

New strains of *Bradyrhizobium* enrich plant biomass nitrogen content in *Crotalaria* for use as a green manure

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ABSTRACT: In spite of the chemical, physical, and biological benefits that *Crotalaria spectabilis* can provide to the soil, it is little used as a green manure crop by farmers. Inoculation with strains of legume-nodulating nitrogen-fixing bacteria that are efficient and competitive may be a strategy to enhance accumulation of N in *C. spectabilis* and stimulate adoption of this green manure crop. The aims of this study were i) to evaluate the symbiotic and agronomic efficiency of new strains of *Bradyrhizobium* on *C. spectabilis* in an oxisol (red latosol) compared to that of noninoculated controls (without and with mineral N) and with the approved strain BR2811, seeking to corroborate possible recommendation as inoculants for this species; and ii) to determine, the contribution of these treatments to N accumulation in the plant of *C. spectabilis* in four periods of cutting for determining possible and ideal periods for its incorporation in the soil. Experiments were carried out in pots and field. Inoculation with the new strains UFLA05-03, UFLA05-09, and UFLA05-14 and with BR2811 on *C. spectabilis* is effective since it increases the production of N-enriched plant biomass when compared to the control without N mineral. However, UFLA05-03 stands out among these strains because it behaves similarly to the control with mineral N both in relation to shoot N accumulation and dry matter just after 150 days in the field.

Key words: *Crotalaria spectabilis*, nodulating nitrogen-fixing bacteria, legume species inoculant, strain selection.

INTRODUCTION

Green manure is able to increase the productive capacity of soils in a sustainable manner, since it improves the soil physical, chemical, and biological characteristics (Aita et al. 2001; Aita and Giacomini 2003; Mercante et al. 2014; Moreira and Siqueira 2006). Among the plant species most used for this practice is *Crotalaria spectabilis*, a Leguminosae that arose in South and North America, whose excellent production of fresh matter (15 to 30 t·ha⁻¹), dry matter (3 to 8 t·ha⁻¹), and estimated N (100 to 160 kg·ha⁻¹) lead to increases of up to 100% in the yield of successive crops (Albuquerque et al. 2013; Nogueira et al. 2019; Souza et al. 2008; Tenelli et al. 2019). The mean flowering period of this species is 120 days, which can be a limiting factor for its use as a green manure crop on the part of producers who do not have that much time between crop seasons. However, it can be used intercropped with other species. For instance, Mendonça et al. (2017) evaluated N dynamics in intercropping between *C. spectabilis* and coffee and found that, of the total of N accumulated (93.42 kg·ha⁻¹) in the soil, 34.10 kg·ha⁻¹ was derived from biological nitrogen fixation (BNF), and 48.8% of the N originating from BNF was transferred to the coffee plants.

The symbiosis of *C. spectabilis* with legume nodulating N₂-fixing bacteria (LNNFB) can provide numerous benefits to plant production (Moreira and Siqueira 2006). Resende et al. (2003) showed that more than 80% of the N in *C. spectabilis* can originate from BNF. However, these benefits can be improved if highly efficient strains are used in its cultivation.



Studies on selection of strains efficient in BNF for *C. spectabilis* are few and are limited to only axenic conditions (Florentino et al. 2014; Rangel et al. 2017). However, promising results in relation to the symbiotic efficiency of *Bradyrhizobium* strains with *C. spectabilis* under axenic conditions indicate that their biotechnological potential should be determined under field conditions (Rangel et al. 2017).

Currently, there are only two strains approved by the Brazilian Ministry of Agriculture (MAPA, Ministério da Agricultura, Pecuária e Abastecimento) as inoculants for *C. spectabilis*, both belonging to the genus *Bradyrhizobium*: BR2003/SEMIA6156 (*B. japonicum*) and BR2811/SEMIA6158 (*B. elkanii*). However, the absence of publications that corroborate this recommendation generates questions regarding the criteria adopted in the recommendation process, which, according to MAPA, was restricted to tests in pots with soil (Brazil 2011). In addition, diverse environmental factors and traits of the symbionts can affect success in symbiosis. Therefore, in selection studies, the LNNFB strains must be tested in regard to their efficiency in fixing N₂ and their competitiveness for infection sites against native microorganisms under diverse edaphic and climatic conditions in the field, with the aim of obtaining suitable inoculants (Moreira and Siqueira 2006).

Given the diverse advantages of the use of *C. spectabilis* as a green manure crop, the lack of articles published on its symbiosis with LNNFB, and lack of information on the best time for cutting and incorporation related to N accumulation in the plant biomass, the hypothesis that *C. spectabilis* benefits from BNF and that its inoculation with new efficient strains of LNNFB will increase the accumulation of N by the plant was worked with.

