Scientific Note

Does the *Bradyrhizobium pachyrhizi* BR 3262 elite strain overcome the native established soil rhizobial population on cowpea nodules occupation?[1]

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ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp.) is a promiscuous crop, nodulating with a wide range of rhizobia strains native to the soil, what often leads to low inoculation responses with selected rhizobia, reducing the efficiency of introduced inoculants. This study aimed to assess the nodule occupancy of the *Bradyrhizobium pachyrhizi* BR 3262 elite strain in two cowpea genotypes (BRS Pujante and BRS Acauã), in soil with established rhizobial population (higher than 10^3 g^{-1} of soil), in a greenhouse. For the BR 3262 nodule occupancy, a strain-specific PCR approach was applied, assessing all the 1,237 nodules obtained from the root crown and the entire root (secondary roots) regions of the inoculated plants. The BR 3262 inoculation increased the root dry mass and nodulation, but not the shoot dry mass. Both genotypes showed a nodular occupancy above 80 % in the crown and secondary roots, indicating a high competitiveness and persistence in the soil.

KEYWORDS: *Vigna unguiculata*, biological nitrogen fixation, rhizobia competitiveness, nodulating bacteria.

RESUMO

A estirpe elite *Bradyrhizobium pachyrhizi* BR 3262 supera a população nativa estabelecida de rizóbios do solo na ocupação de nódulos de feijão-caupi?

O feijão-caupi (*Vigna unguiculata* L. Walp.) é uma cultura promíscua, nodulando com uma ampla gama de estirpes de rizóbio nativas do solo, o que frequentemente leva a baixas respostas de inoculação com rizóbios selecionados, reduzindo a eficiência dos inoculantes introduzidos. Objetivou-se avaliar a ocupação nodular da estirpe elite *Bradyrhizobium pachyrhizi* BR 3262 em dois genótipos de feijão-caupi (BRS Pujante e BRS Acauã), em solo com população de rizóbio estabelecida (superior a 10^3 g^{-1} de solo), em casa-de-vegetação. Para a ocupação de nódulos BR 3262, foi utilizada abordagem de PCR estirpe-específica, avaliando-se todos os 1.237 nódulos obtidos das regiões da coroa radicular e de toda a raiz (raízes secundárias) das plantas inoculadas. A inoculação de BR 3262 aumentou a massa seca da raiz e a nodulação, mas não a massa seca da parte aérea. Ambos os genótipos apresentaram ocupação nodular acima de 80 % na coroa e raízes secundárias, indicando alta competitividade e persistência no solo.

PALAVRAS-CHAVE: *Vigna unguiculata*, fixação biológica de nitrogênio, competitividade de rizóbios, bactérias nodulantes.

Cowpea (*Vigna unguiculata* L. Walp.) is a crucial legume crop grown worldwide in the tropics. Applying inoculants containing selected *Bradyrhizobium* strains can improve cowpea growth, field establishment and yield (Martins et al. 2003, Marinho et al. 2014 and 2017, Boddey et al. 2017). However, cowpea is a promiscuous legume, nodulated by a wide range of tropical native soil rhizobia (Thies et al. 1991, Lira et al. 2015). On the other side of the symbiosis, most of the soil native rhizobia are very competitive for the occupation of nodulation sites. However, they are inefficient in

[1] Received: June 13, 2024. Accepted: Sep. 17, 2024. Published: Oct. 03, 2024. DOI: 10.1590/1983-40632024v5479675.
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nitrogen fixation, what is an additional challenge to tackle by the introduced rhizobial inoculant strains (Thies et al. 1991, Law et al. 2007, Mendoza-Suárez et al. 2021).

