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# *Cross-Feeding* Among Soil Bacterial Populations: Selection and Characterization of Potential Bio-inoculants

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**Abstract**

The biological nitrogen fixation constitutes a strategy to accelerate soil reclamation and the symbiotic systems Rhizobium-legume is the major $N_2$-fixing in which the enzyme carboxymethyl cellulase plays a key role. As many rhizobia species are cellulase negative, the association with cellulolytic bacteria can be a strategy for the recovery of degraded ecosystems. It has been hypothesized that the sharing of resources should mostly be prevalent among phylogenetically and metabolically different species. Accordingly, twenty-seven actinobacteria isolates from Actinobacteria phyla and twenty-six rhizobia isolates from Proteobacteria phyla were selected from the bacterial collection of the Laboratory of Environmental Microbiology of the Federal University of Ceará. The presence of cellulolytic activity was observed for the rhizobia isolates at 28 °C and for actinobacteria isolates at 28, 39, 41, 43 and 45 °C. Rhizobia isolates deficient in cellulase and actinobacteria isolates with enzymatic activity detected at higher temperature were selected and characterized. The antagonism between isolates of two groups was tested and the pairs antagonistic were eliminated. The *cross-feeding* test between actinobacteria and rhizobia isolates was realized in a chemically defined medium containing carboxymethyl-cellulose as the only carbon and energy source. Growth of rhizobia strains in 50% of the pairwise indicated that the cellulose hydrolyzed by actinobacteria was used as substrate for the growth of the rhizobia. The *Bradyrhizobium* strain R10 associated with *Streptomyces* strains A09 and A18 and *Nocardia* A11 are promissory inoculants for recovery of semi-arid regions.

**Keywords:** metabolic interactions, revegetation, actinobacteria, rhizobia, semiarid region

## 1. Introduction

Semiarid environments are characterized by high temperatures, low water availability, high salinity and low nutrient content in the soil which may be attributed to low nitrogen fixing activity (Divito & Sadras, 2014).

Brazil has one of the largest semi-arid areas in the world (about 1 million $km^2$) and about 94% of this region is characterized as having moderate to high susceptibility to desertification resulting from the climatic variations and human activities (Vieira et al., 2015).

Cowpea (*Vigna unguiculata* (L) Walp) is a typical legume of the Brazilian northeast because of its adaptability to severe climatic conditions. The root of this plant is nodulated by a bacterial group generically known as rhizobia (Zilli, Valisheski, Freire Filho, Neves, & Rumjanek, 2004), classified in the Proteobacteria phyla and that act as the primary symbiotic fixer of nitrogen (Seishiro, Motomi, & Wataru, 2013).

Nitrogen fixation is energetically an expensive process, dependent on the available energy from plant residues that contain a high percentage of cellulose. For this reason, the production of cellulolytic enzymes has been considered an indirect mechanism to promote plant growth (Gupta, Parihar, Ahirwar, Snehi, & Singh, 2015). Furthermore, the establishment of rhizobia within legume root nodules requires the disruption of the plant cellular wall (Robledo et al., 2008) and the carboxymethyl cellulase (CMCase) is the enzyme used to establish this symbiosis (Chen, Wei, Chang, & Lin, 2004). However, the cellulolytic activity of rhizobia is very low and there are few species that can fix nitrogen (N) and also use cellulose as their main energy and carbon source (Jiménez-Zurdo, Mateos, Dazzo, & Martínez-Molina, 1996).

Biological nitrogen fixation in arid and semiarid areas is influenced by the higher soil temperatures (Alexandre & Oliveira, 2013). The areas are deficient in soluble phosphorous (Sinaj et al., 2001) and characterized by the presence of antibiotics substances (Naamala, Jaiswal, & Dakora, 2016). Bacterial species nitrogen fixing differ in their tolerance to main environmental stresses (Graham, 1992), therefore, the screening of indigenous strains is imperative for decreasing soil loss and ameliorating adverse edaphic conditions.