Thus, the aims of this study were i) to evaluate the symbiotic and agronomic efficiency of inoculation of three strains of *Bradyrhizobium* on *C. spectabilis* in an oxisol (red latosol) compared to that of noninoculated controls (without and with mineral N) and with the approved strain BR2811, seeking to corroborate possible recommendation as inoculants for this species; and ii) to determine, the contribution of these treatments to N accumulation in the plant biomass of *C. spectabilis* in four periods of cutting for determining possible and ideal periods for its incorporation in the soil.

MATERIALS AND METHODS

Three experiments with *C. spectabilis* for use as a green manure crop were performed from October 2017 to April 2018. The first experiment was conducted to estimate the density of the native populations of nodulating nitrogen-fixing bacteria of *C. spectabilis* in the soil of an experimental area of the Universidade Federal de Lavras, Lavras, MG, Brazil, from which the soil for the experiment in pots was taken and where the field experiment was conducted. The latter two experiments were conducted with the aim of evaluating the symbiotic and agronomic efficiency of the LNNFB, previously selected as based on good results in axenic study (Rangel et al. 2017).

The soil used was classified as an oxisol (red latosol) of known fertility (Table 1) in an area with a history of growing grasses and noninoculated common bean.

Most probable number (MPN) of LNNFB in the soil of the experiment

For estimation of the native populations of LNNFB present in the soil of the experiment, the most probable number (MPN) method was used. Before the field experiment was set up, soil samples were collected from five points of the experimental area in the 0–20 cm layer to compose a single sample that was used for serial dilutions in decimal form. The experiment was conducted in a greenhouse in a completely randomized design, with three replications, using long neck dark bottles with 500 mL capacity containing sterilized Hoagland and Arnon (1950) nutrient solution. Before planting, the *C. spectabilis* seeds were scarified in 98% sulfuric acid for 5 min (Rangel et al. 2017), washed successively in sterilized distilled water, and left to soak for 20 min. After that, they were pregerminated in sterilized Petri dishes containing moistened cotton and incubated for two days at a temperature of 28 °C. After planting, the pregerminated seeds were inoculated with 1 mL of the soil suspensions from the serial dilutions from 10⁻¹ to 10⁻⁶. A positive control was included with the strain BR2811

approved by MAPA for *C. spectabilis*, as well as two negative controls without inoculation (one with 5.25 mg·L⁻¹ and the other with 52.5 mg·L⁻¹ of mineral N).

The experiment was conducted at a mean temperature of 23.6 °C and 73% relative humidity. The plants were collected at 30 days after planting, at which time root nodulation was evaluated considering the presence or absence of nodules in each dilution.

Table 1. Chemical and physical properties of soil samples, taken at the 0.00–0.20 m depth layer, and geographic coordinates of the experimental area in Lavras-MG.

Characteristics ¹		Results	
		Oxisol	Interpretation ²
pH H ₂ O (1:2.5)	-	6.00	M
P	mg·dm ⁻³	28.51	VG
K	mg·dm ⁻³	136.20	VG
Ca	cmol _c ·dm ⁻³	2.38	M
Mg	cmol _c ·dm ⁻³	0.92	G
Al	cmol _c ·dm ⁻³	0.06	VL
H + Al	cmol _c ·dm ⁻³	2.45	L
SB	cmol _c ·dm ⁻³	3.65	G
T	cmol _c ·dm ⁻³	6.10	M
t	cmol _c ·dm ⁻³	3.71	M
m	%	1.62	VL
V	%	59.82	M
Organic matter	dag·kg ⁻¹	1.88	M
Sand	g·kg ⁻¹	520	CS
Silt	g·kg ⁻¹	100	CS
Clay	g·kg ⁻¹	380	CS
Geographic coordinates	21°14'45"S, 44°59'59"W		
Altitude	920 m		
Climate (Köppen)	Cwa (humid subtropical)		

¹pH in water (v/v 1:2.5); P and K: extractor Mehlich⁻¹; Ca, Mg, Al: extractor 1 mol·L⁻¹ KCl; H + Al: potential acidity, extracted by calcium acetate 0.5 mol·L⁻¹ at pH 7. SB: sum of bases; T: cation exchange capacity at pH 7; t: cation exchange capacity; m: aluminum saturation; V: base saturation; OM: organic matter, Walkley-Black method; sand, silt, and clay: pipette method. ²CS: clayey soil, G: good; L: low; M: medium; VG: very good; VL: very low (according to Alvarez et al. 1999).