Four bradyrhizobial strains have been officially selected and are currently used as commercial inoculants in Brazil (Martins et al. 2003, Lacerda et al. 2004, Zilli et al. 2009). Among these strains, *B. pachyrhizi* BR 3262 and *B. yuanmingense* BR 3267 were proven effective inoculants overseas in the African continent (Ghana), where the inoculation with BR 3262 or BR 3267 doubled the grain yields when compared to the non-inoculated treatment, and increased up to 64 % the grain yields when compared with the treatment with 40 kg ha^{-1} of N (Boddey et al. 2017). These findings indicate the high agronomic efficiency and versatility of these Brazilian bradyrhizobial inoculant strains.

Cowpea rhizobial diversity studies in tropical agricultural soils usually obtain more strains belonging to the *B. japonicum* supercluster (Bj-SC) than those within other genetic clusters of *Bradyrhizobium* (Florentino et al. 2010, Jaramillo et al. 2013, Chidebe et al. 2018, Oliveira et al. 2020, Sena et al. 2020). *B. pachyrhizi* BR 3262 belongs to the *B. elkanii* genetic cluster (Be-SC) (Leite et al. 2018), and, theoretically, is not the main microsymbiont associated with cowpea. Moreover, a co-inoculation assay with sterile substrates showed that the nodule occupation of BR 3262 was lower than that of several Bj-SC bacteria isolated from soils in the Brazilian drylands (Nascimento et al. 2021). Despite the presence of BR 3262 in (overall) 30 % of cowpea nodules (occupying more than 50 % of the nodules in seven out of thirty-five BR 3262 plus native Bj-SC co-inoculation treatments), cowpea plants showed a better growth (average of 30 % higher shoot dry mass) than those inoculated with any single strain (BR 3262 or BR 3267).

Despite the low nodule occupancy of BR 3262 when co-inoculated with another bradyrhizobia (equal densities) under sterile substrate conditions, field assays usually indicate the high agronomic efficiency of this bacterium, sustaining a high cowpea growth and yield in the semiarid region of Brazil (Marinho et al. 2014, Xavier et al. 2017). These findings raise questions about the BR 3262 competitiveness in soils with an established rhizobial population.

A dense nodulation in the root "crown", the region where the shoot arises and the older region of the plant root (Schwarz 1972), compared to a sparse nodulation all over the roots, is a proxy for the efficiency of rhizobial inoculation (Cardoso et al. 2009). However, the nodule occupancy of the inoculated strains out of the crown region is usually neglected. Therefore, in this study, it was hypothesized that *B. pachyrhizi* BR 3262 shows a high symbiotic competitiveness in soil, with an established cowpea rhizobial population able to nodulate abundantly both in the crown root region and in the secondary roots of cowpea genotypes. Hence, this study aimed to assess the nodule occupancy of the *B. pachyrhizi* BR 3262 elite strain in two cowpea genotypes, in an agricultural soil densely populated by native rhizobia.

The experiment was implemented in a greenhouse between February and April 2020, within the facilities of the Embrapa Semiárido, in Petrolina, Pernambuco state, Brazil (lat. -9.1354; long. -40.3092). A composite sample of the topsoil (0-0.2 m) of an Ultisol (USDA 1999) and the soil fertility analysis were conducted according to Teixeira et al. (2017) (Table 1). The most probable number

Table 1. Chemical and physical characteristics and cowpea-compatible rhizobial concentration in the topsoil samples used in the experiment.

EC	pH	C	P	K^+	Na^+	Ca^{2+}	Mg^{2+}	Al^{3+}	H + Al	SB	CEC
mS cm^{-1}	H_2O	g kg^{-1}	mg dm^{-3}	$cmol_c$ dm^{-3}							
0.21	6.1	4.9	33.3	0.29	0.05	2.6	1.0	0.0	2.0	3.9	5.9
V	Cu	Fe	Mn	Zn	BD	PD	TP	Sand	Silt	Clay	
%	$cmol_c$ dm^{-3}				kg dm^{-3}		%	g kg^{-1}			
66.8	0.07	0.7	1.4	0.46	1.59	2.5	36.4	902	67	31	
MPN of cowpea compatible rhizobial x 10^3 cells g^{-1} of soil (confidence interval = 95 %)								BRS Pujante 3.731-29.251		BRS Acauã 1.229-6.921	

EC: electrical conductivity; SB: sum of bases; CEC: cation exchange capacity; V: base saturation; BD: bulk density; PD: particle density; TP: total porosity; MPN: most probable number.

approach was applied to estimate the cowpea-compatible rhizobial population following Hungria & Araujo (1994). The sampling site had non-inoculated cowpea in 2018 and kept under fallow in 2019.

