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BIOLOGY

Selection of efficient rhizobial symbionts for *Cratylia argentea* in the cerrado biome

Seleção de rizóbios eficientes na simbiose com *Cratylia argentea* no bioma cerrado

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ABSTRACT

Cratylia argentea *is a leguminous shrub native to the cerrado, which has great potential for forage production and recovery of degraded areas. This study aimed to isolate, characterize, and select efficient rhizobial strains in symbiosis with* ***Cratylia argentea****. Rhizobacteria were isolated from the nodules of 12-month-old plants and cultivated in pots containing cerrado soil. Twenty-five bacterial strains were obtained, which displayed extensive variability with respect to morphological and symbiotic characteristics.* ***Cratylia argentea*** *seeds were planted in pots containing 5kg of cerrado soil and maintained in the greenhouse. The treatments consisted of 25 rhizobial isolates, two controls (without nitrogen and without inoculation), with or without nitrogen fertilization (5mgN·plant^{-1}·week^{-1}), and four replications. Plants were cultivated for 150 days after planting seeds to evaluate nodule number, nodule dry weight, shoot and root dry weight, shoot and root N content, and relative and symbiotic efficiency. Thirteen isolates improved shoot dry weight (up to 65.8%) and shoot nitrogen concentration (up to 76%) compared with those of control treatments. Two isolates, 4 (CR42) and 22 (CR52), conferred higher symbiotic efficiency values of approximately 20%. Therefore, these two rhizobial isolates displayed the highest potential as beneficial inoculants to optimize the symbiotic efficiency for* ***Cratylia*** *and to increase the incorporation of nutrients and biomass into the productive system in the cerrado.*

Key words: *biological fixation, legumes, cerrado.*

RESUMO

Cratylia argentea *é uma leguminosa arbustiva nativa do cerrado, com alto potencial para produção de forragem e recuperação de áreas degradadas. Este trabalho teve como objetivo isolar, caracterizar e selecionar estirpes de rizóbios eficientes na simbiose com* ***C. argentea****. As bactérias foram isoladas de nódulos de plantas com 12 meses de idade, cultivadas em solo de cerrado, em vasos. Foram obtidas 25 estirpes, que apresentaram alta variabilidade em relação às características morfológicas e simbióticas. Sementes de* ***C. argentea*** *foram plantadas em potes contendo 5kg de solo de cerrado, e mantidos em casa de vegetação. Os tratamentos consistiram em 25 isolados, controle (sem N e sem inoculação) e adubação nitrogenada (5mgN·planta^{-1}·semana^{-1}), em quatro repetições. As plantas foram colhidas 150 dias após o plantio para avaliar o número e a massa seca de nódulos, a massa seca e concentração de nitrogênio da parte área e das raízes. Foram calculadas as eficiências relativas e simbióticas. Treze estirpes se destacaram quanto ao acúmulo de massa seca (até 65,8%) e acúmulo de nitrogênio (até 76%), da parte aérea, em relação ao tratamento controle. Dentre estas, as estirpes 4 e 22 apresentaram maiores eficiências simbióticas (em torno de 20%). Portanto, estas estirpes apresentaram potencial para formulação de inoculantes, visando otimizar o processo simbiótico rizóbio-* ***Cratylia*** *e a incorporação de biomassa e nutrientes em sistema produtivos no cerrado.*

Palavras-chave: *fixação biológica, leguminosa, cerrado.*

INTRODUCTION

The cerrado is the second-largest brazilian biome; it covers twelve states and occupies an area equivalent to approximately 25% of the national territory. The cerrado contains an abundant variety of endemic species and is one of the world's biodiversity hotspots, which has made it a global conservation priority (MYERS et al., 2000). However, this biome has undergone deforestation in approximately 50% of its area, and only 30% of its original biodiversity remains. These losses are primarily due to urban

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expansion and the proliferation of agricultural and mining activities.The importance of the cerrado in brazilian agribusiness is significant, considering that this biome accounts for more than 50% of the national grain and meat production (MARQUES et al., 2014).

