IN VITRO ANTAGONISM OF ACTINOBACTERIA AGAINST RHIZOBIA FROM THE SOIL

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ABSTRACT

Studies of bacteria interacting with the environment has historically been focused on strategies to obtain nutrients and resist abiotic stresses. The interbacterial antagonism approach is increasingly highlighted in the scientific literature. Among soil bacteria with potential for interactive studies are actinobacteria and rhizobium. The Brazilian northeast semi-arid is an interesting habitat for these researches mainly due to the extreme characteristics of climate and soil of the region and thus encouraging competition between the organisms present there. The main goal of this research was to evaluate the in vitro antagonism between strains of actinobacteria and rhizobia from soils of the Brazilian semi-arid region. The bacteria strain's used in the research were evaluated using the PAST software for normality tests, ANOVA and multivariate analysis of variance (MANOVA) with a p-value of 0.05. Eleven strains of actinobacteria from soils from Aiuaba - CE, Ubajara - CE and Sete Cidades-PI and seven strains of rhizobium from Quixadá and Cascavel,both located in Ceará, were selected. Yeast – Mannitol - Agar medium was used to visualize the halos of inhibitory effects of actinobacteria. The results revealed that strains A125, A146, A148 and A150 showed inhibitory effects against diazotrophic bacteria, indicating that 36% of the tested strains of actinobacteria showed the production of an inhibitory compound for rhizobia. This study demonstrated the antagonistic activity of actinobacteria against diazotrophic bacteria.
KEYWORDS: Ecological interactions, inhibition, microorganisms.

ANTAGONISMO *IN VITRO* ENTRE ACTINOBACTÉRIAS E RIZÓBIOS DO SOLO DE REGIÃO SEMIÁRIDA BRASILEIRA

RESUMO

O estudo das bactérias interagindo com o ambiente tem focado historicamente em estratégias para obter nutrientes e resistir a estresses abióticos. Assim, a abordagem de antagonismo interbacteriano é cada vez mais destaque na literatura científica. Entre as bactérias do solo com potencial para estudos interativos estão as actinobactérias e os rizóbios. O Semiárido nordestino, por características extremas

de clima e solo, se apresenta um habitat interessante para estas pesquisas, por incentivar a competição entre os organismos presentes. Assim, o objetivo desta pesquisa foi de avaliar o antagonismo *in vitro* entre cepas de actinobactérias e de rizóbios derivados de solos do Semiárido Nordestino. A escolha das cepas de microrganismos foi feita através do software PAST quanto aos testes de normalidade, análise da variância ANOVA e multivariada (MANOVA), com índice de significância de 0,05. Ao final, onze cepas de actinobactérias provenientes de solos dos municípios de Aiuaba - CE, Ubajara - CE e Sete Cidades- PI e sete cepas de rizóbios dos municípios cearenses de Quixadá e Cascavel, foram selecionadas. Para visualização dos halos dos efeitos inibitórios das actinobactérias foi usado o meio YMA com as cepas previamente cultivadas em caldos *Yeast - Manitol* e Caseína - Dextrose. Os resultados revelaram que as cepas A125, A146, A148 e A150 apresentaram efeitos inibitórios frente às bactérias diazotróficas, indicando que 36% das cepas testadas de actinobactérias apresentaram a produção de um composto inibitório para os rizóbios. Por fim, esse estudo demonstrou a atividade antagônica das actinobactérias frente às bactérias diazotróficas.
PALAVRAS-CHAVE: Interações ecológicas, inibição, microrganismos.

INTRODUCTION

The importance of interspecific relationships between living beings has increased with the advances in studies in this area, especially for activities and functional attributes. These attributes are linked to ecological and evolutionary processes, favoring the coexistence or exclusion of species in an environment (NOBRE, 2021). Some ecological relationships can help in bioremediation, biocontrol, aquaculture management and wastewater denitrification. The use of co-cultures containing bacterial consortia is one such example of these applied beneficial ecological relationships of mutualism (KOSINA *et al*., 2021).

