soil, can affect response to inoculation (Moreira and Siqueira 2006). Chada and Polli (1988) and Rodrigues et al. (1994) also observed positive results with native rhizobia in velvet bean in a Red-Yellow Podzolic soil in Itaguai in the state of Rio de Janeiro, Brazil.

Symbiotic efficiency of *Bradyrhizobium* strains in velvet bean

Evaluation of symbiotic efficiency in pots with non-sterile soil at 60 and 100 days after emergency

The averages of NN, NDM, RDM, IRC, SDM, and SNA determined at 60 DAE (Experiment 1) are shown in Table 3, and the contrasts between the averages are shown in Table 4. There was no significant difference among the three controls (W0N, WN, and the BR2811 strain) regarding the variables NN, NDM, SDM, and IRC (Table 4). However, the averages of SDM and SNA from the WN control were higher than those of the BR2811 strain and the W0N control, which were similar to each other. In general, the nodules found were large and red inside, indicating active N_2 fixation (Silva, 2023b). Except for NN from the UFLA05-20 strain, which resembled that of BR2811, the NN results from the UFLA-coded strains were higher than those of the controls, including BR2811. There was no significant difference in NDM and RDM among the treatments evaluated. The IRC of treatments inoculated with the UFLA05-18 and UFLA05-20 strains was higher than that of the WN and BR2811 treatments, and the UFLA05-20 reading was also higher than that of the W0N treatment. The SDM averages of the UFLA05-19 and UFLA05-20 strains were similar to those of the W0N and BR2811 treatments, but the averages of these two UFLA strains did not surpass the average of the WN treatment. The SDM of the UFLA05-18 strain was higher than that of BR2811 and similar to the WN and W0N treatments. The UFLA05-18 and UFLA05-20 strains accumulated the same amount of N in the aerial part as the BR2811 and W0N treatments did. UFLA05-19 stood out in terms of SNA, accumulating the same amount of N as the BR2811 and WN treatments did, surpassing the W0N treatment.

Table 3. Mean values for number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), indirect reading of chlorophyll (IRC), shoot dry matter (SDM), and shoot N accumulation (SNA) of *Stizolobium aterrimum* grown in pots with non-sterile soil (greenhouse), at 60 days after emergence.

Treatment	NN (unit·plant^{-1})	NDM (g·plant^{-1})	RDM (g·plant^{-1})	IRC	SDM (g·plant^{-1})	SNA (mg·plant^{-1})
BR2811	34.75	2.00	7.52	40.60	11.80	339.18
WN	7.75	1.18	8.49	40.45	16.80	510.64
W0N	16.63	2.20	9.34	41.69	13.10	267.41
UFLA05-18	108.13	1.15	7.80	46.86	14.96	292.45
UFLA05-19	76.38	2.16	9.84	45.26	13.01	431.74
UFLA05-20	58.38	0.75	8.86	47.16	11.98	348.54
CV (%)	31.42	25.36	20.04	7.58	11.29	14.56

WN: noninoculated control with mineral N (300 mg·dm^{-3} of NH_4NO_3); W0N: noninoculated control without mineral N; CV: coefficient of variance.

Table 4. Contrast of mean values for number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), indirect reading of chlorophyll (IRC), shoot dry matter (SDM), and shoot N accumulation (SNA) of *Stizolobium aterrimum* grown in pots with non-sterile soil (greenhouse), at 60 days after emergence[1].

NN		NDM■		RDM		IRC		SDM		SNA	
BR2811	WN	BR2811	WN	BR2811	WN	BR2811	WN	BR2811	WN**	BR2811	WN**
BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N
WN	W0N	WN	W0N	WN	W0N	WN	W0N	WN**	W0N	WN**	W0N
UFLA0518**	BR2811	UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518**	BR2811	UFLA0518**	BR2811	UFLA0518	BR2811
UFLA0518**	WN	UFLA0518	WN	UFLA0518	WN	UFLA0518**	WN	UFLA0518	WN	UFLA0518	WN**
UFLA0518**	W0N	UFLA0518	W0N	UFLA0518	W0N	UFLA0518	W0N	UFLA0518	W0N	UFLA0518	W0N
UFLA0519**	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811
UFLA0519**	WN	UFLA0519	WN	UFLA0519	WN	UFLA0519	WN	UFLA0519	WN**	UFLA0519	WN
UFLA0519**	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519**	W0N
UFLA0520	BR2811	UFLA0520	BR2811	UFLA0520	BR2811	UFLA0520**	BR2811	UFLA0520	BR2811	UFLA0520	BR2811
UFLA0520**	WN	UFLA0520	WN	UFLA0520	WN	UFLA0520**	WN	UFLA0520	WN**	UFLA0520	WN**
UFLA0520**	W0N	UFLA0520	W0N	UFLA0520	W0N	UFLA0520*	W0N	UFLA0520	W0N	UFLA0520	W0N