Successful strategies for the recovery of degraded areas recommend that native species of the biome of interest are used for revegetation. For the brazilian cerrado, plants of the legume family (***Fabaceae***, ***Papilionaceae***) are the preferred native species, especially leguminous shrubs and trees. These plants form symbiotic relationships with nitrogen- fixing bacteria, called rhizobacteria, which enhanced the inputs of carbon, nitrogen, and nutrient cycling, and ultimately facilitate the production of greater biomass while reducing the need for nitrogenous fertilizers (CHAER et al., 2011; MARQUES et al., 2014).

Cratylia argentea (Desv.) O. Kuntze, also known as "camaratuba, copada, or cipó-prata", is a legume (***Fabaceae***, ***Papilloneidae***) and is an endemic leguminous species in central Brazil (native of cerrado and caatinga), Bolivia, and Peru (GALDINO et al., 2010; TEIXEIRA et al., 2010; MARQUES et al., 2014; VALLES DE LA MORA et al., 2014). ***Cratylia argentea*** has great potential as a forage plant because it is a deep-rooting shrub with high protein biomass, produces abundant seeds with excellent germination, has strong regrowth capacity, tolerates nutrient-deficient and acidic soils, and is drought resistant owing to its vigorous root development (LASCANO et al., 2002; REYES et al., 2007; ALPALA et al., 2010; CASTILLO-GALLEGOS et al., 2013). These characteristics support its use as aforage plant for agro-pastoral systems and for recovery of degraded areas (MARQUES et al., 2014).

Biological nitrogen fixation (BNF) optimization depends on rhizobial inoculation of the legume seeds during planting. The inoculants should be rhizobial strains with high performance, including strong survival, competitiveness, and host plant root colonization, compared with the performance of native strains at the sites of interest (STRALIOTTO et al., 2002). Edaphic factors such as acidity (pH) and aluminum concentration can limit nodule formation and activity, and plant nitrogen assimilation (HUNGRIA & VARGAS, 2000). CHAER et al. (2011) reported that some N-fixing rhizobial species have preferences and specificities for different legume species, so it is crucial to select optimized strains for specific applications. CHAGAS JR (2010) noted that different rhizobial strains display optimum performance under different environmental conditions, and it is necessary to select rhizobia that are adapted to the environmental conditions in the ecosystem of interest.

Studies on the selection, phenotypic characterization, and availability of rhizobial strains for ***Cratylia argentea*** are limited to a few institutions such as the International Center for Tropical Agriculture (CIAT) in Cali, Colombia, and Embrapa National Center for Research on Agrobiology in Rio de Janeiro, Brazil. In general, the specificity of microsymbionts versus macrosymbionts becomes relevant particularly in studies of new isolates to evaluate their nodulation and symbiotic efficiencies in the cerrado biome. MARQUES et al. (2014) used ***Cratylia argentea*** for recovery of degraded areas in the cerrado, and only one individual in the field was associated with native rhizobia. These previous studies strengthen the relevance of the current research. Thus, the aim of this study was to isolate, characterize, and select optimized rhizobial strains for BNF and symbiosis with ***Cratylia argentea*** utilized for forage production and/or recovery of degraded areas in the brazilian cerrado.

MATERIALS AND METHODS

Rhizobial isolation, cultivation, and phenotypic characterization

These studies were conducted in a greenhouse and in a laboratory at the Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil. Nodules were obtained from ***Cratylia argentea*** seedlings (12 months old, grown in cerrado soil), which were used as trap plants (XAVIER et al., 1997). Colonies were isolated and characterized according to the methods reported by VINCENT (1970) and HUNGRIA & ARAÚJO (1994). The isolated strains were cultured on yeast mannitol agar (YMA; pH 6.8) plates containing Congo red, which were incubated for 96 hours at 28°C before strain characterization. The following phenotypic characteristics were observed: COL, color (light pink, orange, red, or colorless); APP, appearance (homogeneous or heterogeneous); TRANS, transparency (opaque, translucent, or transparent); SHA, shape (circular or irregular); EDG, edge type (full or irregular); ELA, elasticity (low, moderate, or high); ADH, adherence to the platinum loop (adherent or non-adherent); MUC, mucus consistency (mucoid, viscous, watery, orbutyrous); SUR, surface (smooth or rough); GRO, growth period [fast (within 4 days) or slow (more than 4 days)] and growth diameter (Ø) (<1mm, 1-2mm,>2mm). Physiological characteristics were evaluated by transferring microorganisms into medium containing bromothymol blue to measure culture pH (acid, neutral, and alkaline). This protocol enabled selection of 25 strains to test for symbiotic efficiency with the host plant.

