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# *In vitro* co-inoculation of rhizobacteria from the semi-arid aiming at their implementation as bio-inoculants

## Co-inoculação *in vitro* de rizobactérias do semiárido visando sua aplicação como bioinoculante

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## ABSTRACT

The use of nitrogen fertilizers is of paramount importance for the supply of this nutrient to plants. However, the application of these fertilizers brings numerous environmental and health problems. An alternative to these chemical products would be the use of rhizobia — plant growth-promoting rhizobacteria naturally present in the rhizosphere and capable of carrying out biological nitrogen fixation. Through the present work, we propose the co-inoculation of Actinobacteria and rhizobia, aiming at the production of a new bio-inoculant that replaces, at least in part, nitrogen fertilization in legumes. It is expected that Actinobacteria, by producing exoenzymes, enable the growth of rhizobia in non-specific culture media for these microorganisms. Ten strains of Actinobacteria with statistically distinct cellulolytic and xylanolytic activity and seven strains of rhizobia without the aforementioned enzymatic activities were used. A co-inoculation of these microorganisms was performed in culture media containing carboxymethylcellulose (CMC) and xylan as sole carbon sources, and then their compatibility indexes (CI) were calculated. Actinobacteria strains A139 and A145 (both with CI = 0.857 in the medium with CMC and CI = 1 in the medium with xylan) showed remarkable facilitation of rhizobia growth, and had only one antagonistic relation each (both with rhizobia L9 in the medium with CMC). This biological interaction, called cross-feeding, occurs when microorganisms stimulate each other's growth and is promising for prospecting a bio-inoculant, in addition to providing an overview of the ecological relationships that occur between plant growth-promoting rhizobacteria in the semi-arid region.

**Keywords:** actinobacteria; cross-feeding; diazotrophic bacteria; PGPR; rhizobia; *Streptomyces*.

## RESUMO

O uso de fertilizantes nitrogenados é de suma importância para o fornecimento desse nutriente para as plantas. Contudo, a aplicação desses fertilizantes traz inúmeros problemas ambientais e sanitários. Uma alternativa a esses produtos químicos seria o uso de rizóbios — rizobactérias promotoras do crescimento vegetal naturalmente presentes na rizosfera e capazes de realizar a fixação biológica de nitrogênio. Através deste trabalho, nós propomos a co-inoculação de actinobactérias e rizóbios, visando a produção de um novo bioinoculante que substitua, pelo menos em parte, a adubação nitrogenada em leguminosas. É esperado que actinobactérias, pela produção de exoenzimas, possibilitem o crescimento dos rizóbios em meios de cultura inespecíficos para esses microrganismos. Foram utilizadas 10 cepas de actinobactérias com atividade celulolítica e xilanolítica estatisticamente distintas e sete cepas de rizóbios sem as referidas atividades enzimáticas. Uma co-inoculação dos microrganismos foi realizada em meios de cultura contendo carboximetilcelulose (CMC) e xilana como únicas fontes de carbono, e então, calculados seus índices de compatibilidade (IC). As cepas de actinobactéria A139 e A145 (ambas com IC = 0,857 no meio com CMC e IC = 1 no meio com xilana) apresentaram notável facilitação do crescimento dos rizóbios e tiveram apenas relação antagônica cada uma (ambas com o rizóbio L9 no meio com CMC). Essa interação biológica, denominada *cross-feeding*, ocorre quando microrganismos estimulam o crescimento um do outro e se mostra promissora para a prospecção de um bioinoculante, além de fornecer um panorama das relações ecológicas que ocorrem entre as rizobactérias promotoras do crescimento vegetal no Semiárido.