For the plant-inoculation experiment, where the total number of nodules per plant and the shoot and root dry mass were assessed, a 2 (genotypes) x 2 (inoculation) factorial scheme was adopted, in a completely randomized design, with four replications. For the nodule occupancy assessment of the plants inoculated with BR 3262, a completely randomized design, with a 2 (genotypes) x 2 (root regions) factorial scheme, with four replications, was also adopted.

For inoculation, *B. pachyrhizi* BR 3262 was grown in YM liquid medium (Vincent 1970) at 100 rpm, for 5 days, at room temperature (25 ± 2 ºC). The culture broth was centrifuged at 6,000 *g* for 3 min, and the pellet was resuspended in sterile NaCl 0.85 % (w v^{-1}). The procedure was repeated twice, and the final optical density was adjusted to 0.8 in a spectrophotometer (Multiskan GO, Thermo Scientific, USA), at 600 nm. Before inoculation, an aliquot of the broth was serially diluted in triplicates and inoculated in YMA Petri dishes to determine the cell concentration.

Seeds of the BRS Acauã or BRS Pujante cowpea genotypes were surface disinfected (Somasegaran & Hoben 1994), and 5 seeds were sown in pots with 5 L of soil, and those inoculated with *B. pachyrhizi* BR 3262 received 1 mL of broth on each seed. The plants were irrigated with tap water once a day, and, at 10 days after emergence (DAE), the spare plants were cut, and one plant was left per pot. At 36 DAE, the experiment was harvested. The shoots were cut, put into paper bags, oven-dried at 65 ºC under air circulation for 5 days and weighed. The roots were carefully separated from the soil and gently washed with running tap water.

All the nodules were separated from the roots, differing the nodules from the crown (region from the colon and 2-3 cm downward) and the secondary root (the whole root, except the crown) regions. Then, all the nodules were kept under 8 ± 2 ºC until DNA extraction (within 10 days after the experiment harvest). After the nodule's detachment, the roots were dried and weighted, as described for the shoots. Then, all the nodules from the crown and whole root regions were used for the PCR reactions for the inoculated treatments.

The nodule occupancy of the *B. pachyrhizi* BR 3262 strain was assessed by the strain-specific PCR amplification (Osei et al. 2017) with the primers 2645-F (TAGAGGGCTGCTATCATGTC) and 2645-R (GAGATGATTACCGCAATGAG), using all nodules observed in the inoculated plants (1,237 nodules, being 678 and 559 from the BRS Pujante and BRS Acauã, respectively), plus the negative and positive controls. In addition to the nodules from the inoculated cowpea plants and controls, 10 nodules from each non-inoculated pot were randomly chosen, totaling 160 nodules for DNA extraction and PCR.

The nodule's DNA was extracted individually for each nodule. The extraction was conducted by manually macerating each nodule in sterile 1.5 mL centrifuge tubes, using plastic sterile pestles (Osei et al. 2017). To assure the quality of DNA extraction and PCR amplification, positive and negative controls were used, with nodules obtained from cowpea (BRS Pujante) inoculated with *B. pachyrhizi* BR 3262 or *B. yuanmingense* BR 3267, respectively, under gnotobiotic conditions (Nascimento et al. 2021).

The DNA was stored at -4 ºC until the PCR amplifications. The reaction conditions were adopted by Nascimento et al. (2021). The PCR reactions were performed in a Veriti 96 well thermocycler (Applied Biosystems, USA), and the products were subjected to horizontal electrophoresis in 1 % (w v^{-1}) agarose gel at 100 V, for 60 min. A band amplicon around 200 bp in the agarose gel indicated the presence of BR 3262 inside the nodule. Two nodules from positive and two from negative controls were used in each DNA extraction and PCR rounds.