Test for symbiotic efficiency of rhizobial isolates

To evaluate the symbiotic efficiency of the isolated rhizobial strains, soil samples were collected in three different areas of the cerrado in Sete Lagoas. Soil samples were classified as dystrophic dark-red latosol and cerrado. Soil samples were transferred without sterilization into pots with 5kg capacity in a greenhouse (28ºC; natural light in summer, humidity 65%). The experimental treatments were as follows: each selected rhizobial strain, two controls (without nitrogen and without inoculation), and with and without nitrogen fertilization (5mg N·plant^{-1}·week^{-1}) in the form of ammonium nitrate. Seeds were disinfected superficially with alcohol (70%) for 30s, followed by hypochlorite (0.5%) for 10min and sterile distilled water and then inoculated with 2mL of enriched bacterial suspension (10^8cells·mL^{-1}) in YMA medium (For 1L: Yeast extract 1.0g; Mannitol, 10g; Dipotassium phosphate, 0.5g; Magnesium sulfate, 0.2g; Sodium chloride, 0.1g; Calcium carbonate, 1.0g; Agar, 15.0g). Experimental pots were placed using a randomized block design with four replications. Seedlings were thinned to a final density of three plants per pot. All treatments, including controls treatments (without nitrogen and without inoculation), received half strength nutrient solution without nitrogen once a week according to HUNGRIA & ARAÚJO (1994). Plants were irrigated with distilled water when necessary to maintain the humitidy.

Plants were harvested 150 days after seeds were planted. Plants were evaluated for number of nodules (NN), root dry weight (RDW), shoot dry weight (SDW), nodule dry weight (NDW), and N content of shoot and roots. For dry weight measurements, plants were dried in an oven at 60°C until they achieved a constant weight. Relative efficiency (*REf*) of each strain was calculated according to Equation (1):

REf = SDW inoculated treatment / SDW N treatment x 100 (1)

Where SDW = shoot dry weight of the inoculated plants and SDW N treatment = shoot dry weight of plants treated with nitrogen fertilization.

Plant symbiotic efficiency (SE) was calculated according to Equation (2):

SE = [(Ntotal fixed – NtotalCT) / (Ntotal NT – NtotalCT) x 100] (2)

Where N total fixed = total nitrogen content of the inoculated plants; Ntotal CT = total nitrogen content of the control plants without nitrogen fertilization; and N total NT = total nitrogen content of the control plants treated with nitrogen fertilization.

Results were subjected to variance analysis. Averages were compared by the Scott-Knott test (5% probability) using the statistical program SISVAR version 5.3.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization of rhizobia from ***Cratylia argentea***

The ***Cratylia argentea*** trap plants displayed a large NN (59-76 nodules per pot), which were established by natural rhizobial populations in the soil. The start of nodulation was observed at 70 days after seed germination. At that time, nodules were forming primarily in the secondary roots, but also in the main root. These results differed from those reported by OLIVEIRA et al. (1998), who stated that ***Cratylia argentea*** nodulation occurred 100 days after seed germination and only in secondary roots.

Three healthy nodules (those with typical internal pink color) were selected for plating from each plant. The characteristic pink color indicated the presence of leg hemoglobin, which is the protein that transports and releases oxygen in nodules under adequate partial pressure; oxygen is required for nitrogenase activity and nitrogen fixation in mature symbiotic nodules (VINCENT, 1970).