**Palavras-chave:** actinobactérias; bactérias diazotróficas; *cross-feeding*; PGPR; rizóbios; *Streptomyces*.

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## Introduction

Global agricultural production in 2050 will be 60% higher than it was between 2005 and 2007. This implies a greater demand for fertilizers, such as nitrogen (N). The supplementation of these agrochemicals in crops helps ensure the nutrition of 48% of the world's population (Singh, 2018). Yet, using nitrogen fertilizers entails a high monetary and environmental cost. The production of 1 ton of ammonia-based fertilizer demands 949 $m^3$ of natural gas. Using 87% of all energy expended in the fertilizer industry generates 1.6 tons of $CO_2$, which is released into the atmosphere, causing several environmental problems (Beckinghausen et al., 2020). Brazil spent 93.06 kg/ha of nitrogen fertilizers in 2020. This amount is superior to the global average of 72.88 kg of $CO_2$/ha within that same period (FAO, 2022).

Crops only absorb half of the nitrogen added to them. The fertilizer production chain loses a lot of nitrogen throughout its process in synthesis, transport, and waste management. This lost gives rise to the so-called nitrogen pollution (Kanter et al., 2019). Fertilizers dispersion can lead to issues such as greenhouse gas release, soil acidification, eutrophication, biodiversity reduction, and groundwater pollution (Sun et al., 2020; Martínez-Dalmau et al., 2021). This kind of pollution can cause respiratory and heart problems and various types of tumors in humans (Kanter et al., 2019).

Rhizobia are a remarkable class of microorganisms found in the soil. These bacteria establish a symbiotic relationship with legumes and also perform symbiotic nitrogen fixation (SNF). SNF is more efficient than other types of biological nitrogen fixation (BNF) (Wheatley et al., 2020). The use of rhizobia as bio-inoculants to replace nitrogen fertilizers is cheaper, can improve crop yields, reduces atmospheric and water pollution caused by these fertilizers, and saves large amounts of fossil fuels and energy that would be required for the production of agrochemicals (diCenzo et al., 2019).

The survival of plants depends on the community of microorganisms associated with them, especially in environments such as the semi-arid region of Brazil. This can be done in symbiosis or in the rhizosphere, where they change the soil's structure to optimize biological activity (Solans et al., 2021). Bacteria that live in the rhizosphere and stimulate plant growth through one or more mechanisms are called "plant growth-promoting rhizobacteria" (PGPR). They account for 2–5% of the rhizosphere microbiota (Prasad et al., 2019).

Soils of environments in desertification process present an abundance of up to 62% of Actinobacteria. These microorganisms play critical ecological roles in these ecosystems (Lacombe-Harvey et al., 2018; Araujo et al., 2020; Solans et al., 2021). Some Actinobacteria establish endophytic symbiotic relationships with plants in the roots. Actinobacteria act by producing phytohormones or other growth factors in this relationship. This increases resistance to biotic and abiotic stresses, insects, pests, and pathogens in exchange for nutrients and shelter in the host plant (Singh and Dubey, 2019; Bao et al., 2021).

Mixed bio-inoculants containing rhizobia and other PGPR are considered "supreme inoculants" due to their potential for developing new commercial products (Atieno et al., 2020). Several successful examples of co-inoculation between these microorganisms can be found in the literature. For instance, *Bradyrhizobium* shows positive results in plant growth and development when co-inoculated with *Pseudomonas oryzihabitans, Pseudomonas putida, Bacillus megaterium, Bacillus pumillus,* mycorrhizae (*Glomus clarum, Glomus mosseae*, and *Gigaspora margarita*), among others (Jabborova et al., 2021; Sheteiwy et al., 2021; Kumawat et al., 2022; Miljaković et al., 2022).

Cooperation between Actinobacteria and rhizobia affects the nodulation and growth of legumes. Co-inoculation of soybean with *Bradyrhizobium japonicum* and *Streptomyces* sp./*Nocardia* sp. and of alfalfa with *Sinorhizobium meliloti* and *Micromonospora* spp./*Frankia* stimulates nodulation even in soils with high nitrogen levels, which usually inhibit nodulation. These generate examples of this type of successful co-inoculation (Saidi et al., 2021). The prospecting of Actinobacteria and rhizobia-based bio-inoculants, particularly between the genera *Streptomyces* and *Bradyrhizobium*, is well documented in the literature and appears to be promising (Soe and Yamakawa, 2013; Htwe and Yamakawa, 2016; Htwe et al., 2018; Htwe et al., 2019).

