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IN VITRO COINOCULATION BETWEEN ACTINOBACTERIA AND DIAZOTROPHIC NODULATING BACTERIA FROM THE SEMIARID

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ABSTRACT

Purpose: To evaluate the potential of actinobacterial strains from the Brazilian semiarid to establish facilitation relationships with native rhizobia from the same region.

Theoretical framework: The study is based on the ecological and biotechnological importance of soil actinobacteria, producers of biosurfactants and enzymes, and of nitrogen-fixing rhizobia in legumes.

Method: 50 strains of actinobacteria were isolated from soils from Ceará with different levels of anthropization and 19 strains of rhizobia using cowpea. The morphological, cultural and micromorphological characterization of the strains was performed, as well as the evaluation of their enzymatic profiles. *In vitro* facilitation tests were conducted between cellulolytic actinobacteria and non-cellulolytic rhizobia.

Results: The soil areas presented a similar composition of actinobacteria, but strains from the anthropized area showed higher enzymatic activity. Two *Streptomyces* strains promoted the growth of non-cellulolytic rhizobia *in vitro*, indicating potential application as bioinoculants in microbial consortia.

Conclusions: The study contributes to the knowledge of the interaction between beneficial microbial groups from the semiarid region and their possible biotechnological use in agriculture.

Originality/value: Works on coinoculation between actinobacteria and rhizobia from semiarid soils are scarce.

Keywords: Soil Actinobacteria, Rhizobia, Brazilian Semiarid, Microbial Consortia, Bioinoculants.

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COINOCULAÇÃO *IN VITRO* ENTRE ACTINOBACTERIA E BACTÉRIAS NODULADORAS DIAZOTRÓFICAS DO SEMIÁRIDO

RESUMO

Objetivo: Avaliar o potencial de linhagens de actinobactérias do semiárido brasileiro para estabelecer relações de facilitação com a rizobia nativa da mesma região.

Enquadramento teórico: O estudo baseia-se na importância ecológica e biotecnológica das actinobactérias do solo, produtoras de biossurfactantes e enzimas, e da rizobia fixadora de nitrogênio nas leguminosas.

Método: 50 linhagens de actinobactérias foram isoladas de solos cearenses com diferentes níveis de antropização e 19 linhagens de rizóbia com feijão-fradinho. Foi realizada a caracterização morfológica, cultural e micromorfológica das linhagens, bem como a avaliação de seus perfis enzimáticos. Foram realizados testes de facilitação *in vitro* entre a actinobactéria celulolítica e a rizóbia não celulolítica.

Resultados: As áreas de solo apresentaram composição semelhante de actinobactérias, mas as linhagens da área antropizada apresentaram maior atividade enzimática. Duas linhagens de *Streptomyces* promoveram o crescimento da rinizobia não celulolítica *in vitro*, indicando potencial aplicação como bioinoculantes em consórcios microbianos.

Conclusões: O estudo contribui para o conhecimento da interação entre grupos microbianos benéficos da região semiárida e seu possível uso biotecnológico na agricultura.

Originalidade/valor: Trabalhos em coinoculação entre actinobactérias e rizóbia de solos semiáridos são escassos.

Palavras-chave: Actinobactérias do Solo, Rizobia, Semiárido Brasileiro, Consórcio Microbiano, Bioinoculantes.

1 INTRODUCTION

Beneficial soil microorganisms present in agricultural systems play an important role, helping to increase production and reduce the use of chemical fertilizers, pesticides and herbicides. These benefits are made possible through the production of phytohormones (Velrajan & Bhagawati, 2018), production of siderophores (Kumar et al., 2018), phosphate solubilization (Saeid et al., 2018) and nitrogen fixation (Pinheiro et al., 2019).

Microorganisms can be affected by soil use and characteristics, presence of vegetation and environmental factors such as temperature, humidity and availability of nutrients, which can modify the composition, interaction and enzymatic production of the microbial assembly (Xu et al., 2014). In research carried out by Houfani et al., (2019) it was observed that forest and garden soils have greater microbial diversity, and higher endocellulose activity, as well as greater potential for lignocellulose decomposition, when compared to agricultural and desert soils. Among these microorganisms, actinobacteria and rhizobia stand out.