Among the bacterial soil population, the Actinobacteria constitute one of the largest phyla and represents the most abundant phylum in the Brazilian northeastern semiarid region (Kavamura et al., 2013). This bacterial group produces an extensive range of bioactive molecules such as cellulases (Saini, Aggarwal, Sharma, & Yadav, 2015), and also substances active against other microorganisms (Lima, Martins, Siqueira, Soares, & Martins, 2017).

A process that governs the growth and composition of the microbial ecosystem is the *cross-feeding* that involves the metabolic cooperation between microorganisms in partnerships allowing accessing substrates that neither microorganism could metabolize alone (Seth & Taga, 2014; D'Souza et al., 2018). In ecological terms, *cross-feeding* is a type of facilitation where one organism makes the environment more suitable for another (Bronstein, 2009). In nature, bacterial competition for resources is common, but positive interactions among bacteria also occur, especially during the degradation of complex substrates (Wintermute & Silver, 2010). Thus, in a cooperative process in which cellulolytic and nitrogen-fixing microbiota coexist, the cellulose breakdown continues without further depletion of nitrogen and both types are benefited by this process. So, co-inoculation of leguminous plants with cellulolytic actinobacteria and rhizobia is a strategy to enhance nitrogen fixation contributing to revegetation and recuperation of desertified soil. The use of native species is recommended because these bacteria adapt to the specific environmental conditions, which facilitates their survival and the successful nodulation of the host plant (Koskey et al., 2017).

Although association of actinobacterial and rhizobial strains has been described as promoting plant growth (Soe et al. (2012), Htwe and Yamakawa (2015), Soe and Yamakawa (2016), Htwe et al. (2018a, 2018b), studies involving *cross-feeding* between these two groups still are rare, especially in arid and semi-arid environments. Moreover, studies with bacteria isolated from the same environment, maximize resemblance to the natural community and preserves indigenous interactions shaped by co-adaptation/evolution (Stadie, Gulitz, Ehrmann, & Vogel, 2013).

Based on the above, this work aimed to evaluate *in vitro* the metabolic compatibility between strains of actinobacteria and rhizobia isolated from soil in the Brazilian semiarid for selection of potential bioinoculants.

## 2. Material and Methods

This study was approved by the Institute for Biodiversity Conservation (ICMBio) in accordance with Brazilian law (Permit Number: SISBIO38980-3).

The bacterial isolates used (26 rhizobia and 27 actinobacteria) were maintained in the cultural collection of the Laboratory of Environmental Microbiology of the Federal University of Ceará (UFC). They were isolated from soil samples collected in the period of August 20-24, 2012 in Ubajara National Park (UNP), a protected environmental area on the Ibiapaba plateau in northern Ceará, Brazil. The Park is centered at latitude 3º46′ S and longitude 40º54′ W, with altitudes ranging from 800 to 1100 m above sea level (Cunha & Araújo, 2014) and two distinct climate periods: the first half of the year, cooler and wetter, and the second half, hot with virtually no rainfall (IBAMA, 2006).

Rhizobia isolates were captured using cowpea [*Vigna unguiculata* (L.) Walp.] as bait plant and the nodules macerated in agar medium mannitol yeast extract (YMA) (Vincent, 1970). The isolates in which were observed nodules root and the red color were authenticated by planting and inoculation in jars of 300 mL with cowpea (Torres-Júnior et al., 2014). The actinobacteria were isolated in casein dextrose agar (CDA) (Clark, 1965) and the isolates from the two groups were reactivated in the same liquid culture media and inoculated in Petri dishes to verify the purity.

The actinobacteria and rhizobia isolates were inoculated separately in culture medium containing carboxymethylcellulose as only source of carbon and energy. The rhizobia isolates were incubated at 28±2 °C for 10 days and actinobacteria isolates were incubated at 28, 39, 41, 43 and 45 °C to determine the effect of temperature on the enzyme activity. After incubation, Congo red solution was added to each plate and left at rest for 15 minutes at 26 °C. Thereafter, the excess solution was drained and 10 ml of NaCl solution (2M) was added to each dish and left at rest for 30 minutes at 26 °C. The presence of hydrolysis zones around colonies was registered as a positive response (Hankin & Anagnostakis, 1977). Each experiment was carried out

independently by taking four replicates with two repetitions. Non-cellulolytic rhizobia isolates and actinobactéria isolates with enzymatic activity at more elevated temperature were selected to *cross-feeding* test.