In the case of competition, it can be indirect and direct. Competition for resources can decrease the functionality of the community and this interference can end up inhibiting productive competitors (CAVALCANTE, 2017). Competitive exclusion definition was established in the 20th century with the concept that the coexistence of species that exceed limiting factors such as food resources or predators is not possible (GIACOMINI, 2007).

Some mushrooms, as *Pleurotus ostreatus*, effectively degrades lignin and also releases extracellular enzymes. They also present intense activity in the environments that they are established. It was evidenced that this mushroom's antagonistic relationship with other fungi causes a greater production of laccase and genes related to the production of manganese peroxidase, aldo-keto reductase and glutathione S-transferase for its defense. All these compounds are biotechnological tools for applications in industry and in agriculture (ZHONG *et al*., 2017).

Microorganisms are actively present in the soil and participates in biogeochemical cycles, decomposition of organic matter and structuring of the soil. These processes are essential for the health and maintenance of the ecosystem. In addition, degradation of contaminants and more complex substances are also held by soil microorganisms. This action usually occurs in microhabitats because their physical-chemical activity is diversified due to the presence of these organisms in that place. (FRANÇA, 2016).

Rhizobia are a vital contributor to soil microbiota and carry out biotic activities in these environments. They help in the mobilization of nutrients, plant's growth, production of phytohormones and in the control of phytopathogens (HAZARIKA;

THAKUR, 2020). These diazotrophic bacteria are found in the rhizosphere and in symbiosis with plants. They promote nitrogen fixation and metabolic adaptation, such as the inclusion of urea, erythritol and aldehyde metabolism, in addition to glycogen and glutamine synthesis (WHEATLEY *et al*., 2020).

The Brazilian northeastern semi-arid region has a limiting soil, mainly due to its dry and hot climate. As a harsh environment for microbial growth, the presence and activity of these microorganisms represent something to be explored (CAVALCANTE, 2017). This environment has a high level of insolation, resulting in an increase in temperature, in addition to scarce water resources and rainfall. This causes extensive periods of drought. These severe stresses discourage microbial growth in the soil and favor cases of competition (GORLACH-LIRA, COUTINHO, 2007).

It has been reported that the presence of *Bradyrhizobium* and *Rhizobium* have positive impacts on biological nitrogen fixation in soybean plantations (SARANRAJ *et al*., 2021). An inhibitory effect against diazotrophic bacteria can occur in soils with a high presence of actinobacteria, consequently affecting the plant (PEREIRA *et al*., 1999). Because of this factor, analysis of the antagonistic activity in vitro between strains of actinobacteria and of rhizobia from the rhizosphere of the Northeaster's semi-arid region must be carried out in order to test the inhibitory effects of actinobacteria in association with rhizobia.

MATERIAL AND METHODS

MICROORGANISMS

The research's sample had originally 313 strains of actinobacteria and 150 strains of rhizobia. Based on production and enzymatic activity tests, eleven strains of actinobacteria were selected from soils in the cities of Aiuaba - CE (6°7'S to 40°2'W), Ubajara - CE (3°5'S to 40°5'W) and Sete Cidades – PI (8°2'S to 42°4'W) and seven strains of rhizobia from the municipalities of Quixadá (4°6'S to 39°1'W) and Cascavel (4°7'S to 38°14'W). Strains of the two groups of bacteria are stored in the Culture Collection of the Laboratório de Microbiologia Ambiental (LAMAB) in the Department of Biology of the Federal University of Ceará (UFC).