Actinobacteria have a unique potential due to their secondary metabolism and the ability to produce bioactive molecules with extensive medical, industrial and agricultural applications in addition to their diversity and ecological importance (Lewin et al., 2016), while rhizobia are recognized for their ability to produce several enzymes, which allow them to use different substrates (Menéndez et al., 2016).

Diazotrophic bacteria are present in the form of bacilli, aerobic, motile, Gram-negative and can be found both in the free-living form and in association with leguminous plants. The

benefits that rhizobia can bring to agriculture are extensively reported, such as the supply of nitrogen by symbiotic fixation of N_2, which is the most important source of nitrogen in agroecosystems because it is renewable and environmentally sustainable (Zheng et al., 2019). The plant-bacteria symbiosis performed by diazotrophic bacteria is affected by a number of mechanisms and is related to the importance of the cell wall degradation enzyme, cellulase, in the primary infection process and its importance in secondary symbiotic infection and in the strict regulation of its production to establish an effective symbiosis (Robledo et al., 2015).

Actinobacteria are Gram-positive and filamentous bacteria found abundantly in the soil. They can interact with plants through nitrogen fixation, hormone production and protection against plant disease (Singh and Gaur, 2016), in addition, the degradation capacity of actinobacteria is important for the carbon cycle and humus formation in the environment which increases soil nutrition for plant growth. They also actively participate in the degradation of natural lignocellulose, as a result of their ability to synthesize extracellular polymeric hydrolases, such as cellulases, hemicellulases, xylanases, chitinases, pectinases, amylases, peptidases, proteases and keratinases (Saini et al., 2016).

Cellulose is the most abundant organic polymer on earth. Present in plant cell walls, it is mainly found in combination with hemicellulose and xylan. Cellulose degradation is an essential ecosystem service and has a key value in understanding the breakdown of cellulose in the carbon cycle. A great diversity of cellulolytic enzymes is needed for an efficient degradation of cellulose to occur (de Vries et al., 2015).

Coinoculation of actinobacteria, rhizobia and plant growth-promoting rhizobacteria results in increased uptake of nitrogen (N), phosphorus (P) and potassium (K), improving soybean yield and soil quality (Amule et al., 2018). The symbiosis between *Bradyrhizobium* and *Streptomyces griseoflavus* promotes significant increases in plant growth, nodulation, nitrogen fixation, nitrogen, phosphorus and potassium uptake and seed production in mung bean (*Vigna radiata*) and soybean (*Glycine max* L.) (Htwe et al., 2019).

This study tested the hypothesis that actinobacteria from the semi-arid region have the capacity to in vitro cross-feeding with noduliferous diazotrophic bacteria from the same biome. The main goal was to compare the capacity of actinobacteria, isolated from semi-arid soils under different anthropogenic conditions, to establish positive interrelationships with noduliferous diazotrophic bacteria. The results obtained contribute to clarify positive interactions that occur in the edaphic community, as well as highlight the potential of the coinoculation of strains of actinobacteria and rhizobia as future bioinoculants.

2 METHODS

2.1 Research Area

The actinobacterial strains were isolated from soil from the Não Me Deixes Farm, located in the municipality of Quixadá, Ceará -BR. The geographic location is close to 4º49’34’’S and 38º58’9’’W and 210 m above sea level. The climate of Quixadá is classified as Tropical Warm Semi-Arid (BSh, according to the Köppen-Geiger Climate Classification), with an average rainfall of 717.5 mm, which is concentrated in the period from February to April, and an average temperature of 26.6°C. The farm's soil is classified as Argisol equivalent to Lixisol. Soil sampling was carried out in two areas of the farm, an anthropogenic fallow area and a conserved area, belonging to the Private Natural Heritage Reserve (RPPN) recognized by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA).