[1]The treatments are compared by pairs in the row within each analyzed variable. The presence of asterisks in the treatment represents it is significantly greater than the contrasting treatment (*F*-test [$p < 0.05$ or $p < 0.10$]); ** significant effect at 5% probability; *significant effect at 10% probability; WN: noninoculated control with mineral N (300 mg·dm^{-3} of NH_4NO_3); W0N: noninoculated control without mineral N; ■data transformed into $(y+0.5)^{0.5}$.

The results of the assessments carried out at 100 DAE (Experiment 2) are shown in Tables 5 and 6. The three controls had no significant difference for any of the variables. Similar to the results at 60 DAE, the NDM and RDM of the strains were comparable to those of the controls. The NN and IRC results of the UFLA05-18 strain were similar to those of BR2811 and surpassed those of the WN and W0N treatments. The NN results of the UFLA05-19 and UFLA05-20 strains were notable higher than those of the three controls. The SDM of the UFLA05-19 strain was higher than that of the BR2811 and W0N controls and similar to that of the WN control.

Table 5. Mean values for number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), indirect reading of chlorophyll (IRC), shoot dry matter (SDM), and shoot N accumulation (SNA) of *Stizolobium aterrimum* grown in pots with non-sterile soil (greenhouse), at 100 days after emergence.

Treatment*	NN (unit·plant^{-1})	NDM (g·plant^{-1})	RDM (g·plant^{-1})	IRC	SDM (g·plant^{-1})
BR2811	36.50	4.97	31.38	38.43	26.44
WN	23.00	3.87	37.27	33.55	41.64
W0N	17.88	5.88	25.87	33.65	25.26
UFLA05-18	61.13	4.37	22.42	41.80	42.54
UFLA05-19	76.88	2.66	34.50	39.00	45.72
UFLA05-20	90.25	3.392	36.21	38.08	32.26
CV (%)	30.75	18.27	40.40	10.16	26.76

*UFLA05-18, UFLA05-19, UFLA05-20 and BR2811: strains tested; WN: noninoculated control with mineral N (300 mg·dm^{-3} of NH_4NO_3); W0N: noninoculated control without mineral N; CV: coefficient of variance.

Table 6. Contrast of mean values for number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), indirect reading of chlorophyll (IRC), and shoot dry matter (SDM) of *Stizolobium aterrimum* grown in pots with non-sterile soil (greenhouse), at 100 days after emergence[1].

NN		NDM■		RDM		IRC		SDM	
BR2811	WN	BR2811	WN	BR2811	WN	BR2811	WN	BR2811	WN
BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N
WN	W0N	WN	W0N	WN	W0N	WN	W0N	WN	W0N
UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518	BR2811
UFLA0518**	WN	UFLA0518	WN	UFLA0518	WN	UFLA0518*	WN	UFLA0518	WN
UFLA0518**	W0N	UFLA0518	W0N	UFLA0518	W0N	UFLA0518*	W0N	UFLA0518	W0N
UFLA0519**	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519*	BR2811
UFLA0519**	WN	UFLA0519	WN	UFLA0519	WN	UFLA0519	WN	UFLA0519	WN
UFLA0519**	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519**	W0N
UFLA0520**	BR2811	UFLA0520	BR2811	UFLA0520	BR2811	UFLA0520	BR2811	UFLA0520	BR2811
UFLA0520**	WN	UFLA0520	WN	UFLA0520	WN	UFLA0520	WN	UFLA0520	WN
UFLA0520**	W0N	UFLA0520	W0N	UFLA0520	W0N	UFLA0520	W0N	UFLA0520	W0N

[1]The treatments are compared by pairs in the row within each analyzed variable. The presence of asterisks in the treatment represents it is significantly greater than the contrasting treatment (*F*-test [$p < 0.05$ or $p < 0.10$]); **significant effect at 5% probability; *significant effect at 10% probability; WN: noninoculated control with mineral N (300 mg·dm^{-3} of NH_4NO_3); W0N: noninoculated control without mineral N; ■ Data transformed into $(y+0.5)^{0.5}$.