CHROMOGENIC AND MORPHOLOGICAL CHARACTERIZATION

Actinobacteria

The actinobacteria strains were cultured in Casein Dextrose Agar (CDA) medium: 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4*7H_2O$ (magnesium sulfate), 2 g of glucose, 0.01 g of $FeSO_4*7H_2O$ (iron sulfate), 0.2 g of casein (previously dissolved in 10 mL of 0.1 M NaOH), 15 g of agar, 2.5 mL of nystatin to one liter of distilled water (KUSTER; WILLIAMS, 1964, ARIFUZZAMAN *et al*., 2010). Thereafter, the strains were incubated in a biochemical oxygen demand chamber (BOD) at 28°C for ten days and confirmatory tests such as chromogenic characterization with observation of their aerial and reverse mycelium were carried out according to Wink (2012) together with the RAL colors chart. A microscopic analysis was performed using the microculture technique for micromorphological characterization according to Kern; Blevins (2003) with adaptations. The culture was inoculated on the sides of a cube of CDA medium contained on a slide and covered by a coverslip, along with cotton moistened and then incubated for 10 days at 28ºC, after which the coverslip was relocated on a new slide, stained with cotton blue dye, sealed and observed under a Zeiss light microscope at 1000x magnification.

Rhizobia

The isolation and cultivation of the rhizobia was carried out using the Yeast Mannitol Agar (YMA) culture medium: 10g of Mannitol, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4*7H_2O$ (magnesium sulfate), 0.1 g of NaCl (sodium chloride), 0.5 g of yeast extract, 15 g of agar, 2.5 mL of nystatin for one liter of distilled water and for the use of a pH reaction indicator, a solution of bromothymol: 1.1 g of KOH (potassium hydroxide), 100 ml of distilled water and 0.5 g of bromothymol blue, with 5 ml of the stock solution added per liter (VINCENT, 1970). After that, the strains were incubated in the BOD at 28°C for seven days and confirmatory tests were performed analyzing the cultural variables (modification of the pH of the medium, growth time, mucus production, colony color) and all strains were identified using the sequencing of 16S rRNA genes, which data are disclosed by Sousa (2020).

STATISTICAL ANALYSIS AND STRAINS SELECTION

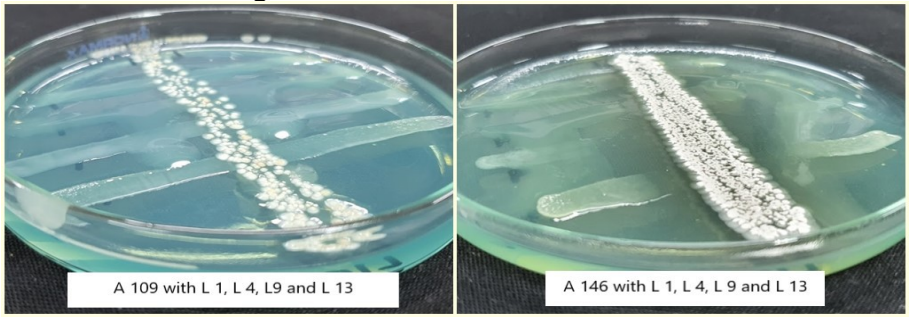
The enzymatic tests were performed in quadruplicate at least two times for each strain. The tests results were submitted to normality test using ANOVA and multivariate analysis of variance (MANOVA). Both actinobacteria and rhizobia data were submitted to the chi-square test. To analyze the normality of the data, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used, and the homogeneity of variance was performed using the Levene test. All these steps were used to verify the assumptions of the applied statistical tests.

All statistical tests were performed in the SPSS program (IBM Corp. Released 2011). With a significance value of less than 5% validating the tests performed, the choice of actinobacteria were the strains: A108, A109, A125, A136, A139, A143, A144, A145, A146, A148 and A150 and the rhizobia strains were: L1, L4, L9, L13, L15, L24 and L27.