All statistical analyses were carried out in R environment v. 4.3.2, using the interface RStudio v. 2023.12.1+402 (R Core Team 2021). Before the one-way analysis of variance (Anova), the data assessed the normal distribution of the residuals (errors) by the Shapiro-Wilk test and the variance homogeneity by the Lavene test. Afterwards, Anova was performed, and the two-tailed t-test compared significant variables. All analyses used the "stats" package in the base R (R Core Team 2021). The plots were built using the "ggplot2" R package (Wickham 2016), and the statistical comparisons within the plots were made using the "ggpubr" R package (Kassambara 2023).

The most probable number analysis of the cowpea-compatible rhizobial community in the soil revealed that the confidence interval of viable rhizobial cells for BRS Pujante was between 3.731 and 29.251 x 10^3 nodulating bacterial cells (NB) per gram of soil (Table 1). The BRS Acauã confidence interval ranged from 1.229 to 6.921 x 10^3 NB g^{-1}. The colony counting of the OD_{600} culture broth showed that *B. pachyrhizi* BR 3262 was inoculated at 2.1 (± 0.3) x 10^9 cells mL^{-1}. Agricultural soils from the semiarid region of Brazil are usually densely colonized by effective rhizobial strains able to nodulate a promiscuous crop like cowpea (Martins et al. 1997, Leite et al. 2009, Sena et al. 2020). The soil used in the current experiment was cropped with non-inoculated cowpea two years before the soil sampling. The abundant nodulation of the non-inoculated treatments indicated that, after one fallow year, the rhizobial population remained high, even within one year without legume cultivation, indicating the rhizobial strains' adaptability and cowpea infection ability from the Brazilian drylands.

The BR 3262 inoculation increased the number of cowpea nodules, and the assessed plant genotype also influenced the nodulation. However, no interaction was observed between the BR 3262 inoculation and the plant genotype used (Table 2), showing that cowpea genotypes (BRS Pujante and BRS Acauã) respond to inoculation with BR 3262 strains in the same way (increasing nodulation), despite the different magnitudes. The bradyrhizobial inoculation increased the number of nodules in the cowpea ($p < 0.05$) (Figures 1A and 1B), what indicates the high competitiveness of this inoculant strain in the soil, with an established population of adapted cowpea rhizobia. The same pattern in the total number of nodules was also observed in the root dry mass, since the inoculation of BR 3262 increased the total root weight (Figures 1C and 1D). Considering the plant genotypes, the BRS Pujante's roots showed a higher dry mass than those from BRS Acauã ($p < 0.1$). Also, roots of inoculated cowpea were heavier than those without BR 3262 inoculation ($p < 0.05$). No significant differences were observed among all the sources of variation for shoot dry mass.

Cowpea inoculation with bradyrhizobia, even with the strains used in commercial inoculation, is not a "done deal" for plant growth and nodulation in tropical soils, since the community of native strains, smaller in numbers but actually competitive, usually impairs the inoculant strains to nodulate and promote the host-plant growth (Martins et al. 2003). The response of cowpea inoculation to rhizobial strains is variable, ranging from increment in plant growth and/or yield (Marinho et al. 2014, Silva et al. 2023) to no effects on cowpea development (Almeida et al. 2010, Marinho et al. 2014, Xavier et al. 2017), indicating that the interaction among plant, microbial strain and the environmental conditions influence on the inoculant performance. The root growth promotion and increased nodulation indicate the efficiency of the BR 3262 strain, which is not expected for a Be-SC strain.