To discard possible antagonism between selected actinobacteria and rhizobia isolates each actinobacteria was inoculated with each one of the rhizobia isolates. Accordingly, the plates were incubated for 5 days at 28 °C and inhibitory activity was confirmed by the appearance of a clear zone (inhibition zone) extending from the colony of the actinobacteria to the rhizobia growth border (Lima et al., 2017). The pairwise in which actinobacteria isolate inhibited the rhizobia isolate were discarded. All tests were performed in triplicate with three repetitions.

The cultural characterization of the cellulolytic actinobactéria selected was performed by observation of the aerial and reverse mycelium with the use of a color chart (Wink, 2012). Morphological characterization was carried out by micro cultivation under a Zeiss light microscope at 1000 magnification and for the production of melanin the strains were grown in medium with and without tyrosine (Lima et al., 2017). Tolerance to pH (4, 5, 6, 7, 8 and 9), sodium chloride (0, 5, 10, 20, 25 and 35 g $L^{-1}$) and temperature (41, 43 and 45 °C) was evaluated using the CDA culture medium (Priyadarshini et al., 2016). Moreover, the use of carbon sources (arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose), with glucose (positive control) and water (negative control) (Shirling & Gottlieb, 1966) and nitrogen sources (potassium nitrate, L-histidine, Arginine) using as positive control (L-asparagine) and medium without nitrogen as negative control (Basak & Majumdar, 1973) were determined. Production of lipase (Sierra, 1957) amylase (Tole, Fawade, & Patil, 2016) and xylanase (Tasia & Melliawati, 2017) was evaluated in culture medium containing the inducers Tween 80, starch and xylan, respectively.

Non-cellulolytic Rhizobia isolates were analyzed using morph-cultural characteristics (growth rate, color and slime production) and resistance to antibiotics Amikacin (AMI 30), Amoxicillin (AMC 30), Ampicillin (AMP10), Aztreonam (ATM 30), Cephalothin (CFL 30), Cefepime (CPM 30), Cefoxitin (CFO 30), Ceftadizime (CAZ 30), Ceftriaxone (CRO 30), Ciprofloxacin (CIP 05), Chloramphenicol (CLO 30), Gentamycin (GEN 10), Piperacillin (PIT 110) and Tetracycline (TET 30) (Mueller, Skipper, Shipe, Grimes, & Wagner, 1988). Moreover, the use of carbon sources (sucrose, glucose, arabinose, rhamnose, fructose and xylose), amylase, and phosphate solubilization were determined according to Shirling and Gottlieb (1966), Tole et al (2016) and Pikovskaya (1948), respectively. Tolerance to sodium chloride (0, 5, 10, 20, 30, 40 and 50 g $L^{-1}$), pH (4 and 10) and temperature (39, 41, 43 and 45 °C) was performed in YMA medium (Kulkarni & Nautiyal, 2000). The strains BR 3486 (*Burkholderia phymatum*), INPA 03-11B-*Bradyrhizobium* sp. (BR 3301), UFLA 03-84-*Bradyrhizobium* sp (BR 3302), BR 3267 (*Bradyrhizobium* sp.), BR 3262 (*Bradyrhizobium* sp.), BR 2003, and BR 2801 (*Bradyrhizobium elkanii*) were used as references.

For the selection of the most promising rhizobia isolates for each characteristic evaluated was established a range of values of 0-5 in the total maximum of 22 scores (Table 1).