Microorganisms such as Actinobacteria and rhizobia are usually not found isolated in their habitat and interact with each other in either a cooperative or antagonistic manner (Fields et al., 2021). Co-inoculation of these two microorganisms would first depend on their metabolic compatibility. Given the ability of these rhizobacteria to enhance plant growth in adverse conditions, this study aimed to co-inoculate Actinobacteria and rhizobia isolated from Brazilian semi-arid zones and test their *in vitro* metabolic compatibility, with the goal of developing a bio-inoculant that can reduce the demand for nitrogen fertilizers in the future.

## Material and Methods

The microorganisms used in this study were obtained from the culture collection of the Laboratory of Environmental Microbiology (LAMAB) of the Universidade Federal do Ceará (UFC). The collection comprised 313 strains of Actinobacteria and 150 strains of rhizobia that were previously studied for their extracellular xylanolytic and cellulolytic activity and had their enzymatic indices (EIs) determined by using the methodology of Bandeira et al. (2022).

The Supplementary Material presents more information on Actinobacteria's cellulolytic and xylanolytic activity tests. The selection criterion for Actinobacteria was based on statistical tests with their respective EIs. The data were first submitted to the normality test (Kolmogorov-Smirnov) and homoscedasticity test (Levene). Afterward, multivariate analysis of variance (MANOVA) was performed by contemplating the EIs of xylanolytic and cellulolytic activities, followed by the Tukey test, which differentiated the groups of strains with higher EIs ($p < 0.05$). The software used for the analyses was the IBM Statistical Package for Social Sciences (SPSS) (version 20).

MANOVA showed a statistically significant difference between the EIs of Actinobacteria strains for cellulolytic ($F = 61.802$; $p < 0.000$) and

xylanolytic (F = 127.704; p < 0.000) activity. Through the Tukey test, it was possible to differentiate and select a group of 10 strains (A108, A109, A125, A136, A139, A144, A145, A146, A148, A150) that obtained the highest EIs for cellulolytic and xylanolytic activity, simultaneously. A micromorphological evaluation previously determined the genus of the selected strains according to Santos et al. (2019) (Table 1). The statistical tests performed are also found in the Supplementary Material.

Strains A108, A109 and A125 were obtained from soils of the Ecological Station of Aiuaba (CE), strains A136 and A139, from the Ubajara National Park (CE), and strains A143, A144, A145 and A146 were isolated from the Sete Cidades National Park (PI). Strain A148 was isolated from the soil surrounding the Sete Cidades National Park. The authorization to collect soil samples in these preservation areas was obtained under the research project CNPq/ICMBio/FAP's Nº18/2017, proceeding 421350/2017.2.

The rhizobia were isolated by Pinheiro et al. (2014), had their enzymatic activity evaluated by Sousa (2020), and were identified by the sequencing of the 16S rRNA gene by Silva (2020). The genetically identified strains L1, L4, L9, L13, L15, L24, and L27 were selected due to the absence of cellulolytic and xylanolytic activity (Table 2).

**Table 1 – Actinobacteria strains from the Brazilian semi-arid obtained from the Laboratory of Environmental Microbiology (LAMAB) culture collection.**

| Strain | Genus |
|---|---|
| A108 | *Streptomyces sp.* |
| A109 | *Nocardia sp.* |
| A125 | *Streptomyces sp.* |
| A136 | *Streptomyces sp.* |
| A139 | *Streptomyces sp.* |
| A143 | *Streptomyces sp.* |
| A144 | *Streptosporangium sp.* |
| A145 | *Streptomyces sp.* |
| A146 | *Streptosporangium sp.* |
| A148 | *Streptomyces sp.* |

Source: the author.