ANTAGONISM

Each antagonism test was made from the inoculation of selected strains in a Petri dish containing culture medium in order to visualize the formation of halos related to the inhibitory effects of actinobacteria. Yeast Mannitol Agar medium was used with the chosen strains of rhizobia and actinobacteria previously cultivated in YM and CD broths and Cavalcante's (2017) technique was applied with some adaptations. In Petri dishes containing the YMA medium, the rhizobia strains were inoculated with sterile swabs. The actinobacteria strains were inoculated horizontally and perpendicularly of the rhizobium strain. One actinobacterium and three or four rhizobia were inoculated for each plate. After the procedure, the inoculated strains were incubated in the BOD for seven days at 28°C and after this period, the antagonistic activity was analyzed.

FIGURE 1: Antagonism test



Source: Authors

Figure 1 shows the negative result for antagonistic activity (A 109) and the positive result due to the formation of halos (A 146)

RESULTS AND DISCUSSION

The characterization of the actinobacteria strains provided information about each colony through the colors of the aerial and vegetative mycelium. Yellow and white colors prevailed in the aerial mycelia of the strains analyzed, but some strains showed red, gray and brown coloration. Similar colors were observed on the vegetative mycelium with the exception of gray. The strains behaved in a similar way regarding their morphology. Strains belonging to the Sete Cidades region (A143 to A150) showed similarities in their morphologies and dominance of the genus Streptomyces (TABLE 1).

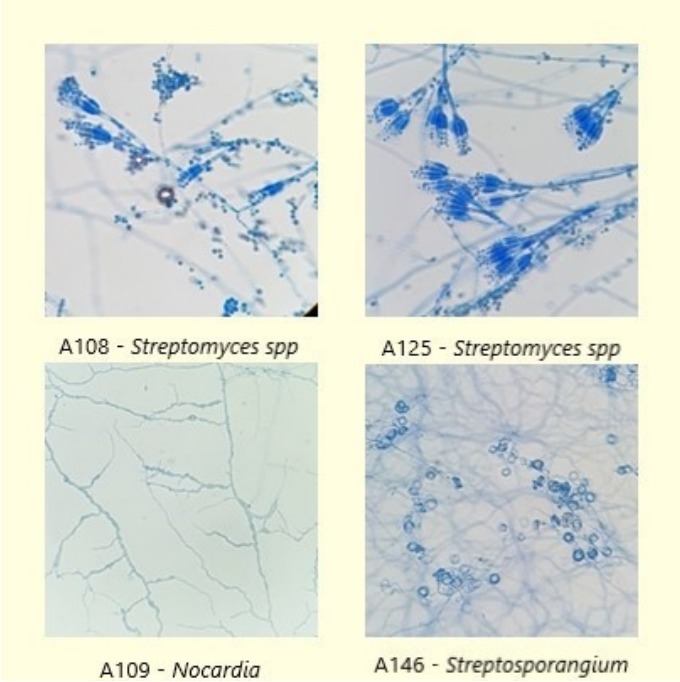
TABLE 1 - Morphological characteristics and genus identification of actinobacteria

Actinobacteria	Morphology	Mycelium		Genus
		Aerial	Vegetative	
A108	Fasciculated spore chain	White	Red	*Streptomyces*
A109	Cocos	Grey	White	*Nocardia*
A125	Fasciculated spore chain	Brown	Brown	*Streptomyces*
A136	Straight and short spore chain	Yellow	Yellow	*Streptomyces*
A139	Straight and short spore chain	Red	Red	*Streptomyces*
A143	Fasciculated spore chain	Yellow	Yellow	*Streptomyces*
A144	Fasciculated spore chain	Yellow	Yellow	*Streptosporangium*
A145	Fasciculated spore chain			*Streptomyces*
A146	Spore wall	White	Yellow	*Streptosporangium*
A148	Straight and short spore chain	White	White	*Streptomyces*
A150	Straight and short spore chain	White	White	*Streptomyces*

Source: Authors

It was reported by Lima *et al*. (2017), that actinobacteria strains of the genus Streptomyces have a greater in vitro inhibition against diazotrophic bacteria. This bacteria genus massive presence in Brazilian semi-arid soils was described, together with the genus Nocardia, corroborating the current study in which most strains of actinobacteria were classified based on micromorphological characterization (FIGURE 2) as Streptomyces spp (73%), Streptosporangium spp (18%) and Nocardia spp (9%).