Table 1. Scores for phenotypic characteristics of rhizobia isolates

| Rate growth | Score | Temperature (ºC) | Score | NaCl (g $L^{-1}$) | Score | No. of Antibiotics | Score | Carbon sources | Score | Phosphorous solubilization | Score | pH | Score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Slow | 0 | < 39 | 1 | < 10 | 0 | 0 | 0 | 0 | 0 | + | 1 | 4 | 1 |
| Fast | 1 | 39 | 2 | 10 | 1 | 01-03 | 1 | 1-3 | 1 | - | 0 | 10 | 1 |
| - | | 41 | 3 | 20 | 2 | 04-06 | 2 | 4-6 | 2 | - | - | - | - |
| - | | 43 | 4 | 30 | 3 | 07-09 | 3 | 7-9 | 3 | - | - | - | - |
| - | | 45 | 5 | 40 | 4 | 10-12 | 4 | - | - | - | - | - | - |
| - | | - | - | 50 | 5 | 13-14 | 5 | - | - | - | - | - | - |

Molecular characterization of cellulolytic actinobacteria and non-cellulolytic rhizobia isolates was partially performed (Pereira, Ibanez, Rosenblueth, Etcheverry, & Martinez-Romero, 2011).

*Cross-feeding* assay was realized according to Cuesta et al. (2012) with modifications. In the experimental design each rhizobial strain deficient for the production of cellulase was paired with an actinobacteria strain still capable of producing cellulase at the highest temperature tested. Accordingly, actinobacteria strains were inoculated separately in carboxymethylcellulose agar in the form of spots and incubated at 28±2 °C for 10 days. After, one milliliter of culture of each rhizobia isolate previously cultivated in YM medium for seven days, was transferred to micro tubes with a capacity of 1.5 mL, centrifuged at 12,000 rpm for 10 minutes, and the precipitate resuspended two times. A droplet of each purified rhizobia culture was distributed around each colony of actinobacteria at a distance of 0.5 to 2.0 cm. The growth of rhizobia strains closer to the actinobacteria was the

parameter to diagnose for the occurrence of positive metabolic interaction. Moreover, the assay was performed in duplicate with two repetitions.

Compatibility Index was calculated as the number of compatible pairs detected divided by all possible pairwise interactions.

CI = nº of compatible pairs/all pairwise interactions.

**3. Results and Discussion**

In the present work, at 28 °C, 18 over 27 (66.7%) of the actinobacteria isolates presented cellulolytic activity but, at 39 °C only 7 (25.9%) isolates were still positive, at 41 °C six of the seven isolates maintained this characteristic and at 43 °C and 45 °C none isolate produced cellulase. Thus, the actinobacteria isolates A06, A09, A11, A12, A13 and A18 were selected for posterior *cross feeding* test with rhizobia isolates cellulase negative. These isolates were amylase and lipase positive, xylanase negative and able to use all carbon and nitrogen sources tested. The isolates tolerated the maximum temperature evaluated (45 °C), and sodium chloride concentration of 35 g $L^{-1}$. Except A12 that did not tolerate the pH 4 and 9, the others were able to develop in these extreme values. The isolates were identified on the basis of morphological characteristics (Table 2) and the A18 confirmed the identification on the basis of genotypic characteristics.

Table 2. Morphological features of spore chains and aerial and reverse mycelium of the actinobacteria isolates producers of cellulase at 41 °C and the identification at the genus level (Shirling & Gottlieb, 1966)

| Actinobacterial Isolates | Morphology | Color | | Melanin | Genus |
|---|---|---|---|---|---|
| | | Aerial mycelium | Reverse pigments | | |
| A06 | Spiral spore chain | Cream | Cream | (-) | *Streptomyces* |
| A09, A18 | Spiral spore chain | Brown | Gray | (+) | *Streptomyces* |
| A11, A12, A13 | Short straight spore chain | Brown | Blue | (+) | *Nocardia* |

With respect to rhizobia only three isolates grew in the medium supplemented with carboxymethylcellulose, and twenty-three isolates (88.4%) were unable to grow, confirming the inability of this bacterial group to degrade cellulose (Jiménez-Zurdo et al., 1996).

The results of antagonistic activity of the selected cellulolytic actinobacteria isolates on the growth of 23 rhizobia strains deficient in the production of cellulase (118 pairwise combinations) showed that the growth of rhizobia isolates was inhibited only in 14 pairs (Table 3), corresponding to 11.9%. These results confirm that antagonism should mostly be prevalent among phylogenetically and metabolically similar species (Russel, Røder, Madsen, Burmøllea, & Sørensen, 2017). In addition, as these bacteria were isolated from the same environment, they were presumed to have interacted with each other in the soil and over time promoting coexistence between these groups. The fourteen antagonistic pairwise (Table 3) were eliminated from further *cross-feeding* test.