**Table 2 – Rhizobia strains from the Brazilian semi-arid were acquired from the Laboratory of Environmental Microbiology (LAMAB) culture collection.**

| Strain | Species |
|---|---|
| L1 | *Bradyrhizobium elkanii* |
| L4 | *Bradyrhizobium elkanii* |
| L9 | *Rhizobium tropici* |
| L13 | *Bradyrhizobium kavangense* |
| L15 | *Bradyrhizobium japonicum* |
| L24 | *Bradyrhizobium yuanmingense* |
| L27 | *Bradyrhizobium iriomotense* |

Source: Silva (2020).

The rhizobia strains were isolated from Quixadá (4°58'S to 39°1'W) and Cascavel (4°7'S to 38°14'W), in Ceará, and from Jardim de Angicos (5°39'S to 35°58'W) and Santana do Mato (5°57'S to 36°39'W), in Rio Grande do Norte. The authorization to collect soil samples was also obtained within the framework of the already-mentioned research project.

An *in vitro* co-inoculation was performed to investigate the capacity of metabolic cooperation (facilitation) between the strains of Actinobacteria and rhizobia following the methodology of Silva et al. (2019), with some modifications. To this end, two culture media were used, each containing a carbon source: carboxymethylcellulose (CMC; 5 g $L^{-1}$ of CMC, 0.5 g $L^{-1}$ $MgSO_4$, 0.5 g $L^{-1}$ of KCl, 3 g $L^{-1}$ of $NaNO_3$, 0.01 g $L^{-1}$ of $FeSO_4$, 1 g $L^{-1}$ of $K_2HPO_4$, 15 g $L^{-1}$ of agar, 2 mL $L^{-1}$ of Nystatin 100,000 UI/$mL^{-1}$, pH 6) and xylan (XY; 1 g $L^{-1}$ of xylan obtained from wood, 0.5 g $L^{-1}$ $MgSO_4$, 1 g $L^{-1}$ of yeast extract, 0.5 g $L^{-1}$ of $NaNO_3$, 0.01 g $L^{-1}$ of $FeSO_4$, 1 g $L^{-1}$ of $K_2HPO_4$, 15 g $L^{-1}$ of agar, 2 mL $L^{-1}$ of Nystatin 100,000 UI/$mL^{-1}$, pH 6.5). The duplicate *spot*-inoculation with the Actinobacteria was conducted in the culture media mentioned above and incubated for seven days in a bio-oxygen demand (BOD) incubator at 28°C. At the end of the seven days, the plates were evaluated for the presence of contamination and adequate growth. Contaminated and/or non-growing plates were discarded and redone.

The rhizobia were then purified for inoculation. An amount of 1 mL from each rhizobium was transferred into YM (yeast manitol) broth (10 g $L^{-1}$ of mannitol, 0.5 g $L^{-1}$ of $K_2HPO_4$, 0.2 g $L^{-1}$ $MgSO_4$, 0.1 g $L^{-1}$ of NaCl, 0.5 g $L^{-1}$ of yeast extract, 5 mL $L^{-1}$ of 0.5% bromothymol blue in 0.2 N KOH) into a sterile microtube and centrifuged in a Marconi MA 1,800 centrifuge at 9,261 x g (10500 RPM) for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of sterile distilled water and homogenized in a vortex shaker model Phoenix AP56. This process was done twice, in duplicate, until the purified rhizobia suspended in distilled water were obtained.

For the actual co-inoculation, 10 µL of the purified rhizobia were inoculated near the Actinobacteria spots. Growth was re-evaluated after seven days of incubation in a BOD-type inoculator at 28°C. The growth of rhizobia colonies was considered positive.

The CI was calculated from the ratio between the number of compatible pairs and the number of possible pairs. For Actinobacteria, the compatibility index was abbreviated as ACI. As for rhizobia, the acronym adopted was RCI.