FIGURE 2: Spore chain and genus identification of actinobacteria



Source: Authors

The rhizobia were analyzed through their phenotypic characteristics, where 43% alkalized and 57% neutralized the culture medium. The consistency of the formed mucus was between viscous, butyric and most was gummy and all showed a slow growth, most with a white colony coloring (TABLE 2). The comparison with the 16S rRNA sequences with the bacteria presents in the GenBank® database resulted in the identification of *Bradyrhizobium* and one as *Rhizobium tropici*, which data was disclosed by Sousa (2020).

TABLE 2 - Phenotypic and physiological characteristics of rhizobia strains

Rizobhia	Species	pH	Growth time	Mucus	Colony color
L1	*Bradyrhizobium elkanii*	Neutral	Slow	Viscous	White
L4	*Bradyrhizobium elkanii*	Alkali	Slow	Viscous	White
L9	*Rhizobium tropici*	Alkali	Slow	Butyric	White
L13	*Bradyrhizobium kavangense*	Neutral	Slow	Rubbery	White
L15	*Bradyrhizobium japonicum*	Neutral	Slow	Rubbery	White
L24	*Bradyrhizobium yuanmingense*	Neutral	Slow	Rubbery	White
L27	*Bradyrhizobium iriomotense*	Alkali	Slow	Rubbery	Yellow

Source: Authors

Antagonism results revealed that strains A125, A146, A148 and A150 showed inhibitory effects against diazotrophic bacteria. It means that 36% of the tested strains of actinobacteria showed the production of an inhibitory compound for rhizobia. These bacteria are a source of antibiotics and have the potential to control

various phytopathogens, in addition to promoting plant growth (CHAURASIA *et al*., 2018).

TABLE 3 - Results obtained from antagonistic tests

	L1	L4	L9	L13	L15	L24	L27
A108	-	-	-	-	-	-	-
A109	-	-	-	-	-	-	-
A125	-	-	+	-	-	-	+
A136	-	-	-	-	-	-	-
A139	-	-	-	-	-	-	-
A143	-	-	-	-	-	-	-
A144	-	-	-	-	-	-	-
A145	-	-	-	-	-	-	-
A146	+	+	+	+	+	-	-
A148	-	-	+	+	-	+	+
A150	-	-	+	-	-	-	+

The (+) signs indicate that there was a halo formation, showing an inhibitory effect and the (-) signs indicate that there was no halo formation. The nomenclature A refers to actinobacteria and the nomenclature L refers to rhizobia.
Source: Authors

The tests showed that all diazotrophic bacteria were inhibited by one or more strains of actinobacteria. This also shows an antagonism relationship between the strains, which was also reported by Cavalcante (2017). It was reported by Lima *et al* (2017) that actinobacteria of the genus Streptomyces showed antagonistic interaction with the species *Rhizobium tropici* and *Bradyrhizobium yuanmingense*. There are also researches of antagonistic activity in *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* that were used as inoculants in soybean, and some harmful problems have been reported for soybean in Brazilian Cerrado soils (PEREIRA *et al*., 1999).

As already mentioned in this study, the soil of the semi-arid region presents an area conducive to competition between the microorganisms present in that place. However, our results can contradict that statement. We had more strains that presented a good relationship when inoculated together than the formation of a halo related to antagonism. Some strains (A146 and A148) obtained different antagonistic effects with different strains of rhizobia.

CONCLUSION

This study demonstrated the antagonistic activity of actinobacteria against diazotrophic bacteria. This serves as a basis for future in vivo tests on co-inoculation of the two strains of bacteria, aiming to evaluate this effect on plants. This would serve as a significant contribution to the production of bioinoculant, combined with biotechnological techniques, in favor of agriculture.

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