Table 3. Antagonism among strains of actinobactéria and of rhizobia isolates from Brazilian semiarid

| Actinobactéria isolates | Rhizobia isolates | | | | | |
|---|---|---|---|---|---|---|
| | R01 | R03 | R06 | R07 | R11 | R14 |
| A06 | + | + | + | + | - | - |
| A09 | - | - | + | - | - | - |
| A11 | - | - | + | + | + | + |
| A12 | - | - | - | - | - | + |
| A13 | - | - | + | - | + | + |
| A18 | + | - | - | - | - | - |

*Note.* (+): presence of antagonistic activity; (-): absence of antagonistic activity.

It is widely accepted that actinobacteria are prolific producers of natural bioactive compounds, such as antibiotics (Tiwari & Gupta, 2012), and that different rhizobia strains show different degrees of susceptibility (Obaton, 1971), due to genetic variation in target genes and resistance genes acquired through horizontal gene transfer (Bhargava, Murthy, Kumar, & Rao, 2016). Studies *in vitro* on the effects of metabolites of five actinobacteria strains on the growth of five rhizobia strains (25 pairwise combinations) showed that the growth

of rhizobia strains was inhibited in 16 pairs, corresponding to 64%. Antagonism was also observed by Patel (1974) between actinobacteria and 12 strains of rhizobia from soil samples. This author reported that about 23-70% of the actinobacteria inhibited the rhizobia strains. Van Schreven (1964), Damirgi and Johnson (1966), Foo and Varma (1976), Pugashetti et al. (1992), Pereira (1999), and Gregor (2003) have also described the antagonistic action of actinobacteria on rhizobia. More recently, Rassem and David (2017) tested three isolates of actinobacteria in a dual culture against three rhizobium isolates, out of 9 combinations of antagonistic assay 8 showed that actinobacteria executed an inhibitory effect on growth of rhizobium isolate. The antagonistic effect of 14 actinobacteria on 5 rhizobia obtained from soil samples of the same climatic region of the present work registered that of the 70 pairwise combinations 15 (21.4%) presented antibiosis (Lima et al., 2017). In this work, the authors isolated the strains of actinobacteria and rhizobia of soil of different regions of Brazilian semiarid, which may explain the higher rate of antagonism towards present study.

Generally, the low rainfall, extremes of temperature, pH and salinity of the semiarid environs are restraining factors to the growth and activity of the nitrogen-fixing bacteria. Therefore, the selection of native strains with better characteristics may improve the efficiency of process (Marulanda, Porcel, Barea, & Azcon, 2007; Marulanda, Barea, & Azcon, 2009). In this perspective, non-cellulolytic rhizobia isolates were phenotypically characterized and classified into seven groups (Table 4).

Table 4. Grouping of non-cellulolytic rhizobial isolates according to the phenotypic characteristics