## Results and Discussion

Figure 1 illustrates the results obtained from the co-inoculations between Actinobacteria and rhizobia. Both positive (1A) and negative (1B) results were obtained. Antagonistic relationships (1C), perceived as negative, were also observed.

In the medium with CMC, A144 and A148 showed positive results with all tested rhizobia (ACI = 1). A108, A139 and A145 presented an ACI of 0.857, having positive results with 6 of the 7 strains of the tested

rhizobia. The L1 strain presented growth with 9 of the 10 tested Actinobacteria (RCI = 0.9) (Table 3).

In the medium with xylan, A109, A139 and A145 had positive results with all tested rhizobia (ACI = 1). Strains A125 and A143 presented ACIs of 0.857, showing positive results with 6 of the 7 rhizobia strains. The L4 presented growth with 9 of the 10 tested Actinobacteria (RCI = 0.9) (Table 4).

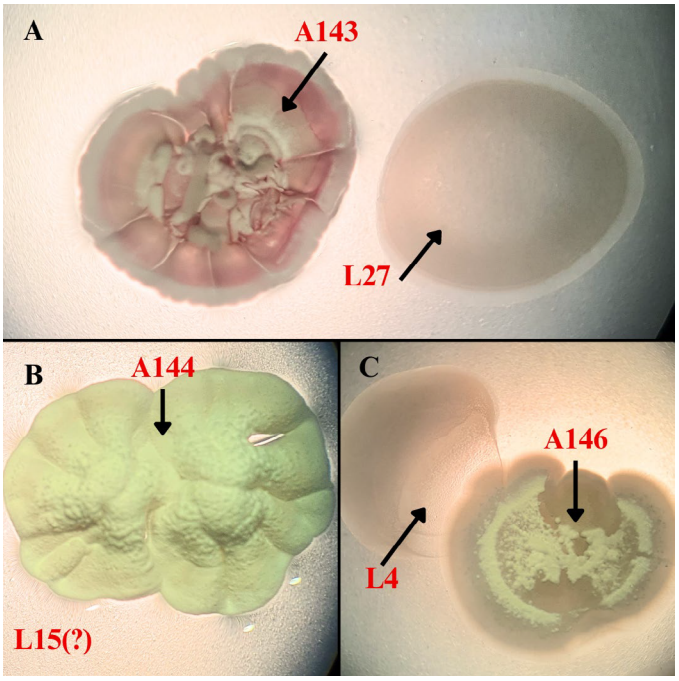


**Figure 1 – Examples of co-inoculation test results. (A) Interaction between L27 rhizobia and A143 Actinobacteria strains in the medium with xylan. (B) Absence of interaction between the L15 rhizobia (inoculated on the left side of the image, where there was no growth) and A144 Actinobacteria strains in the medium with xylan. (C) Antagonistic interaction between L4 rhizobia and A146 Actinobacteria strains in the medium with CMC. The Actinobacteria produced a halo of rhizobia growth inhibition.**

SNF is an energetically expensive process and relies on cellulose-rich plant residues to obtain this energy. Moreover, the rhizobium must break through the plant cell wall to colonize it and nodulate. Although the production of exocellulase is necessary to colonize the roots of legumes and perform SNF, it is not common for rhizobia to present this metabolic apparatus (Silva et al., 2019). It became evident that rhizobia require assistance to colonize the roots, considering the mixed composition of lignocellulose (a major constituent of plant cell walls, which is cellulose and hemicellulose-rich; Wu et al., 2019) and the inability of rhizobia to degrade these carbohydrates. This assistance can be provided through a microorganism that facilitates the rhizobia growth in the presence of carbon sources which they cannot assimilate. Therefore, we propose using Actinobacteria isolated from the same site as the rhizobia to research cross-feeding, considering that studies with bacteria isolated from the same environment are closer to the natural community, thus, more faithfully representing in situ interactions (Stadie et al., 2013).