Phenotypic characterization of the rhizobial isolates detected substantial morphological variability among different colonies for all evaluated parameters except transparency and surface (Figure 1). All isolates were translucent and had smooth surfaces. The observed colonies exhibited predominantly light pink color (76%), circular shape (100%), full edge (100%), and mucoid consistency (60%). Only 16% of isolates displayed elasticity, whereas 92% of isolates easily adhered to the platinum loop. Morphological characteristics provide important information that can be used to determine the identity and group classification of isolated strains (VINCENT, 1970), symbiotic efficiency between rhizobia and host plants, and rhizobial diversity in a particular ecosystem community (SANTOS et al., 2007). CHAGAS JR et al. (2010) proposed that analyses of morphological and physiological differences between microorganisms is the crucial first step for identifying new taxonomic groups, and results can be used to guide subsequent

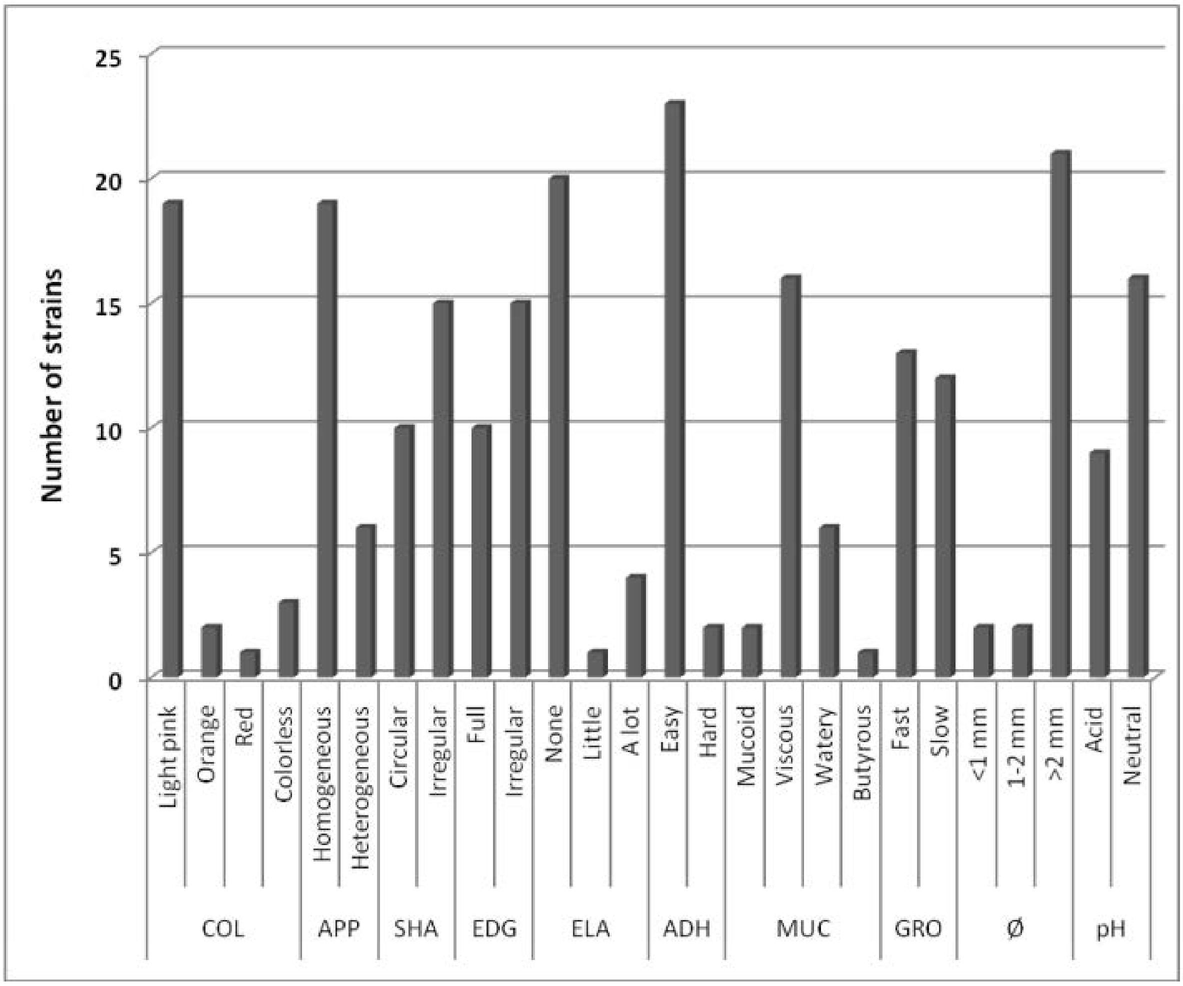

Figure 1 - Distribution of morphological characteristics for rhizobial isolates obtained from Cratylia argentea root nodules: color (COL); appearance (APP); shape (SHA); edge type (EDG); elasticity (ELA); adherence to the platinum loop (ADH); mucus consistency (MUC); growth period (GRO); colony diameter (Ø); and pH.

analyses such as molecular genetic properties. In the present research, morphological analysis resulted in the selection of 25 rhizobial strains (Table 1). These strains displayed morphological and physiological differences, and were evaluated in symbiosis with ***Cratylia argentea*** plants.