The cowpea genotype and root region influenced the total nodulation, with a positive interaction between both variables (Figure 2A). The BRS Pujante secondary roots had an average of 137 nodules $plant^{-1}$, higher than the number of nodules

Table 2. Summary of the analysis of variance (one-way Anova) of *Bradyrhizobium pachyrhizi* BR 3262 nodule occupancy, total nodulation, number of nodules, shoot and root dry mass of cowpea, in soil with established rhizobial population.

Source of variation	Df	Mean of squares	p-value
BR 3262 nodule occupancy			
Cowpea genotype	1	4.29	0.673
Root region	1	375.54	0.002
Cowpea genotype * root region	1	0.13	0.941
Residuals (error)	12	22.90	
Total nodulation (nodules $plant^{-1}$ or root $region^{-1}$)			
Cowpea genotype	1	9,457.56	0.000
Root region	1	15,190.56	0.000
Cowpea genotype * root region	1	7,014.06	0.001
Residuals (error)	12	379.31	
Number of nodules $plant^{-1}$			
Cowpea genotype	1	31,064.06	0.000
Inoculation	1	4,257.56	0.029
Cowpea genotype * inoculation	1	333.06	0.502
Residuals (error)	12	695.10	
Shoot dry mass $plant^{-1}$			
Cowpea genotype	1	0.68	0.539
Inoculation	1	3.43	0.182
Cowpea genotype * inoculation	1	1.23	0.414
Residuals (error)	12	1.71	
Root dry mass $plant^{-1}$			
Cowpea genotype	1	7.52	0.056
Inoculation	1	14.27	0.013
Cowpea genotype * inoculation	1	5.28	0.101
Residuals (error)	12	1.67	

Df: degrees of freedom.