| Groups | Isolates | pH | Growth rate | Mucus | Colony color | Resistance (number of of antibiotics) | NaCl (g $L^{-1}$) | Temperature (º C) |
|---|---|---|---|---|---|---|---|---|
| 1 | R16 | Acid | Fast | Butyric | Yellow | 1 | 20 | 41 |
| | R21 | Acid | Fast | Butyric | Yellow | 2 | 10 | 41 |
| | R22 | Acid | Slow | Butyric | Yellow | 2 | 50 | 41 |
| | R15 *Burkholderia* sp. (R 15) | Acid | Fast | Butyric | Yellow | 0 | 10 | 41 |
| 2 | R17 | Acid | Fast | Butyric | White | 1 | 10 | 41 |
| | R19 | Acid | Fast | Butyric | White | 1 | 3 | 41 |
| | R20 | Acid | Fast | Butyric | White | 2 | 10 | 39 |
| | R23 | Acid | Fast | Butyric | White | 2 | 10 | 41 |
| | R24 | Acid | Fast | Butyric | White | 0 | 10 | 41 |
| | R25 | Acid | Fast | Butyric | White | 0 | 10 | 41 |
| | R05 | Acid | Fast | Butyric | White | 0 | 10 | 39 |
| | R09 | Acid | Fast | Viscous | White | 0 | 10 | 41 |
| | *Burkholderia phymatum* (BR-3486) | Acid | Fast | Butyric | White | 1 | 10 | 41 |
| 3 | R18 | Acid | Fast | Butyric | Yellow | 3 | 20 | 45 |
| 4 | R26 | Acid | Fast | Butyric | Yellow | 2 | 50 | - |
| 5 | R07 | Acid | Slow | Butyric | Yellow | 10 | 30 | 39 |
| 6 | R01 | Neut | Slow | Viscous | White | 13 | 3 | - |
| | R03 | Neut | Slow | Viscous | White | 11 | 3 | - |
| | R06 | Neut | Slow | Viscous | White | 12 | 3 | - |
| | R11 *Bradhyrhizobium retamae* | Neut | Slow | Viscous | White | 11 | 3 | - |
| | R12 | Neut | Slow | Viscous | White | 12 | 3 | - |
| | R14 *Bradhyrhizobium retamae* (R11) | Neut | Slow | Butyric | White | 12 | 10 | 39 |
| | *Bradyrhizobium* sp. (BR 3262) | Acid | Slow | Viscous | White | 13 | 3 | - |
| | *Bradyrhizobium* sp. (BR-3301) | Acid | Slow | Viscous | White | 11 | 3 | 39 |
| | *Bradyrhizobium elkani* (BR2801) | Neut | Slow | Viscous | White | 12 | 3 | - |
| | *Bradyrhizobium elkani* (BR2003) | Neut | Slow | Butyric | White | 9 | 3 | - |
| 7 | R04 | Alkali | Slow | Viscous | White | 12 | 10 | 45 |
| | R10 | Alkali | Fast | Viscous | White | 13 | 20 | 45 |
| | *Bradyrhizobium* spp. (BR-3302) | Acid | Slow | Viscous | White | 14 | 10 | 45 |

Although the isolates of Group 6 were distinguished by the higher resistance to antibiotics (Resistance > 11), they were less tolerant to high temperatures and to salinity (Table 4), except R14 that tolerate 10 g $L^{-1}$ of NaCl. It

was also noted that 83% of the isolates of this group suffered antagonistic effect of selected cellulolytic actinobacteria (Table 3) and all showed slow growth. The R11 isolate was identified on the basis of genotype characteristics as *Bradyrhizobium retamae* (Table 4), confirming the similarity of the isolates from Group 6 with the utilized reference strains.

The greatest diversity of rhizobia for the use of carbon, ability to solubilize phosphate and grow in extreme pH were also factors that can positively affected the biological nitrogen fixation (Table 5). The isolated R20 of the Group 2 was the only phosphate solubilizing (Table 5), characteristic very important since the semi-arid soil is deficient in soluble phosphorous in forms of phosphate ion (P).

Moreover, the R15 was identified on the basis of genotype characteristics as *Burkholderia* sp suggesting that the isolates of the Group 1 can also be identified as of the same genus (Table 4).