Symbiotic efficiency of LNNFB

The experiments to evaluate the symbiotic efficiency of the LNNFB were conducted in pots with soil and in the field. In the two experiments, three strains of *Bradyrhizobium* (UFLA05-03, UFLA05-09, and UFLA05-14) that proved to be efficient in previous study (Rangel et al. 2017), and the strain *Bradyrhizobium* BR2811 (strain approved by MAPA for *C. spectabilis*) were tested, as well as two noninoculated controls, without mineral N (W0N) and with mineral N (WN). The inoculants were prepared in liquid 79 medium at 28 °C and constant shaking (110 rpm) for 96 h (log phase of the bacterial growth). For field experiments, the inoculum was transferred to turf sterilized in an autoclave at the proportion of 3:2 (w:v) of turf and inoculum. The quality of the inoculants was monitored by counting the number of colony-forming units (CFU), respecting the minimum legal number of 10⁹ per mL (pots) or g (field) of inoculant. The mean rainfall and temperature during the growing period are shown in Fig. 1.

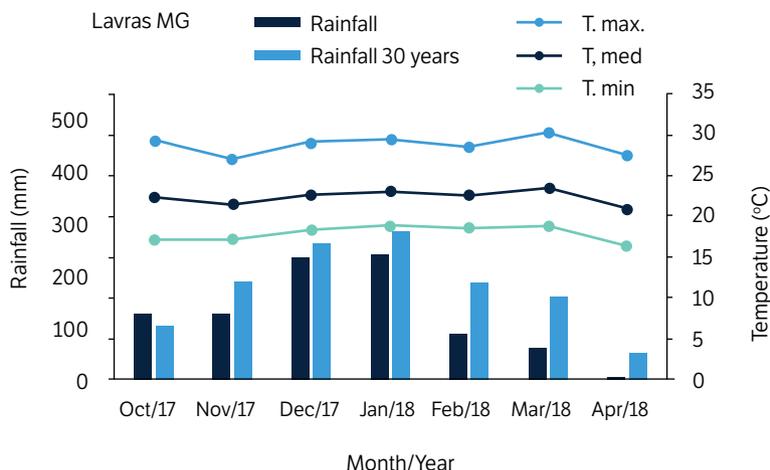


Figure 1. Monthly variation of maximum, mean and minimum temperatures and rainfall during the period of conducting the experiments and the average of the last 30 years. The pot experiment lasted from October 2017 to March 2018 and the field experiment from November 2017 to April 2018.

Experiment in pots with non-sterile soil

The experiment was conducted in a greenhouse in the period from October 2017 to March 2018. A randomized block experimental design was used with four replications for each one of the two periods of evaluation. The plants were grown in 3 dm³ pots (evaluated at 60 days after sowing [DAS]) and in 5 dm³ pots (evaluation at flowering at 140 DAS). The soil used for filling the pots was collected from the tillable layer (0–20 cm); clods were broken up, and the soil was homogenized and sieved through a 4 mm mesh size.

The fertilization adopted in the pots in all six treatments (element, quantity in mg·dm⁻³: P, 300; K, 200; Ca, 200; Mg, 50; S, 50; B, 0.8; Cu, 1.5; Fe, 2; Mn, 3; Mo, 0.10; and Zn, 4) followed the recommendations of Malavolta et al. (1989). The soluble sources used were calcium phosphate monobasic (monohydrate) Ca(H₂PO₄)₂·H₂O; potassium chloride KCl; magnesium sulfate heptahydrate (MgSO₄)₇·H₂O; boric acid H₃BO₃; copper sulfate pentahydrate (CuSO₄)₅·H₂O; iron (III) chloride FeCl₃; manganese chloride tetrahydrate (MnCl₂)₄·H₂O; sodium molybdate dihydrate (Na₂MoO₄)₂·H₂O; and zinc sulfate heptahydrate (ZnSO₄)₇·H₂O. The control treatment without inoculation and with mineral N received 300 mg·NH₄NO₃·dm⁻³, divided into three applications (at planting and at 10 and 20 DAS).

Preparation of the seeds followed the same procedure adopted in 2.1 (MPN). Four seeds were sown per pot and, at five days after emergence (DAE), the plants were thinned, leaving two seedlings per pot. In the treatments with bacterial strains, each seed received 1 mL of inoculant.

The pots were irrigated daily to maintain soil moisture near field capacity (60% of total pore volume). The mean values of minimum and maximum temperatures registered during the period of conducting the experiment were 19.4 and 31.5 °C, respectively.