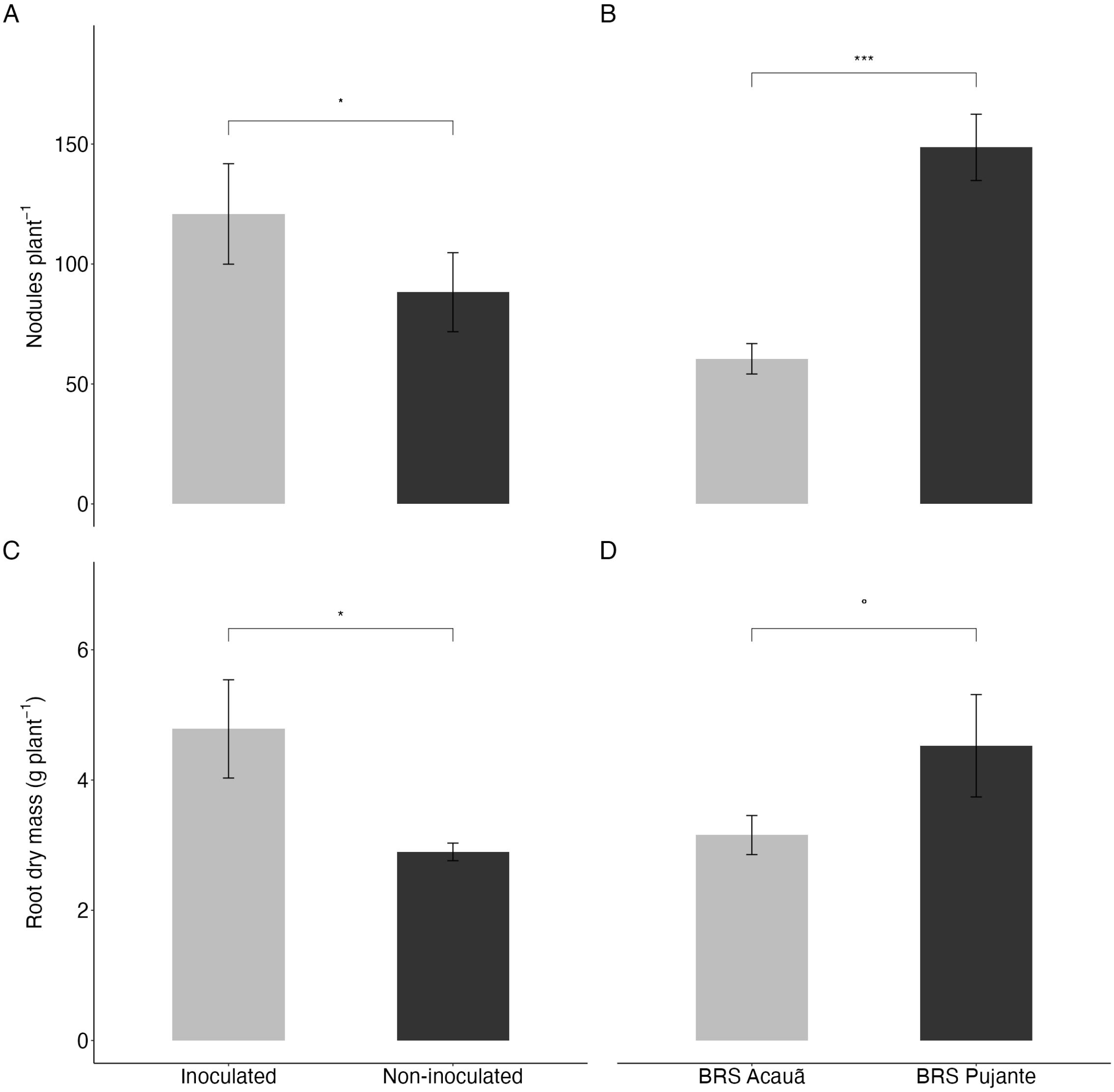

Figure 1. Effects of BR 3262 inoculation and cowpea genotype on the number of nodules (A and B) and root dry mass (C and D), in soil colonized by an effective cowpea rhizobial population. Bars represent the errors (n = 4). *** p value < 0.001; * p value < 0.05; o: p value < 0.1.

in the BRS Pujante crown (33 nodules plant^{-1}) and the secondary roots of the BRS Acauã genotype (46 nodules plant^{-1}). The number of nodules between the root regions of the BRS Acauã did not differ, as did the average of nodules per crown, when comparing both genotypes. So, these results show that the superior nodulation of the BRS Pujante over the BRS Acauã originated from secondary roots.

Regarding the BR 3262 nodule occupancy analysis by applying the PCR-specific molecular marker, the quality control treatments showed the efficiency of all nodule DNA extraction and PCR rounds, since no amplifications were observed using the negative controls and non-inoculated plants. In addition, all positive controls amplified the desired amplicon size. After analyzing the 1,237 nodules from the inoculated plants, it was observed that the root region influenced the BR 3262 nodule occupancy ($p < 0.001$), since the nodules from the crown region reached the average of 91.5 % positive BR 3262 amplicons, while, in the secondary roots, the average of 81.4 % was observed (Figure 2B).

In the present experiment, BR 3262 was inoculated under a high cell concentration (10^9 CFU mL^{-1}) and competed against an established population of 10^3 cells g^{-1} of soil. The massive inoculation of