The rhizobial strains come from soil samples from Quixadá (4°58'S to 39°1’W) and Cascavel (4°7'S to 38°14'W), in Ceará - BR, and in Rio Grande do Norte- BR, in the

municipalities of Jardim de Angicos (5°39'S to 35°58'W) and Santana do Mato (5°57'S to 36°39'W) (Pinheiro et al., 2015).

2.2 Microorganisms

Actinobacteria strains were isolated from soil using the spread plate technique and the casein dextrose agar (CDA) medium (Clark, 1965). The rhizobia 'strains were isolated using cowpea [*Vigna unguiculata* (L.) Walp.] as a symbiotic plant and the nodules obtained were macerated in agar medium mannitol yeast extract (YMA) (Vincent, 1970). The 50 strains of actinobacteria, 22 from the anthropized area and 28 from the conserved area, and 19 strains of rhizobia were added to the Culture Collection of the Laboratory of Environmental Microbiology (LAMAB) of the Department of Biology of the Federal University of Ceará (UFC).

2.3 Soil Chemical Analysis

The chemical analysis of the soil was carried out according to Teixeira et al., (2017). The soil attributes evaluated were pH, potential acidity (H^+ Al), Ca^{2+} and Mg^{2+}, exchangeable aluminum (Al^{3+}), Phosphorus (P), sodium (Na^+) and potassium (K^+). pH was measured in H_2O (1:2.5) by potentiometry. Potential acidity was extracted with buffered calcium acetate at pH 7.0 and determined by titration. Calcium and magnesium were extracted with a 1M KCl solution and determined by atomic absorption spectrometry. Exchangeable aluminum was extracted with 1M KCl solution and determined by titration. P, Na^+ and K^+ were extracted with Mehlich 1 solution, with P determined by colorimetry and K^+ and Na^+ by flame photometry.

2.4 Cultural Characterization of Microorganisms

Chromogenic characterization of actinobacteria was performed by observing aerial and reverse mycelium after growth of the strains in Petri dishes. The RAL color chart was used and the colonies were classified as velvety, concentric, radial, umbonate, and convex according to Augustine et al., (2013). Micromorphological characterization was performed by means of microculture according to Kern & Blevins (2003), with some modifications. The culture was inoculated onto one side of a cube of CDA medium contained on a microscope slide and covered with a cover slip. Sterile moistened cotton was used to provide moisture for the culture to grow, which was then incubated for 14 days at 28ºC. After this period the cover slip was relocated on a new microscopic slide, stained with cotton blue and observed under a Zeiss light microscope at 1000 magnification.

The characterization of rhizobia strains was carried out through cultural variables (modification of the pH of the medium, growth time, mucus production, colony color), and physiological variables such as tolerance to high temperatures (39, 41, 43 and 45ºC), tolerance NaCl (1, 2, 2.5, 3, 10 and 20 g L^{-1}) and pH (4 and 10) all being tested in YMA medium. The rhizobia resistance to antibiotics was also analyzed, such as Amikacin (AMI 30), Amoxicillin (AMC 30), Ampicillin (AMP10), Aztreonam (ATM 30), Cephalotin (CFL 30), Cefepime (CPM 30), Cefoxitin (CFO 30), Ceftadizime (CAZ 30), Ceftriaxone (CRO 30), Ciprofloxacin (CIP 05), Chloramphenicol (CLO 30), Gentamicin (GEN 10), Piperacillin (PIT 110) and Tetracycline (TET 30) (Mueller et al., 1988). Also, amylase production (Alariya et al., 2013), xylanase (Kumar et al., 2012), celullase (Couri & Farias 1995) and phosphate solubilization (Nautiyal, 1999). Standard strains BR 3486 (*Paraburkholderia phymatum*), INPA 03-11B-*Bradyrhizobium* sp. (BR 3301), BR 3302 (*Bradyrhizobium viridifuturi*), BR 3267 (*Bradyrhizobium yuanmingense*), BR 3262 (*Bradyrhizobium pachyrhizi*), BR 2003

(*Bradyrhizobium elkanii*) and BR 2801 (*Bradyrhizobium elkanii*) were used. All strains were identified using 16S rRNA gene sequencing.