In nature, bacteria often compete for numerous limiting factors, such as more favorable habitats, minerals, and various nutrients. For this reason, these microorganisms developed numerous strategies to allow growth and reproduction under these conditions, such as the secretion of toxins and antibiotics, which provide an advantage for the growth of the bacteria that produced them. Generally, these antagonistic relationships often overlap with neutral relationships, which in turn, overlap with positive relationships, such as cross-feeding (D'Souza et al., 2018).

A low phylogenetic proximity between two groups in co-culture entails a lower probability of overlapping growth requirements among these microorganisms. Consequently, low donor-recipient kinship decreases but does not eliminate the extent of competition for resources between the co-inoculated bacteria. Therefore, the intended group's growth is facilitated (Mitri and Foster, 2013). The Rhizobiaceae family, which houses the rhizobia, consists of rod-shaped Gram-negative bacteria.

**Table 3 – In vitro facilitation among Brazilian semi-arid strains of Actinobacteria and rhizobia in the medium with carboxymethylcellulose.**

| Rhizobia | Actinobacteria | | | | | | | | | | RCI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A108 | A109 | A125 | A136 | A139 | A143 | A144 | A145 | A146 | A148 | |
| L1 | + | + | + | + | + | + | + | + | - | + | 0,9 |
| L4 | + | - | + | A | + | + | + | + | A | + | 0,7 |
| L9 | - | - | - | - | A | - | + | A | - | + | 0,2 |
| L13 | + | - | - | - | + | - | + | + | - | + | 0,5 |
| L15 | + | + | + | A | + | - | + | + | A | + | 0,7 |
| L24 | + | - | - | + | + | - | + | + | + | + | 0,7 |
| L27 | + | - | - | + | + | + | + | + | + | + | 0,8 |
| ACI | 0,857 | 0,286 | 0,429 | 0,429 | 0,857 | 0,429 | 1 | 0,857 | 0,286 | 1 | |

+: presence of facilitation (positive result); -: absence of facilitation (negative result); A: antagonistic interaction, considered a negative result; ACI: Actinobacteria compatibility index; RCI: Rhizobia compatibility index.
Source: the author.

Their main characteristic is the formation of symbiotic nodules with legumes, where they perform biological nitrogen fixation (Kuykendall, 2015; Wheatley et al., 2020). Actinobacteria are filamentous, Gram-positive microorganisms capable of forming aerial and substrate mycelium and sporulation (Jose et al., 2021). The physiological, morphological and phylogenetic dissimilarity between these microorganisms justifies the results we obtained, in which Actinobacteria were able to facilitate the growth of rhizobia. The A139 and A145 Actinobacteria strains have a more significant potential for developing a bio-inoculant due to their intense compatibility with the rhizobia in the two tested culture media. The L1 rhizobia strain showed the best growth when co-inoculated with Actinobacteria in all tested media. These results are presented graphically through the heatmaps in Figure 2.

**Table 4 – In vitro facilitation between Brazilian semi-arid strains of Actinobacteria and rhizobia in the medium with xylan.**

| Rhizobia | Actinobacteria | | | | | | | | | | RCI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A108 | A109 | A125 | A136 | A139 | A143 | A144 | A145 | A146 | A148 | |
| L1 | + | + | + | A | + | + | + | + | A | + | 0,8 |
| L4 | + | + | + | + | + | + | + | + | + | A | 0,9 |
| L9 | - | + | - | - | + | + | + | + | - | - | 0,5 |
| L13 | - | + | + | A | + | A | + | + | A | - | 0,5 |
| L15 | - | + | + | A | + | + | - | + | A | A | 0,5 |
| L24 | + | + | + | + | + | + | - | + | A | + | 0,8 |
| L27 | + | + | + | + | + | + | - | + | A | A | 0,7 |
| ACI | 0,571 | 1 | 0,857 | 0,429 | 1 | 0,857 | 0,571 | 1 | 0,143 | 0,286 | |

+: presence of facilitation (positive result); -: absence of facilitation (negative result); A: antagonistic interaction, considered a negative result; ACI: Actinobacteria compatibility index; RCI: Rhizobia compatibility index.
Source: the author.