Collections of the experiment in pots with soil occurred at 60 and 140 DAS. For each period of evaluation, all the plants from each plot (two plants per pot) were collected, considering the four replications of each treatment. The following determinations were made: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), shoot N content (SNC) and shoot N accumulation (SNA). To obtain the NDM and SDM, the nodules and shoots were placed in a forced air circulation laboratory oven at 60 °C until obtaining constant weight. The SNC was determined by the semimicro Kjeldahl (total nitrogen) method (Sarruge and Haag 1979). The SNA was calculated by multiplying the SDM by the SNC and dividing by 100.

Field experiment

The field experiment was conducted in the rainy crop season (November 2017 to April 2018) so to evaluate growth parameters of *C. spectabilis* in different times. A split-plot in time arrangement was adopted, consisting of four

collection times (60, 90, 120 and 150 DAS) in each one of the six treatments already described, with four replications for each split treatment.

Each experimental unit (10.8 m²) was constituted by four 4.5 m rows, spaced at 0.6 m, and the center rows were used for data collection. Soil tillage consisted of plowing, disking, and furrowing for demarcation of the rows. None of the plots received base fertilization since this is not a common practice for green manure crops in the region. The mineral nitrogen control treatment (WN) received 70 kg-N-urea-ha⁻¹, applying half at sowing and the other half in side dressing at 20 DAE.

Seeds were sown manually immediately after seed inoculation, adopting the density of 35 seeds per linear meter (Peché Filho et al. 2014). Weeds were controlled by manually weeding and ants were controlled by ant bait whenever necessary. No other control of pests or diseases was necessary.

A five-plant sample per split-plot was collected in the central rows (2–3), randomly, in each collection period for determination of NN, NDM, SDM, SNC and SNA. The methods of these evaluations followed those described in the experiments in pots with soil. For calculation of SDM e SNA in t-ha⁻¹ and kg-ha⁻¹, respectively, the expected stand of 437,500 plants-ha⁻¹ was used.

Statistical analyses

Evaluation of population density of native LNNFB was interpreted according to the table of McCrady (Döbereiner et al. 1995). Analysis of variance was carried out on the data from the two efficiency experiments after the tests of normality (Shapiro–Wilk), homoscedasticity of variances (Breusch–Pagan), and independence (Durbin–Watson) had first been carried out on the data on the R software (R Development Core Team 2019). To fulfill the presuppositions of analysis of variance, the data of all the variables were first transformed into log (y). When there was a significant effect by the F test ($p < 0.05$ or $p < 0.10$ [Brazil 2011]), the mean values of the treatments (strains tested) were contrasted with those of the controls of each trial (WN, W0N and BR2811) by Dunnett's test at the same level of significance. Controls were also evaluated between them.

RESULTS

Evaluation of the density of native populations of LNNFB

The native community of LNNFB in the soil of the experiment (used in trials in pots with soil and in the field) able to nodulate *C. spectabilis* was 2.5×10^3 cells per gram of soil.

Evaluation of symbiotic efficiency in pots with non-sterile soil

There was a significant effect of the treatments at 60 DAS, being the mean values and the contrast presented in Tables 2 and 3, respectively. There was no significant difference for NN between the treatments tested. For NDM, the UFLA05-03 strain exhibited mean similar to that of BR2811 and superior to that of the W0N and WN controls. The mean of the UFLA05-09 strain exceeded only the mean value of WN, and was similar to that of the other controls. The mean of the UFLA05-14 strain did not differ from that of the WN or W0N controls, and also did not differ from that of the BR2811 strain. There was no difference between the mean values of W0N and of the other controls, and BR2811 exceeded the results of WN. For SDM, the new strains UFLA05-03, UFLA05-09 and UFLA05-14 did not differ from controls. The BR2811 strain did not differ from W0N and both were lower than WN. For SNA, the UFLA05-03 strain had a mean value higher than that of the W0N control and of the BR2811 strain, with values statistically similar to that of the WN treatment. The mean values obtained by the UFLA05-09 and UFLA05-14 strains did not differ from those of the WN, W0N or BR2811 controls. The BR2811 did not differ from W0N, and both were lower than WN.

Table 2. Mean values for number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM) and shoot N accumulation (SNA) of *C. spectabilis* cultivated in pots with non-sterile soil (greenhouse), at 60 DAS.

Treatments ¹	NN (unit-plant ⁻¹)	NDM (mg-plant ⁻¹)	SDM (g-plant ⁻¹)	SNA (mg-plant ⁻¹)
BR2811	473.75	287.00	11.16	309.22
WN	241.34	107.12	19.15	522.57
WON	188.75	175.50	11.15	297.21
UFLA05-03	243.00	468.37	13.44	477.35
UFLA05-09	352.87	281.00	11.39	402.58
UFLA05-14	214.10	280.75	11.82	352.19
CV(%)	29.08	9.06	6.45	4.16

¹UFLA05-03, UFLA05-09, UFLA05-14 and BR2811: strains tested; WN: noninoculated control with mineral N (300 mg-dm⁻³ of NH₄NO₃); WON: noninoculated control without mineral N.