2.5 Enzyme Profile

Production of amylase (Alariya et al., 2013), xylanase (Kumar et al., 2012) and cellulase (Couri and Farias 1995) were evaluated, where the degradation of the compounds: starch, xylan and cellulose contained in the medium were observed. Actinobacteria strains were incubated at temperatures of 28º, 39º, 41º, 43º and 45 °C to determine the effect of temperature on enzymatic activity. The enzymatic index (IE) was calculated using the equation: IE = Diameter in mm of the halo of hydrolysis / Diameter in mm of the halo of the colony.

2.6 *In Vitro* Cross-Feeding

Actinobacteria and rhizobia strains were inoculated separately in a culture medium containing carboxymethylcellulose sodium salt as the only source of carbon and energy (Couri & Farias 1995), The presence of hydrolysis zones around the colonies was recorded as a positive response. Each experiment was carried out independently. Actinobacteria strains that showed a statistically distinct enzyme index along with non-cellulolytic rhizobia strains were selected for the cross-feeding test.

Actinobacteria strains were inoculated separately on carboxymethylcellulose agar in "spots" and incubated at 28 °C for 10 days. One milliliter of culture of each rhizobia strain previously cultivated in YM medium (yeast-mannitol extract) for seven days was transferred to microtubes, centrifuged at 9659.52 g for 10 minutes and the precipitate resuspended twice. One drop of each purified rhizobia culture was distributed around each actinobacteria colony at a distance of 2 cm. A positive result was considered when the growth of rhizobia colonies occurred.

The Compatibility Index was calculated as the number of compatible pairs detected divided by all possible pairs: CI = number of compatible pairs / all interactions between pairs.

2.7 Statistical Analysis

All tests were performed in quadruplicate, with two trials, totaling eight replications. Qualitative data for the characterization of actinobacteria and rhizobia were submitted to the Chi-square test. Data normality was assessed using the Kolmogorov-Smirnov test and the homogeneity of variance using the Levene test, which we used to verify the assumptions of the applied statistical tests. The enzymatic index was submitted to a Studart t test for unpaired data to compare the mean values of each area and an analysis of variance to compare the means between enzymes and between strains, using Tukey's test as post hoc.

Data on the effects of temperature on enzymatic activity were evaluated using the Pearson Correlation Coefficient. A 95% confidence interval was established. The SPSS program was used for all tests performed.

3 RESULTS AND DISCUSSION

3.1 Soil Properties

The soil in both areas can be classified as strongly acidic soils since they showed low pH values and a low cation exchange capacity (T). The anthropized area is classified as low fertility and susceptible to desertification (Table 1).

Table 1: Chemical attributes of soils from the Não Me Deixes Farm, Quixadá, CE - Semi-arid region, Brazil.

Areas	pH (H2O)	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}	SB	Al^{3+}	H+ Al^{3+}	T	V	P	EC	N	MO	Textural class
		----------	----------	----------	$cmol_c\ kg^{-1}$	----------			---------%	---------	mg.kg	dS/m	g/kg	g/kg	
Conserved	4,5	2,4	0,6	0,33	0,12	3,45	0,1	7,4	10,9	31,8	8,98	2.64	0,68	11,17	Areia Franca
Anthropized	5,17	1,81	0,51	0,19	0	2,51	0	1,21	3,7	67,5	3,48	0.55	0,85	2,1	Areia Franca

Source: Authors (2021).

3.2 Microorganisms Characterization

The 50 selected strains were culturally characterized by the color of aerial and reverse mycelium. White and gray colors were predominant in the aerial mycelium in both areas, while in the reverse mycelium yellow was predominant in the anthropized area and gray in the conserved area. Radial texture and the genus *Streptomyces* (Table 2) were also the most present in both areas. There was no significant difference between the strains of the two areas analyzed in the characteristics of morphology ($p = 0.307$), aerial mycelium color ($p = 0.396$), reverse mycelium color ($p = 0.066$), texture ($p = 0.907$) and genus ($p = 0.851$).