Regarding the greenhouse results, the experimental controls only differed for SDM and SNA at 60 DAE. At that collection time, the BR2811 strain and the W0N control had similar means, and both were lower than the WN control. This result can be explained by the fact that at 60 DAE there was still mineral N available for plant development. As velvet bean has a long period until flowering, the BR2811 and native rhizobia strains were not yet fully contributing to BNF, hence the superiority of the WN control.

The lack of response of RDM to treatments may be related to the limited size of the pots, impairing root development, since velvet bean has a considerable volume of roots. The limitation imposed by pot size may also have affected the IRC result for the UFLA05-20 strain, which had higher values than the controls at 60 DAE, but not at 100 DAE, in which the strain resembled the controls. In contrast, the UFLA05-18 strain maintained its superiority in IRC in relation to the WN control, both at harvest times and in pot sizes. This strain outperformed native rhizobia results and resembled BR2811 at 100 DAE compared to 60 DAE. These results are important because they demonstrate the efficiency of UFLA05-18 under limiting conditions, such as restricted space for root development.

In the greenhouse, the SDM production of UFLA05-18 surpassed that of BR2811 at 60 DAE, but not at 100 DAE, equaling the other controls tested in the two harvest periods. Just as in the field, UFLA05-18 produced more SDM than the WN and native rhizobia did; the size of the pots may also have affected this variable.

As for SNA, the better results obtained by WN compared to the other treatments, except for UFLA05-19, in the harvest at 60 DAE can be explained by the relatively recent fertilization with mineral N in the pots. Due to technical problems, it was not possible to present the SNA data at 100 DAE, but the UFLA05-18 strain accumulated N, as well as the W0N and BR2811 controls at 60 DAE; and at 100 DAE, it had high IRC values, exceeding the values from native rhizobia, the nitrogen control, and strain BR2811.

Evaluation of the symbiotic efficiency of *Bradyrhizobium* strains in the field

The results of symbiotic efficiency of the strains inoculated on velvet bean at 170 DAS are presented in Tables 7 and 8. There was no significant difference among the three contrasting controls for any of the variables. In general, the nodules found were large and red inside, indicating active N_2 fixation (Kemmelmeier and Silva, 2023). The NN of the UFLA05-20 strain was higher than that of all the controls. The RDM averages of the UFLA05-19, UFLA05-20, and UFLA04-212 strains exceeded that of the WN. SDM production with inoculation of the UFLA05-18 strain was equivalent to BR2811 and superior to the WN and W0N controls. The UFLA05-18 strain also led to nitrogen accumulation in the aerial part (496.19 kg·ha^{-1}) higher than the accumulation in the BR2811, WN, and W0N controls.

Table 7. Mean values of number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), and shoot N accumulation (SNA) of *Stizolobium aterrimum* grown in a conventional planting area, evaluated at 170 days after sowing (at flowering).

Treatment*	NN (unit·plant^{-1})	NDM (mg·plant^{-1})	SDM (t·ha^{-1})	SNA (kg·ha^{-1})
BR2811	5.75	186.16	8.81	314.35
WN	2.41	59.50	7.99	279.61
W0N	6.66	180.16	7.92	203.14
INPA104A	7.66	140.83	7.57	225.31
UFLA03-144	2.33	108.91	5.87	289.96
UFLA04-212	7.25	295.08	6.29	176.31
UFLA05-18	6.50	253.75	11.72	496.19
UFLA05-19	8.41	335.00	8.15	289.49
UFLA05-20	15.16	571.91	11.12	386.36
CV (%)	37.49	18.78	26.13	32.56

*BR2811, INPA104A, UFLA03-144, UFLA04-212, UFLA05-18, UFLA05-19, and UFLA05-20: strains tested; WN: noninoculated control with mineral N (70 kg·N-urea·ha^{-1}); W0N: noninoculated control without mineral N.

Table 8. Contrast of mean values of number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), and shoot N accumulation (SNA) of *Stizolobium aterrimum* grown in a conventional planting area, evaluated at 170 days after sowing (at flowering)!.