Table 3. Contrast of mean values for number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM) and shoot N accumulation (SNA) of *C. spectabilis* cultivated in pots with non-sterile soil (greenhouse), at 60 DAS.

NN		NDM		SDM		SNA	
BR2811	WN	BR2811*	WN	BR2811	WN**	BR2811	WN**
BR2811	WON	BR2811	WON	BR2811	WON	BR2811	WON
WN	WON	WN	WON	WN**	WON	WN**	WON
UFLA0503	BR2811	U0503	BR2811	UFLA0503	BR2811	UFLA0503**	BR2811
UFLA0503	WN	U0503**	WN	UFLA0503	WN	UFLA0503	WN
UFLA0503	WON	U0503*	WON	UFLA0503	WON	UFLA0503**	WON
UFLA0509	BR2811	U0509	BR2811	UFLA0509	BR2811	UFLA0509	BR2811
UFLA0509	WN	U0509*	WN	UFLA0509	WN	UFLA0509	WN
UFLA0509	WON	U0509	WON	UFLA0509	WON	UFLA0509	WON
UFLA0514	BR2811	U0514	BR2811	UFLA0514	BR2811	UFLA0514	BR2811
UFLA0514	WN	U0514	WN	UFLA0514	WN	UFLA0514	WN
UFLA0514	WON	U0514	WON	UFLA0514	WON	UFLA0514	WON

The treatments are compared by pairs on the line within each analyzed variable. The presence of asteristics in the treatment represents it is significantly greater than the constringing 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 NH₄NO₃); WON: noninoculated control without mineral N.

In collection of plants at flowering (140 DAS), there was significant difference among the treatments only for the variables NN and NDM (Tables 4 and 5). The NN obtained from the UFLA05-14 strain exceeded that from WN and did not differ statistically from that of the other controls. For NN, the results of BR2811 exceeded the results of WN, and the results of WON did not differ from the other controls. The NDM of the strain UFLA05-14 exhibited a mean value superior to that of the native LNNFB (WON) and did not differ from that of the other controls evaluated. The UFLA05-03 strain had a lower mean value than that of the BR2811 strain and did not differ from the mean values of the other controls. Furthermore, for NDM, there was no significant difference between the WN and BR2811 controls, and both were higher than the results of WON.

Table 4. Mean values for number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM) and shoot N accumulation (NAS) of *C. spectabilis* cultivated in pots with non-sterile soil (greenhouse), at 140 DAS.

Treatments ¹	NN (unit-plant ⁻¹)	NDM (mg-plant ⁻¹)	SDM (g-plant ⁻¹)	SNA (mg-plant ⁻¹)
BR2811	621.37	1948.50	144.16	3493.16
WN	144.87	1725.75	144.41	4205.92
WON	389.50	624.50	109.95	2876.00
UFLA05-03	189.75	719.25	135.84	3460.61
UFLA05-09	409.37	886.25	144.11	3948.54
UFLA05-14	978.62	1981.12	138.57	4151.63
CV(%)	11.30	6.34	6.37	3.80

¹UFLA05-03, UFLA05-09, UFLA05-14 and BR2811: strains tested; WN: noninoculated control with mineral N (300 mg-dm⁻³ of NH₄NO₃); WON: noninoculated control without mineral N.

Table 5. Contrast of mean values for number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM) and shoot N accumulation (NAS) of *C. spectabilis* cultivated in pots with non-sterile soil (greenhouse), at 140 DAS.

NN		NDM		SDM		SNA	
BR2811**	WN	BR2811	WN	BR2811	WN	BR2811	WN
BR2811	WON	BR2811**	WON	BR2811	WON	BR2811	WON
WN	WON	WN**	WON	WN	WON	WN	WON
UFLA0503	BR2811	U0503	BR2811*	UFLA0503	BR2811	UFLA0503	BR2811
UFLA0503	WN	U0503	WN	UFLA0503	WN	UFLA0503	WN
UFLA0503	WON	U0503	WON	UFLA0503	WON	UFLA0503	WON
UFLA0509	BR2811	U0509	BR2811	UFLA0509	BR2811	UFLA0509	BR2811
UFLA0509	WN	U0509	WN	UFLA0509	WN	UFLA0509	WN
UFLA0509	WON	U0509	WON	UFLA0509	WON	UFLA0509	WON
UFLA0514	BR2811	U0514	BR2811	UFLA0514	BR2811	UFLA0514	BR2811
UFLA0514**	WN	U0514	WN	UFLA0514	WN	UFLA0514	WN
UFLA0514	WON	U0514**	WON	UFLA0514	WON	UFLA0514	WON

The treatments are compared by pairs on the line within each analyzed variable. The presence of asteristics 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 ($300 \text{ mg} \cdot \text{dm}^{-3}$ of NH_4NO_3); WON: noninoculated control without mineral N.