Table 2: Morphological characteristics and identification of actinobacteria genus.

Area	Actinobacteria	Morphology	Color		Strains texture	Genus
			Aerial mycelium	Reverse pigments		
Anthopized	QX1	Short straight spore chain	White	Yellow	Radial furrows	*Streptomyces*
	QX2	Short straight spore chain	Beige	Yellow	Concentric	*Streptomyces*
	QX3	Short straight spore chain	Brown	Yellow	Radial furrows	*Streptomyces*
	QX4	Short straight spore chain	Beige	Yellow	Radial furrows	*Streptomyces*
	QX5	Cocci	Brown	Brown	Concentric	*Micromonospora*
	QX7	Cocci and Bacilli	Brown	Yellow	Radial furrows	*Nocardia*
	QX9	Cocci	Cream	Grey	Radial furrows	*Nocardia*
	QX10	Flexuous spore chains	Yellow	Yellow	Radial furrows	*Streptomyces*
	QX12	Flexuous spore chains	Yellow	Yellow	Radial furrows	*Streptomyces*
	QX59	Short straight spore chain	Pink	Pink	Cottony	*Streptomyces*
	QX60	Spiral spore chain	Grey	Cream	Cottony	*Streptomyces*
	QX61	Spore wall	White	Yellow	Concentric	*Streptosporagium*
	QX62	Short straight spore chain	White	Cream	Cottony	*Streptomyces*
	QX63	Spiral spore chain	Brown	Brown	Radial furrows	*Streptomyces*
	QX64	Cocci	Grey	Grey	Cottony	*Micromonospora*
	QX65	Cocci	Grey	Yellow	Cottony	*Nocardia*
	QX67	Fascicied spore chain	Grey	Yellow	Umbonate	*Streptomyces*
	QX68	Spiral spore chain	Grey	Yellow	Radial furrows	*Streptomyces*
	QX70	Short straight spore chain	Grey	Yellow	Radial furrows	*Streptomyces*
	QX71	Bacilli	Grey	Grey	Concentric	*Nocardia*
	QX75	Bacilli	White	Yellow	Cottony	*Nocardia*
	QX76	Flexuous spore chains	White	Cream	Cottony	*Streptomyces*

Conserved	QX13	Flexuous spore chains	Grey	Yellow	Radial furrows	*Streptomyces*
	QX14	Flexuous spore chains	Brown	Cream	Cottony	*Streptomyces*
	QX15	Bacilli	White	White	Radial furrows	*Nocardia*
	QX16	Spiral spore chain	Grey	Grey	Concentric	*Streptomyces*
	QX17	Fascicied spore chain	Brown	Cream	Concentric	*Streptomyces*
	QX19	Short straight spore chain	Grey	Grey	Radial furrows	*Streptomyces*
	QX21	Cocci	Grey	Grey	Cottony	*Micromonospora*
	QX23	Short straight spore chain	Purple	Purple	Cottony	*Streptomyces*
	QX24	Coccobacilli	Orange	Brown	Radial furrows	*Nocardia*
	QX25	Short straight spore chain	Grey	Yellow	Radial furrows	*Streptomyces*
	QX27	Cocci	Brown	Yellow	Radial furrows	*Nocardia*
	QX28	Cocci	Grey	Beige	Radial furrows	*Nocardia*
	QX29	Coccobacilli	Grey	Grey	Concentric	*Nocardia*
	QX30	Fascicied spore chain	White	Yellow	Umbonate	*Streptomyces*
	QX31	Straight spore chain	Grey	Grey	Concentric	*Streptomyces*
	QX32	Fascicied spore chain	Grey	Grey	Cottony	*Streptomyces*
	QX33	Spore wall	Grey	Grey	Radial furrows	*Streptosporagium*
	QX47	Straight spore chain	Grey	Grey	Cottony	*Streptomyces*
	QX48	Fascicied spore chain	Cream	Cream	Cottony	*Streptomyces*
	QX49	Flexuous spore chains	Grey	Grey	Radial furrows	*Streptomyces*
	QX50	Bacilli	White	Cream	Cottony	*Nocardia*
	QX52	Straight spore chain	Yellow	Grey	Umbonate	*Actinomadura*
	QX53	Cocci	Grey	Grey	Concentric	*Micromonospora*
	QX54	Fascicied spore chain	White	Cream	Radial furrows	*Streptomyces*
	QX55	Short straight spore chain	Grey	Yellow	Radial furrows	*Streptomyces*
	QX56	Spore wall	White	Pink	Cottony	*Streptosporagium*
	QX57	Cocci	Grey	Beige	Concentric	*Nocardia*
	QX58	Bacilli	Grey	Grey	Concentric	*Nocardia*