NN■		NDM□		SDM		SNA	
BR2811	WN	BR2811	WN	BR2811	WN	BR2811	WN
BR2811	W0N	BR2811	W0N	BR2811	W0N	BR2811	W0N
WN	W0N	WN	W0N	WN	W0N	WN	W0N
INPA104A	BR2811	INPA104A	BR2811	INPA104A	BR2811	INPA104A	BR2811
INPA104A	WN	INPA104A	WN	INPA104A	WN	INPA104A	WN
INPA104A	W0N	INPA104A	W0N	INPA104A	W0N	INPA104A	W0N
UFLA03-144	BR2811	UFLA03-144	BR2811	UFLA03-144	BR2811	UFLA03-144	BR2811
UFLA03-144	WN	UFLA03-144	WN	UFLA03-144	WN	UFLA03-144	WN
UFLA03-144	W0N	UFLA03-144	W0N	UFLA03-144	W0N	UFLA03-144	W0N
UFLA04-212	BR2811	UFLA04-212	BR2811	UFLA04-212	BR2811	UFLA04-212	BR2811
UFLA04-212	WN	UFLA04-212*	WN	UFLA04-212	WN	UFLA04-212	WN
UFLA04-212	W0N	UFLA04-212	W0N	UFLA04-212	W0N	UFLA04-212	W0N
UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518	BR2811	UFLA0518*	BR2811
UFLA0518	WN	UFLA0518	WN	UFLA0518*	WN	UFLA0518**	WN
UFLA0518	W0N	UFLA0518	W0N	UFLA0518*	W0N	UFLA0518**	W0N
UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811	UFLA0519	BR2811
UFLA0519	WN	UFLA0519*	WN	UFLA0519	WN	UFLA0519	WN
UFLA0519	W0N	UFLA0519	W0N	UFLA0519	W0N	UFLA0519	W0N
UFLA0520*	BR2811	UFLA0520	BR2811	UFLA0520	BR2811	UFLA0520	BR2811
UFLA0520*	WN	UFLA0520**	WN	UFLA0520	WN	UFLA0520	WN
UFLA0520*	W0N	UFLA0520	W0N	UFLA0520	W0N	UFLA0520*	W0N

[!]The treatments are compared by pairs in the row within each analyzed variable. The presence of asterisks in the treatment represents it is significantly greater than the contrasting treatment (F-test [$p < 0.05$ or $p < 0.10$]); **significant effect at 5% probability; *significant effect at 10% probability; WN: noninoculated control with mineral N (300 mg·dm^{-3} of NH_4NO_3); W0N: noninoculated control without mineral N; ■ Data transformed into $(y+0.5)^{0.5}$ and □ log (y).

The UFLA05-20 strain showed SNA similar to the BR2811 and WN controls, and superior to that of the W0N control. Although the UFLA05-20 strain did not differ from the controls regarding SDM, it accumulated 386.36 kg·ha^{-1} of nitrogen, surpassing the average of native rhizobia and equaling the mineral nitrogen control (WN) and the BR2811 strain. This efficiency may not have occurred under controlled conditions due to the size of the pots restricting the root system, as previously reported. An indication of this efficiency, however, was observed in the IRC result at 60 DAE when the strain showed higher values than the controls. Another important indicator was the high nodulation shown by this strain in both harvest times under controlled conditions, as well as in the field.

In a strain selection study for *S. aterrimum* in Pernambuco, Brazil, Santos et al. (2017) pre-selected five isolates in a greenhouse for field evaluation. The symbiotic efficiency of these strains was confirmed by the superior or similar nitrogen accumulation compared to the experimental controls: 80 kg·ha^{-1} of mineral N, inoculation with a mixture of the SEMIA 6156 (CPAC IJ, BR2003) and SEMIA 6158 (CPAC42-BR2811) strains, and a control without inoculation and without added N (native strains), at 45 days of growth. However, none of the strains tested surpassed the 140 kg·ha^{-1}-N mark, unlike our results, in which UFLA05-18 accumulated 496.19 kg·ha^{-1} of N and UFLA05-20 accumulated 386.36 kg·ha^{-1} of N at flowering (170 DAS), This difference in values may be related to the harvest time, as the peak of BNF occurs at flowering (Moreira and Siqueira 2006). Like the current study, strain selection experiments need to be conducted until flowering so that the full potential of BNF can be measured.

The strains efficient in other hosts (INPA104A, UFLA03-144, and UFLA04-2012) did not provide positive and significant effects on any of the parameters evaluated.

Therefore, this highlights the importance of inoculation with efficient strains in BNF, such as UFLA05-18 and UFLA05-20 (field results) and UFLA05-20 (pot results). Much of the N accumulation in velvet bean comes from the BNF (Wutke et al. 2014, Rangel et al. 2017, Santos et al. 2017, Costa et al. 2020); however, this accumulation is not always obtained only from the performance of native communities, making it necessary to use inoculants containing selected strains.