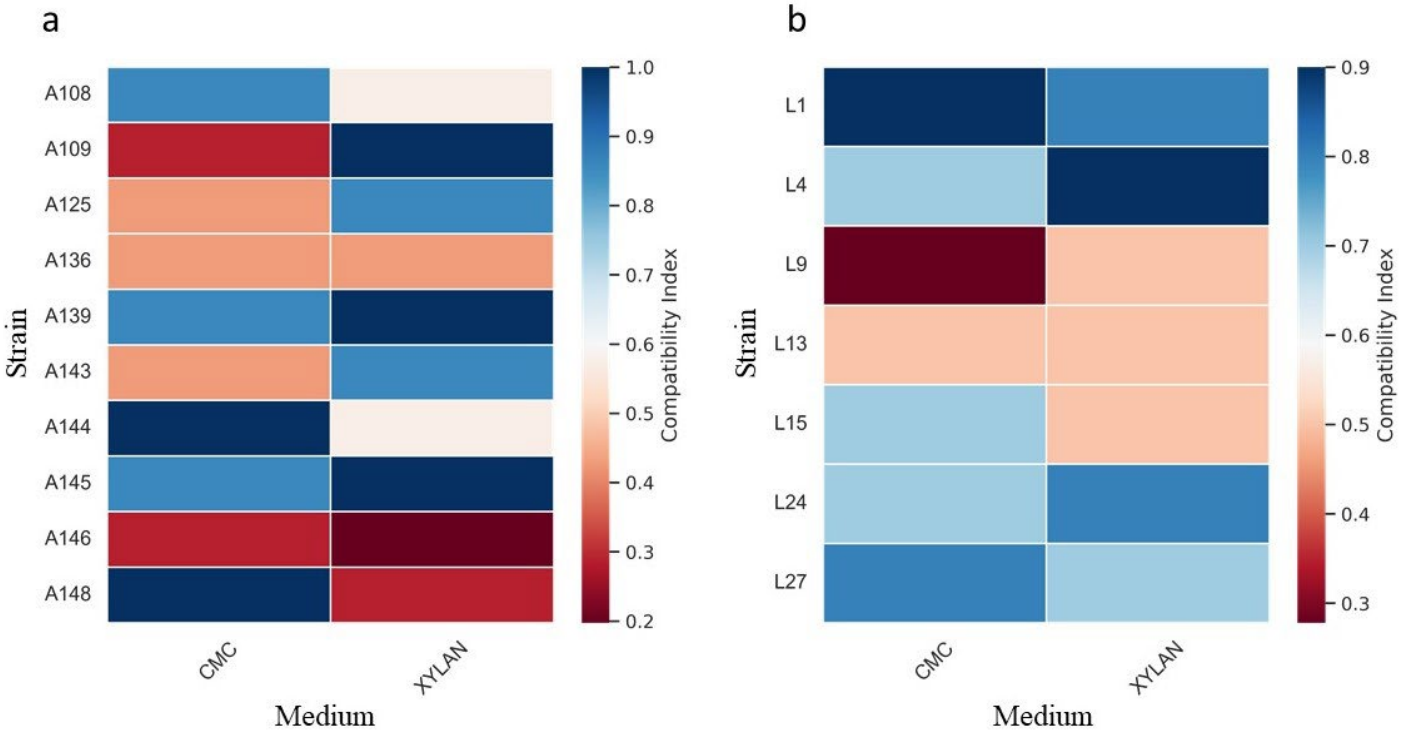


**Figure 2 – Heatmaps illustrating the compatibility indices of (A) Actinobacteria and (B) rhizobia in media with carboxymethylcellulose and xylan. The X axis represents the strains, while the Y axis represents the culture media used.**
CMC: carboxymethylcellulose.
Source: the author.