Growth rate of 25 selected strains differed, with 13 showing fast growth (up to 4 days) and 12 showing slow growth (more than 4 days). These results indicated that isolated strains that are competent to nodulate ***Cratylia argentea*** display significant metabolic diversity. Another interesting finding was that 14 of the isolated strains maintained the pH of the culture medium at approximately 7.0, whereas nine strains slightly acidified the culture medium (pH approximately 6.0), and two strains strongly acidified the culture medium (pH approximately 4.0). NORRIS (1965) proposed that rhizobial acid release functions as an adaptive mechanism to adverse conditions. Our results are consistent with those of TEIXEIRA et al. (2010), who evaluated rhizobial strains of ***Cratylia mollis*** in caatinga soil and reported that most isolates had rapid growth and did not alter the medium pH.

Selection of efficient rhizobia in symbiosis with ***Cratylia argentea***

Table 1 presents the morphological, physiological, and symbiotic characteristics (nodulation, growth, and nitrogen content) of ***Cratylia argentea*** plants subjected to different treatments and

Table 1 - Physiological characteristics of ***Cratylia argentea*** (three plants per pot) that were subjected to control treatment (CT), treatment with 25 rhizobial strains (1 to 25), and nitrogen fertilizer treatment (NT). Mean* values for number of nodules per pot (NN)**, nodule dry weight per pot (NDW), shoot dry weight per pot (SDW), root dry weight per pot (RDW), shoot nitrogen content per pot (NSH), and root nitrogen content per pot (NRO). The relative efficiency (REf) and symbiotic efficiency (SE) for each test is presented.