The present study showed good results of a symbiosis of velvet bean with the strains UFLA05-18, UFLA05-19, and UFLA05-20 in pots with non-sterile soil and in the field, and these results corroborate those observed by Rangel et al. (2017) under axenic conditions, which also provided important information about the behavior of these strains under conditions of competition and stress. These strains were isolated from *S. aterrimum* nodules from an area contaminated with arsenic; and they were resistant to important antibiotics (except UFLA05-19), were moderately resistant to Cd (UFLA05-18), produced indole acetic acid and siderophores (except UFLA05-18), and were able to solubilize Ca phosphate (UFLA05-20) (Table 2). In Rangel et al.'s (2017) study, these strains were efficient in accumulating N in the aerial part of *S. aterrimum*, surpassing the results of the mineral nitrogen control and BR2811. Our results were not limited to axenic conditions, which indicates that these strains have biotechnological potential for this green manure crop.

Other *Bradyrhizobium* strains (UFLA05-03, UFLA05-09, and UFLA05-14) of the same origin (Rangel et al. 2017) showed excellent symbiotic efficiency in another green manure species, *C. spectabilis* (Silva et al. 2021). On that occasion, the authors demonstrated the biotechnological potential to produce inoculants from the three strains evaluated and showed that the cultivation of *C. spectabilis* inoculated with the strains, especially with UFLA05-09, can lead to earlier harvest and incorporation of this green manure already at 30 days before flowering, still ensuring a considerable contribution of N to the soil. Tests like this, from different harvest periods of velvet bean, inoculated with the strains used in the present study, would be useful for evaluating the possibility of incorporating green manure before flowering, since we have already seen from the current results that the strains enrich the phytomass of velvet bean.

The symbiotic potential of selected strains can also be affected by soil and climate conditions. Therefore, the recommendation would be to evaluate the efficiency of these strains under different conditions before submitting them to MAPA for approval for inoculant production (Brasil 2011). For that reason and considering the biotechnological potential of the UFLA strains presented here, new tests should be conducted under different soil and climate conditions.

CONCLUSION

Native soil rhizobia are effective in symbiosis with velvet bean, both in pots (100 DAE) and in the field, but their nitrogen-fixing efficiency is below that achieved through inoculation with UFLA05-19 in pots (60 and 100 DAE) and UFLA05-18 and UFLA05-20 in the field.

The strains UFLA05-18 and UFL 05-20 show biotechnological potential for production of inoculants for velvet bean, as they increase yield and nitrogen accumulation in the crop phytomass under field conditions. UFLA05-20 is superior to native rhizobia, while UFLA05-18 is superior to all three controls (native rhizobia, BR2811, and mineral nitrogen), which were similar to each other.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Conceptualization: Silva, J. S., Oliveira, D. P., Botrel, E. P. and Moreira, F. M. S.; **Data curation:** Silva, J. S. and Oliveira, D. P.; **Formal analysis:** Silva, J. S., Oliveira, D. P., and Rufini, M.; **Investigation:** Silva, J. S., Oliveira, D. P., Rufini, M., Silva Júnior, C. L., Lamounier, C. J. G., and Oliveira, P. A. C.; **Methodology:** Silva, J. S., Oliveira, D. P. and Botrel, E. P.; **Project administration:** Silva, J. S. and Moreira, F. M. S.; **Resources:** Silva, J. S. and Moreira, F. M. S.; **Supervision:** Silva, J. S. and Moreira, F. M. S.; **Original – draft writing:** Silva, J. S.; Visualization: Silva, J. S. and Moreira, F. M. S.; **Writing – review and editing:** Silva, J. S., Oliveira, D. P., Rufini, M., and Moreira, F. M. S.

DATA AVAILABILITY STATEMENT

Suplementary data are available at:

Thermopluviometricgraph. Monthly variation of maximum, mean and minimum temperatures and rainfall during the period of conducting the experiments.
http://repositorio.ufla.br/jspui/handle/1/59147

Images of Velvet beannodule attached to the plant root (a) and of nodules already detached and sanitized (b).
http://repositorio.ufla.br/jspui/handle/1/59146

Images of the sections (a,b,d,e) and surface (c) of Velvet bean nodules enlarged by a stereomicroscope.
http://repositorio.ufla.br/jspui/handle/1/59145

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