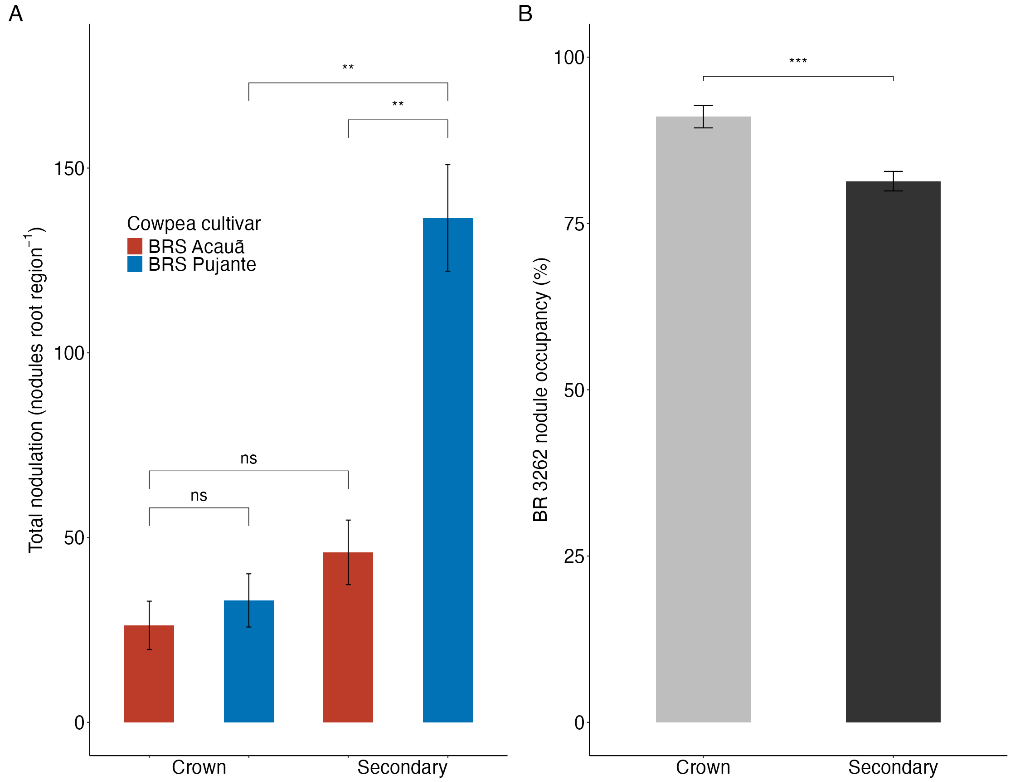

Figure 2. Total cowpea nodulation on the crown and secondary root regions (A) and percentage of nodule occupation by BR 3262 in both plant regions of inoculated cowpeas (B). Bars are the errors (n = 4). *** p value < 0.001; ** p value < 0.01; ns: non-significant.

BR 3262 probably gave a competitive advantage to this strain, when compared with the soil native population. However, even though being 1 million times smaller than the inoculated population, the soil bacteria occupied almost 10 % of the nodules in the crown region and nearly 20 % of the nodules on the secondary roots, indicating a high competitiveness of soil rhizobia. An occupation of more than 80 % of the crown nodules is found in experiments analyzing the competitiveness of highly efficient and competitive strains (Martins et al. 2003, Ndungu et al. 2018), indicating the high ability of BR 3262 to occupy cowpea nodules and promote plant growth.

Another interesting finding is the presence of BR 3262 in almost 80 % of the secondary roots' nodules, indicating that this strain can persist in the soil over the plant growth, since the secondary roots are younger than the crown region, pointing out the ability of BR 3262 to densely infect cowpea root hairs in the initial root development, in the first root emerged after germination (the region that derives the root crown) and continue the infection of cowpea root hairs over the plant vegetative development, what is a desirable characteristic of an efficient inoculant strain (Martins et al. 2003).

Isolation studies from nodules of cowpea growing in tropical agricultural soils, particularly in soil from the Brazilian drylands, usually retrieve much more Bj-SC than Be-SC strains (Leite et al. 2009, Oliveira et al. 2020, Sena et al. 2020), sustaining the argument that cowpea "prefers" to nodulate with Bj-SC, in detriment of Be-SC. The competition experiment of Bj-SC against BR 3262 strains performed by Nascimento et al. (2021) reinforces this hypothesis. However, with a larger inoculated population of *B. pachyrhizi* BR 3262 under actual field conditions, this inoculant strain can overcome the established soil bacteria and form effective nodules in cowpea, what certainly contributes to the outstanding agronomic performance of this Brazilian inoculant strain.

Finally, *B. pachyrhizi* BR 3262 overcomes the established soil population. It densely occupies the BRS Pujante and BRS Acauã root nodules in the crown and secondary root regions, indicating a high competitiveness and persistence in the soil over cowpea vegetative growth.

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