Test/Strain	------NN------	----NDW (g)---	---SDW (g)----	----RDW (g)-----	----NSH (g)-----	-----NRO (g)-----	REf (%)	SE (%)
CT	67 ^{b}	0.27 ^{b}	5.96 ^{c}	4.59 ^{a}	0.17 ^{c}	0.08 ^{c}	–	–
1	133 ^{a}	0.50 ^{a}	8.40 ^{b}	6.31 ^{a}	0.24 ^{b}	0.11 ^{b}	44.63	10.92
2	137 ^{a}	0.43 ^{b}	8.12 ^{b}	6.31 ^{a}	0.23 ^{b}	0.14 ^{b}	43.15	12.29
3	119 ^{a}	0.61 ^{a}	9.51 ^{b}	6.67 ^{a}	0.26 ^{b}	0.14 ^{b}	50.5	15.59
4	116 ^{a}	0.61 ^{a}	9.88 ^{b}	6.73 ^{a}	0.28 ^{b}	0.15 ^{b}	52.48	19.59
5	95 ^{a}	0.54 ^{a}	9.66 ^{b}	6.17 ^{a}	0.29 ^{b}	0.13 ^{b}	51.32	17.85
6	77 ^{b}	0.37 ^{b}	7.12 ^{c}	5.95 ^{a}	0.22 ^{b}	0.14 ^{b}	37.81	10.77
7	79 ^{b}	0.57 ^{a}	8.70 ^{b}	5.73 ^{a}	0.26 ^{b}	0.12 ^{b}	46.23	14.8
8	92 ^{a}	0.51 ^{a}	6.67 ^{c}	7.51 ^{a}	0.23 ^{b}	0.18 ^{b}	35.43	17.19
9	104 ^{a}	0.50 ^{a}	8.17 ^{b}	5.89 ^{a}	0.23 ^{b}	0.13 ^{b}	43.42	12.23
10	95 ^{a}	0.52 ^{a}	8.42 ^{b}	6.33 ^{a}	0.25 ^{b}	0.15 ^{b}	44.71	14.93
11	75 ^{b}	0.49 ^{a}	7.37 ^{c}	6.40 ^{a}	0.25 ^{b}	0.14 ^{b}	39.16	15.17
12	40 ^{c}	0.22 ^{b}	4.30 ^{d}	2.25 ^{b}	0.13 ^{c}	0.05 ^{d}	22.85	–6.88
13	80 ^{b}	0.66 ^{a}	7.48 ^{c}	5.70 ^{a}	0.24 ^{b}	0.12 ^{b}	39.72	11.35
14	106 ^{a}	0.63 ^{a}	7.58 ^{c}	5.83 ^{a}	0.25 ^{b}	0.14 ^{b}	40.26	14.52
15	49 ^{c}	0.27 ^{b}	4.34 ^{d}	1.90 ^{b}	0.13 ^{c}	0.04 ^{d}	23.04	–7.61
16	100 ^{a}	0.63 ^{a}	7.01 ^{c}	5.86 ^{a}	0.23 ^{b}	0.13 ^{b}	37.25	12.28
17	83 ^{b}	0.59 ^{a}	6.90 ^{c}	5.40 ^{a}	0.22 ^{b}	0.13 ^{b}	36.66	11.47
18	107 ^{a}	0.71 ^{a}	8.16 ^{b}	5.60 ^{a}	0.26 ^{b}	0.13 ^{b}	43.37	13.7
19	81 ^{b}	0.58 ^{a}	7.84 ^{b}	5.68 ^{a}	0.25 ^{b}	0.12 ^{b}	41.65	12.45
20	68 ^{b}	0.53 ^{a}	7.11 ^{c}	5.38 ^{a}	0.23 ^{b}	0.12 ^{b}	37.77	11.08
21	85 ^{a}	0.55 ^{a}	8.72 ^{b}	5.72 ^{a}	0.29 ^{b}	0.12 ^{b}	46.32	17.1
22	102 ^{a}	0.60 ^{a}	9.01 ^{b}	6.07 ^{a}	0.30 ^{b}	0.14 ^{b}	47.89	20.1
23	49 ^{c}	0.27 ^{b}	7.56 ^{c}	1.79 ^{b}	0.21 ^{b}	0.03 ^{d}	40.15	0.5
24	113 ^{a}	0.67 ^{a}	6.55 ^{c}	6.07 ^{a}	0.22 ^{b}	0.13 ^{b}	34.8	10.58
25	94 ^{a}	0.52 ^{a}	8.77 ^{b}	5.49 ^{a}	0.24 ^{b}	0.10 ^{b}	46.61	10.73
NT	0 ^{d}	–	18.82 ^{a}	7.57 ^{a}	0.98 ^{a}	0.26 ^{a}	–	–

*Means with the same letter in the column do not differ significantly by the Scott-Knott test at 5% probability.
**The number of nodules was transformed to log x+1 for statistical analysis.

harvested 150 days after germination. There were significant differences ($P<0.05$) between treatments for all parameters. The NN ranged between 40 and 137 per pot, and displayed various sizes and shapes. The majority of nodules were spherical, except for plants treated with nitrogen fertilization, which did not produce nodules. In general, individual nodule structures were produced, although a few siamese nodules also could be observed. These results are consistent with those of SCHEFFER-BASSO et al. (2000) for nodules of ***Adesmia araujoi*** (aloe), which also grows in acidic and low-fertility soils. Soil characteristics significantly influenced the rhizobial population in the soil (BALA et al., 2003). These results provided further evidence to support isolation and selection of specific ***Cratylia argentea*** rhizobia in cerrado soil.