Table 5. Biochemical characteristics of non-cellulolytic rhizobial isolates

| Rhizobia isolates | Source of carbon | | | | | | | | Amylase | Phosphate solubilizing | pH | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Suc[1] | Ino[2] | Ara[3] | Xyl[4] | Man[5] | Fruct[6] | Rham[7] | Galac[8] | | | 4 | 10 |
| R01 | + | + | + | + | + | + | + | + | + | - | + | - |
| R03 | + | + | + | + | + | + | + | + | + | - | + | + |
| R04 | + | + | + | + | + | + | + | + | - | - | + | + |
| R05 | + | + | + | + | + | + | + | + | + | - | + | + |
| R06 | + | + | + | + | + | - | + | + | + | - | + | + |
| R07 | + | + | + | + | + | + | + | + | + | - | - | + |
| R09 | + | + | - | - | + | - | - | - | + | - | + | + |
| R10 | + | + | + | + | + | + | + | + | - | - | - | + |
| R11 | + | + | + | + | + | + | + | + | + | - | + | + |
| R12 | + | + | + | + | + | + | + | + | + | - | + | + |
| R14 | + | + | + | + | + | + | + | + | + | - | + | + |
| R15 | + | + | + | + | + | + | + | + | - | - | + | + |
| R16 | + | + | + | + | - | + | - | + | - | - | + | + |
| R17 | - | + | - | - | + | + | + | + | - | - | + | - |
| R18 | - | + | - | + | - | - | - | - | + | - | - | + |
| R19 | - | + | - | - | - | - | - | + | + | - | + | + |
| R20 | + | + | + | + | + | + | + | + | + | + | + | + |
| R21 | - | - | - | - | + | - | + | - | + | - | + | + |
| R22 | + | + | + | + | + | + | + | + | - | - | + | + |
| R23 | + | + | + | + | + | + | + | + | + | - | + | + |
| R24 | + | + | + | + | + | + | + | + | + | - | + | + |
| R25 | + | + | + | + | + | + | + | + | + | - | + | + |
| R26 | + | + | + | + | + | + | + | + | + | - | + | - |

*Note*. [1]Sucrose; [2]Inositol; [3]Arabinose; [4]Xylose; [5]Manose; [6]Fructose; [7]Rhamnose; [8]Galactose.

Table 6. Classification of rhizobia isolates according to the values established in the Table 1

| Rhizobia | Growth rate | Temperature | Resistance antibiotics | NaCl | Carbon sources | Phosphate solubilization | pH | Total |
|---|---|---|---|---|---|---|---|---|
| *Group 1* | | | | | | | | |
| R15 | 1 | 3 | 0 | 1 | 3 | 0 | 2 | 10 |
| R16 | 1 | 3 | 1 | 2 | 2 | 0 | 2 | 11 |
| R21 | 1 | 3 | 1 | 1 | 1 | 0 | 2 | 9 |
| R22 | 0 | 3 | 1 | 5 | 3 | 0 | 2 | 14 |
| *Group 2* | | | | | | | | |
| R05 | 1 | 2 | 0 | 1 | 3 | 0 | 2 | 9 |
| R09 | 1 | 3 | 1 | 1 | 2 | 0 | 2 | 10 |
| R17 | 1 | 3 | 1 | 1 | 2 | 0 | 1 | 9 |
| R19 | 1 | 3 | 1 | 0 | 1 | 0 | 2 | 8 |
| R20 | 1 | 2 | 1 | 1 | 3 | 1 | 2 | 11 |
| R23 | 1 | 3 | 1 | 1 | 3 | 0 | 2 | 11 |
| R24 | 1 | 3 | 0 | 1 | 3 | 0 | 2 | 10 |
| R25 | 1 | 3 | 0 | 1 | 3 | 0 | 2 | 10 |
| *Group 3* | | | | | | | | |
| R18 | 1 | 5 | 1 | 2 | 1 | 0 | 1 | 11 |
| *Group 4* | | | | | | | | |
| R26 | 1 | 1 | 1 | 5 | 3 | 0 | 1 | 12 |
| *Group 5* | | | | | | | | |
| R07 | 0 | 2 | 4 | 3 | 3 | 0 | 1 | 13 |
| *Group 6* | | | | | | | | |
| R01 | 0 | 1 | 5 | 0 | 3 | 0 | 1 | 10 |
| R03 | 0 | 1 | 4 | 0 | 3 | 0 | 2 | 10 |
| R06 | 0 | 1 | 4 | 0 | 3 | 0 | 2 | 10 |
| R11 | 0 | 1 | 4 | 0 | 3 | 0 | 2 | 10 |
| R12 | 0 | 1 | 4 | 0 | 3 | 0 | 2 | 10 |
| R14 | 0 | 2 | 4 | 1 | 3 | 0 | 2 | 12 |
| *Group 7* | | | | | | | | |
| R04 | 0 | 5 | 4 | 1 | 3 | 0 | 2 | 15 |
| R10 | 1 | 5 | 5 | 2 | 3 | 0 | 1 | 17 |

Considering that the maximum score of 22 corresponds to 100%, it was established that the isolates with a percentage greater than or equal to 70% of the maximum value (≥ 15 scores) would be selected. Thus, the isolates, R04 and R10 of the group 7 (Table 6) were selected for *cross-feeding* test with cellulolytic actinobacterial strains already selected.