Evaluation of agronomic efficiency in the field

The effect of the treatments on SDM and SNA depended on the time of collection, with significant differences occurring in the collections carried out at 60 and 150 DAS (Tables 6–8). At 60 DAS for SDM, the UFLA05-03 strain exceeded W0N and BR2811, but not WN control; the UFLA05-09 strain had a mean value similar to those of the controls W0N and BR2811, but was lower than that of the WN control; and the UFLA05-14 strain had a mean value lower than those of all the controls evaluated. The WN control was higher than the mean values of the other controls; BR2811 was similar to W0N. At 150 DAS (full flowering), the strains UFLA05-03 and UFLA05-14 had mean SDM values higher than those of the three controls; the UFLA05-09 strain was higher than the W0N control, and was similar to the other controls. The BR2811 and WN did not differ from each other, both were higher than the mean values of W0N.

Table 6. Mean values of shoot dry matter (SDM) and shoot N accumulation (SNA) of *C. spectabilis* cultivated in conventional planting area, evaluated at 60, 90, 120 and 150 DAS.

Treatments ¹	SDM ($\text{t} \cdot \text{ha}^{-1}$)	SDM ($\text{g} \cdot \text{plant}^{-1}$)	SNA ($\text{kg} \cdot \text{ha}^{-1}$)	SNA ($\text{mg} \cdot \text{plant}^{-1}$)
60 DAS				
BR2811	2.07	4.73	84.69	193.58
WN	3.40	7.78	133.00	304.00
WON	2.24	5.13	81.67	186.67
UFLA05-03	2.77	6.34	97.45	222.74
UFLA05-09	2.22	5.07	83.85	191.67
UFLA05-14	1.74	3.98	66.44	151.87
90 DAS				
BR2811	7.15	16.35	317.23	725.09
WN	5.78	13.21	205.86	470.53
WON	5.49	12.56	174.62	399.13
UFLA05-03	7.64	17.46	231.36	528.82
UFLA05-09	6.72	15.37	232.27	530.91
UFLA05-14	5.91	13.52	161.72	369.65

Continue...

Table 6. Continuation...

Treatments ¹	SDM (t·ha ⁻¹)	SDM (g·plant ⁻¹)	SNA (kg·ha ⁻¹)	SNA (mg·plant ⁻¹)
120 DAS				
BR2811	18.99	43.40	488.40	1116.35
WN	16.07	36.73	416.92	952.97
WON	17.80	40.69	415.74	950.27
UFLA05-03	19.93	45.56	613.43	1402.13
UFLA05-09	20.03	45.78	697.70	1594.75
UFLA05-14	16.36	37.40	442.49	1011.40
150 DAS				
BR2811	30.95	70.74	876.83	2004.18
WN	31.55	72.11	771.61	1763.68
WON	20.59	47.07	620.54	1418.37
UFLA05-03	40.41	92.37	1293.24	2955.97
UFLA05-09	34.39	78.60	1095.49	2503.98
UFLA05-14	37.65	86.05	1186.45	2711.88

¹UFLA05-03, UFLA05-09, UFLA05-14 and BR2811: strains tested; WN: noninoculated control with mineral N (70 kg-N-urea·ha⁻¹); WON: noninoculated control without mineral N.

Table 7. Contrast of mean values for shoot dry matter (SDM) of *C. spectabilis* cultivated in conventional planting area, evaluated at 60, 90, 120 and 150 DAS.

Shoot dry matter (SDM)							
60 DAS		90 DAS		120 DAS		150 DAS	
BR2811	WN*	BR2811	WN	BR2811	WN	BR2811	WN
BR2811	WON	BR2811	WON	BR2811	WON	BR2811*	WON
WN*	WON	WN	WON	WN	WON	WN*	WON
UFLA0503*	BR2811	U0503	BR2811	UFLA0503	BR2811	UFLA0503*	BR2811
UFLA0503	WN*	U0503	WN	UFLA0503	WN	UFLA0503*	WN
UFLA0503*	WON	U0503	WON	UFLA0503	WON	UFLA0503*	WON
UFLA0509	BR2811	U0509	BR2811	UFLA0509	BR2811	UFLA0509	BR2811
UFLA0509	WN*	U0509	WN	UFLA0509	WN	UFLA0509	WN
UFLA0509	WON	U0509	WON	UFLA0509	WON	UFLA0509*	WON
UFLA0514	BR2811*	U0514	BR2811	UFLA0514	BR2811	UFLA0514*	BR2811
UFLA0514	WN*	U0514	WN	UFLA0514	WN	UFLA0514*	WN
UFLA0514	WON*	U0514	WON	UFLA0514	WON	UFLA0514*	WON