In agriculture, bio-inoculants have several functions, such as the inhibition of phytopathogens growth, production of siderophores, nitrogen fixation, production of phytohormones, and phosphate solubilization. These inoculants can also reduce the damage of chemical fertilizers (Chaudhary and Shukla, 2020). Several microorganisms are already used as biofertilizers, such as those of the genera *Pseudomonas, Bacillus, Phyllobacterium* and *Rhodococcus*, in addition to rhizobia, such as *Azorhizobium, Sinorhizobium* and *Bradyrhizobium*, and Actinobacteria, such as *Mycobacterium, Frankia, Arthrobacter* and *Streptomyces* (Mahanty et al., 2016).

The research conducted by Htwe et al. (2019) indicated that a biofertilizer based on *Bradyrhizobium japonicum* SAY3-7, *Bradyrhizobium elkanii* BLY3-8, and *Streptomyces griseoflavus* P4 has positive effects on the growth of mung beans, cowpeas and soybeans. Especially when inoculated with mung beans and soybeans, this bio-inoculant improves growth, nodulation, nitrogen fixation, NPK absorption and seed yield in a greenhouse experiment. Considering the *in vitro* metabolic compatibility between strains A139 and A145 (*Streptomyces* sp.) and L1 (*Bradyrhizobium elkanii*), it is evident that an *in vivo* co-inoculation is necessary to advance in the prospecting of this bio-inoculant. However, it is possible to suggest that *in vitro* co-inoculation is an essential step to eliminate possible antagonistic pairs before proceeding to *in vivo* experiments. Furthermore, this research with growth-promoting rhizobacteria isolated from the semi-arid zone provided an overview of the interactions between these microorganisms from an ecological standpoint.

Despite the limited understanding of the action mechanisms of various bacteria, studies indicate that the use of different microorganisms together allows, in addition to better growth, effective plant protection. The bioprospecting of these microorganisms must be multicomponent due to the environment's considerable variability to produce optimal degrees of microorganism cooperation and the desired outcome for the plant. Combining bacterial strains in a single formulation improves efficacy and reliability, allowing for greater culture specificity, and appears to be highly promising (Zardak et al., 2018; Kour et al., 2022).

## Conclusion

Actinobacteria stimulated the growth of rhizobia in culture media not specific to these bacteria. Strains A139 and A145 (*Streptomyces* sp.) presented the highest compatibility with rhizobia in the tested media. The L1 rhizobia strain (*Bradyrhizobium elkanii*) had the best growth when co-inoculated with Actinobacteria in these media. The association of these pairs of microorganisms (A139+L1 and A145+L1) in the rhizosphere environment may stimulate plant growth and act as a potential new bio-inoculant. Therefore, *in vivo* studies using combinations of A139 and A145 Actinobacteria strains with the L1 rhizobia strain are required to assess whether the promising *in vitro* results may be reproduced when associated with plants. In view of the current literature and the results obtained with this research, it is clear that a co-inoculation of *Streptomyces* and *Bradyrhizobium* has excellent potential for the development of a biofertilizer that promotes greater nitrogen fixation efficiency, thus reducing the demand for nitrogen fertilizers.

## Contribution of authors:

MESQUITA, A. F. N.: Writing — original draft; Writing — review & editing; Investigation; Data curation; Methodology. RIBEIRO, G. A. L.: Investigation; BANDEIRA, L. L.: Conceptualization; Supervision; Writing – review & editing; CAVALCANTE, F. G.: Conceptualization; Supervision; Validation; Methodology; MARTINS, S. C. S.: Funding; Acquisition; Resources; Writing — review & editing; MARTINS, C. M.: Supervision; Funding; Resources; Writing — review & editing.

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