An understanding of microbial metabolism must come from the properties of individual strains in pure culture to the combined interactions of microbial strains in co-culture (Wintermute & Silver, 2010). Thus, each one of the 6 cellulolytic actinobacterial strains selected was co-cultured with each one of the 2 selected rhizobial strains deficient in the production of this enzyme totaling 12 combinations and 24 experimental units (two repetitions), in quadruplicate.

The *Nocardia* strains A12 and A13 were incompatible with R04 and R10 *Bradyrhizobium* strains, while *Streptomyces* strains A06 and A09 and *Nocardia* strain A11 were compatible. The *Bradyrhizobium* strain R10 was compatible with the same strains and also with *Streptomyces* strain A18 (Figure 1), confirming the hypothesis that phylogenetically and metabolically different bacterial species promote the sharing of resources.

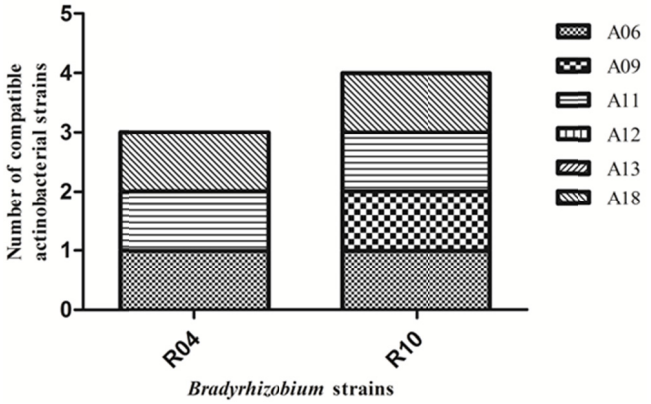


Figure 1. *Cross-feeding* among *Bradyrhizobium* strains R04 and R10 and *Streptomyces* strains (A06, A09 and A18) and *Nocardia* strains (A11, A12 and A13)

Like this, the compatibility index (nº of compatible pairs/all pairwise interactions) for *Bradyrhizobium* strains R04 and R10 was 0.5 and 0.7, respectively, confirming the superiority of *Bradyrhizobium* R10 (Table 6). Thus, the highest index associated with the best performance suggest that, at first instance, the *Bradyrhizobium* R10 strain to be preferentially chosen to test its association with *Nocardia* strain A11 and *Streptomyces* strains A06, A09 and A10. As the strain *Streptomyces* A18 already was identified at the genotypic level, it would initially be chosen to test in consortium with the *Bradhyrhizobium* R10 as inoculants for cowpea bean in an environmentally controlled room and later under field conditions.

The effect *in vitro* and under field conditions of actinobacterial strains on the growth of strains of rhizobia was reported by Htwe et al. (2018ab), Htwe and Yamakawa (2015), Xuyen et al. (2016) and Sahur et al. (2018) and the results indicated that the bacterial pairs can be useful as effective inoculants in the future.

In pioneering work, Jesus (2013) evaluated the metabolic compatibility among nine actinobacteria strains that degrade humus and six diazotrophic bacterial strains from the culture collection of the State University in Rio de Janeiro, Brazil. The author used the same method of this work and observed that 44% of the pairs were compatible (44.4%). The lower percentage compared to the present work may be due to the greater chemical complexity of humus in relation to cellulose.

It is important to emphasize that the present study provided a better understanding of the interactions between actinobacteria and rhizobia in semiarid soils, which may be a key to developing strategies for manipulating these microbial communities, with agricultural and environmental implications.

## 4. Conclusions

*In vitro cross-feeding* between actinobacteria and rhizobia strains from semiarid environment proved to be an effective tool for selection bio inoculants.

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