Values for accumulation of SDW and RDW ranged from 4.30-9.88 and 1.79-7.51g per pot, respectively, depending on the isolated strain (Table 1). The highest values for SDW and RDW correspond to biomass increases of 65.8% and 63.6%, respectively, compared with those of controls (without nitrogen fertilizer and without inoculation). However, these values represented 52.5% of the SDW and 88.9% of the RDW, respectively, of ***Cratylia argentea*** plants treated with nitrogen fertilizer.

Nitrogen accumulation in the shoot (NS) and in the root (NR) ranged from 0.13-0.30 and 0.03-0.18g per pot, respectively. The highest values were equivalent to increases of 76% and 125%, respectively, compared with those of the control treatment, and equivalent to 31% and 69%, respectively, compared with those plants treated with nitrogen fertilizer.

The Scott-Knott test ($P<0.05$) indicated that 13 isolated strains (1, 2, 3, 4, 5, 7, 9, 10, 18, 19, 21, 22, and 25) showed significantly greater SDW compared with those of other isolates and controls, although the SDW values were significantly lower than those of plants receiving nitrogen fertilizer. There were no significant differences among RDW values for any of the isolates or controls either with or without nitrogen fertilizer. The 13 isolates had NS and NR values that were significantly lower than those of plants treated with nitrogen fertilizer, but were higher than those of the controls. These combined results indicate that specific rhizobial strains can enhance BNF in ***Cratylia argentea***. Therefore, it is important to genetically characterize new rhizobial isolates with specific host and environmental preferences to increase cerrado biomass production and promote natural reparation of environmentally degraded cerrado areas.

We computed Pearson correlations for total dry weight (TDW=SDW+RDW) and total nitrogen (NT) (0.911), for TN and the number of nodules (NN) (0.575), and for TDW and NN (0.607); all correlations were positive and significant. These results are in agreement with those reported by DÖBEREINER (1966), which suggested that nodulation can be used as an indicator of BNF efficiency. By contrast, OLIVEIRA et al. (1998) reported no significant correlation between the number of nodules and the fixed nitrogen content in ***Cratylia argentea*** tissues.

Table 1 presents the estimates for relative BNF efficiency based on our results for dry weight accumulation, symbiotic efficiency (SE), and plant nitrogen accumulation. The highest relative efficiency was 52.5%, which was computed for plants inoculated with isolated strain 4. The highest symbiotic efficiencies were computed for plants inoculated with isolated strain 4 (19.6%) and strain 22 (20.1%). The correlation between the relative BNF efficiency and the SE was positive and highly significant (r=0.80, $P<0.01$).

Strains with higher SE (strains 4 and 22) have growth rates that are significantly different than those of strains with negative SE (strains 12 and 15). Strains with higher SE grew rapidly, whereas strains 12 and 15 grew slowly. SANTOS et al. (2007) proposed that fast growth could confer a competitive advantage over others oil microbiota, which could partly explain our results.

Our combined results indicated that the selection of rhizobial strains for this legume is a promising practice to optimize ***Cratylia***-rhizobial interactions, fixed organic nutrient concentrations, and biomass forage production in the cerrado environment. Results highlighted the potential of two selected strains (strains 4 and 22) to optimize efforts for legume biomass production and environmental reparation in the brazilian cerrado. The protocol developed in this study can be used as a guide for rhizobial strain selection to optimize BNF in ***Cratylia argentea***.

CONCLUSION

There was extensive variability among rhizobial strains associated with ***Cratylia argentea*** in cerrado soil, which varied with respect to morphology, SE, and relative BNF efficiency. Most of the tested isolates maintained the soil at neutral pH. Further testing and genetic characterization should be performed for 13 isolated strains that enhanced shoot biomass production (strains 1, 2, 3, 4, 5, 7, 9, 10, 18, 19, 21, 22, and 25) and particularly for two strains, 4 (CR42) and 22 (CR52), that also had the highest symbiotic efficiencies. The 13 selected strains are promising for future field trials even in different local cerrado environments which aimed to optimizing BNF in the ***Cratylia argentea***-rhizobia interaction.

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