The treatments are compared by pairs on the line within each analyzed variable. The presence of asteristics in the treatment represents it is significantly greater than the constringing 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 (70 kg N-urea ha⁻¹); WON: noninoculated control without mineral N.

In relation to the SNA variable (Tables 6 and 8) at 60 DAS, the SNA provided by the strains UFLA05-03 and UFLA05-09 was similar to those of the controls WON and BR2811, but not exceeding that of WN. The SNA of the UFLA05-14 strain was lower than that of all the controls. The WN control was greater than the mean values of the other controls, and BR2811 was equal to the results of the WON. In the last collection period, at 150 DAS (full flowering), all the new strains tested led to higher SNA than the WON control; the UFLA05-03 strain also outperformed the results of the controls the BR2811 strain and of WN, whereas the UFLA05-09 and UFLA05-14 strains presented similar SNA averages of BR2811 and WN. The BR2811 was equal to WN, and both were greater than the results of WON.

Table 8. Contrast of mean values for shoot N accumulation (SNA) of *C. spectabilis* cultivated in conventional planting area, evaluated at 60, 90, 120 and 150 DAS.

Shoot N accumulation (SNA)							
60 DAS		90 DAS		120 DAS		150 DAS	
BR2811	WN*	BR2811	WN	BR2811	WN	BR2811	WN
BR2811	WON	BR2811	WON	BR2811	WON	BR2811*	WON
WN*	WON	WN	WON	WN	WON	WN*	WON
UFLA0503	BR2811	U0503	BR2811	UFLA0503	BR2811	UFLA0503*	BR2811
UFLA0503	WN*	U0503	WN	UFLA0503	WN	UFLA0503*	WN
UFLA0503	WON	U0503	WON	UFLA0503	WON	UFLA0503*	WON
UFLA0509	BR2811	U0509	BR2811	UFLA0509	BR2811	UFLA0509	BR2811
UFLA0509	WN*	U0509	WN	UFLA0509	WN	UFLA0509	WN
UFLA0509	WON	U0509	WON	UFLA0509	WON	UFLA0509*	WON
UFLA0514	BR2811*	U0514	BR2811	UFLA0514	BR2811	UFLA0514	BR2811
UFLA0514	WN*	U0514	WN	UFLA0514	WN	UFLA0514	WN
UFLA0514	WON*	U0514	WON	UFLA0514	WON	UFLA0514*	WON

The treatments are compared by pairs on the line within each analyzed variable. The presence of asteristics in the treatment represents it is significantly greater than the constringing 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 (70 kg-N-urea-ha⁻¹); WON: noninoculated control without mineral N.

DISCUSSION

In the soil where the experiments were set up, native LNNFB was found capable of nodulating *C. spectabilis*. This may be related to the edaphic and climatic conditions that allowed them to establish themselves and survive. The mean density (2.5×10^3 cells per gram of soil) obtained in the current study is within the range found in other studies with promiscuous grain legumes (Oliveira et al. 2019; Soares et al. 2006 a, b) and with pigeon pea, a species also used as a green manure crop (Rufini et al. 2016) in the same region as the experimental area.

As already explained, in greenhouse evaluations for the collection made at 60 DAS, there was a significant difference among the treatments for the NDM, SDM and SNA variables, which did not occur in the evaluations made at 140 DAS. The results of the last collection may have been affected by space limitations for the roots due to the dimensions of the pots. Although the plants were well developed, it was necessary to wait for them to reach the flowering stage to perform the evaluation. If it was not for the limitation of space for plant development, good results would likely have been observed in the second collection since good performance of the strains was observed in the field in a similar period.

The strains UFLA05-03, UFLA05-09, and UFLA05-14 provided high SDM and SNA production in the field, in the collection made at 150 DAS (flowering); their results were superior to those of the native LNNFB, and even superior or equal to those of the control with mineral N and of the reference strain BR2811. These results indicate that these strains have high BNF efficiency when inoculated on *C. spectabilis*, mainly the strain UFLA05-03. Therefore, they have the potential to be used in the production of inoculants for *C. spectabilis*; however, new tests are recommended in different edaphoclimatic conditions.

The strains UFLA05-03, UFLA05-09 and UFLA05-14 also showed high symbiotic efficiency with *C. spectabilis* in studies in pots with sterilized nutrient solution (Rangel et al. 2017). These strains, which were isolated from nodules of the plant species itself present in an area contaminated with arsenic (gold mining area), had dry matter weight and shoot N accumulation higher than or equal to that of the control with mineral N and the strain approved as an inoculant, BR2811. The authors concluded that these strains had biotechnological potential and suggested confirmation of their efficiency under more complex growing conditions, such as those carried out in this study.

When each treatment is evaluated separately, an increase is found in SDM and in SNA over time, i.e., the quantity of dry matter produced, as well as the N accumulated in this material, increases at each collection made, reaching the maximum values at flowering. There are advantages in growing this green manure crop for this period of time, which, although it

is long, would avoid infestation of the area by *C. spectabilis* due to seed dispersal after fructification. In addition, in this period, the three strains tested and also BR2811 showed efficiency in symbiosis with *C. spectabilis*. The contribution of the BNF promoted by *C. spectabilis* had also been shown by the ^{15}N technique. Mendonça et al. (2017) evaluated N levels in leaves of coffee intercropped with species of green manure crops. The results showed that the total N accumulation by *C. spectabilis* was $93.42 \text{ kg}\cdot\text{ha}^{-1}$, of which $34.1 \text{ kg}\cdot\text{ha}^{-1}$ was derived from BNF, which, in turn, contributed approximately 50% of the N transferred to the coffee plant. Thus, the authors concluded that *C. spectabilis* is a leguminous crop with high potential for transferring N to the coffee plant and recommended its use in intercropping with coffee. Considering that the seeds of *C. spectabilis* were not inoculated and that fertilization through the soil likely inhibited the action of native LNNFB, there is reason to believe that even better results could have been obtained with the use of efficient strains.

The results of SNA of the native LNNFB indicate that they were efficient in BNF up to the 120 DAS. However, a LNNFB community efficient in BNF is not always the rule under the diverse edaphic and climatic conditions in Brazil. Resende et al. (2003) estimated that BNF contributed $16 \text{ kg}\cdot\text{ha}^{-1}$ of N to the biomass of *C. spectabilis* grown for 71 days, but these plants had not been inoculated with LNNFB of proven efficiency. It can therefore be concluded that the BNF occurring through the native community present in the soil presented low efficiency when compared to the results obtained in this study by the new strains. This comparison shows the importance of ensuring the inoculation of the green manure crop with strains of proven efficiency, since an efficient native community cannot always be relied upon.

According to the results obtained in this study, the inoculation with efficient strains did not increase the development of the plants in the evaluations that preceded the flowering, not interfering in the cut period of the plants since they did not differ from the W0N, WN and BR2811 controls. This was probably due to the very low precipitation during this period (Fig. 1). However, ensuring the inoculation of the green manure crop with strains of proven efficiency is necessary since the soils do not always have efficient native LNNFB. In addition, the practicality and the low cost of inoculation should also be considered. Thus, the results of the present study show that the strains tested (mainly UFLA05-03) are efficient in BNF and promote the growth of *C. spectabilis*, and the practice of inoculating this species is recommended for enriching its shoots in N especially at flowering.

CONCLUSION

The greatest gains in the development and nutrition of plants using the new strains were obtained in flowering at the field experiment.

All the new strains tested, as well as BR2811, were efficient in fixing N_2 in *C. spectabilis*. However, inoculation of *C. spectabilis* seeds with the UFLA05-03 strain is a real alternative for increasing production of biomass enriched with N to serve as a green manure crop and more tests must be developed with a view to its approval as inoculant by MAPA.

AUTHORS' CONTRIBUTION

Conceptualization: Silva J. S., Oliveira D. P., Rufini M. and Moreira F. M. S.; **Formal Analysis:** Silva J. S. and Oliveira D. P.; **Funding Acquisition:** Moreira F. M. S.; **Investigation:** Silva J. S., Oliveira D. P., Rufini M., Silva C. L., Baptista M. V. D. G. B., Silva L. C., Aragão O. O. S. and Pereira T. A.; **Methodology:** Silva J. S., Oliveira D. P., Rufini M. and Moreira F. M. S.; **Project Administration:** Silva J. S. and Moreira F. M. S.; **Supervision:** Moreira F. M. S.; **Visualization:** Silva J. S., Oliveira D. P. and Aragão O. O. S.; **Writing – Original Draft:** Silva J. S., Oliveira D. P., Rufini M. 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

Data will be available upon request.

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