Source: Authors, 2021.

Fifty strains of actinobacteria were isolated in this study (22 are from the anthropized area and 28 from the conserved area). There was no diversity of genera between the areas (Figure 1; p = 0.851), with a predominance of the genus *Streptomyces* in both study areas and the genus *Actinomadura* identified only in the conserved area.

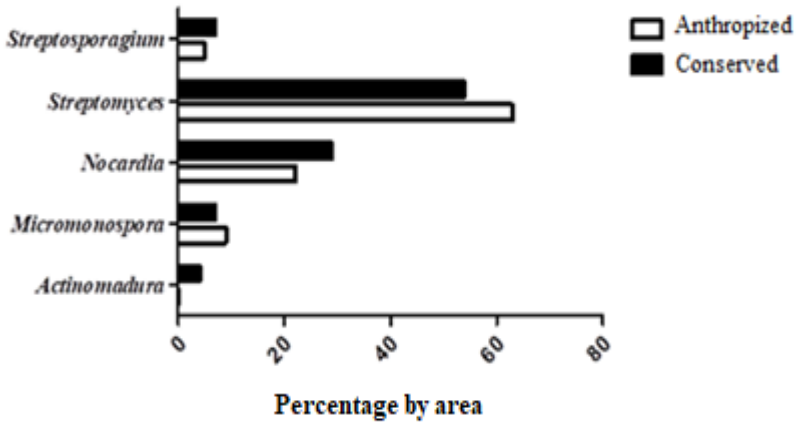

Figure 1: Percentage of genera by study area
Source: Authors, 2021.

The 19 rhizobia strains, together with the seven standard rhizobia strains, were analyzed through phenotypic characteristics, where 78% neutralized or alkalized the culture medium. Of all the rhizobia strains analyzed, 67% had buttery or gummy consistency, 85% were tolerant to

high temperatures and high concentrations of salts. Although they tolerate alkalinity well, the strains were susceptible to the acidity of the medium (Table 3). Comparing the bacterial 16S rRNA sequences with those available in the GenBank® database, 18 strains were identified as Bradyrhizobium while one was identified as *Rhizobium tropici*. The strains showed no statistical difference among themselves in the analyzed characteristics (p=0.395).

Table 3: Phenotypic and physiological characteristics of rhizobia strains

Rhizobium Isolates	Espécie	pH	Growth rate	Mucus	Colony color	Temperature (°C) †	pH 4 †	pH 10 †	NaCl (g L^{-1})
L1	*Bradyrhizobium elkanii*	Neut	Slow	Viscous	White	-	+	+	3
L4	*Bradyrhizobium elkanii*	Alkali	Slow	Viscous	White	39	+	-	3
L6	*Bradyrhizobium vignae*	Neut	Slow	Gummy	White	45	-	+	3
L7	*Bradyrhizobium elkanii*	Acid	Fast	Butyric	Yellow	43	-	+	20
L9	*Rhizobium tropici*	Alkali	Slow	Butyric	White	39	-	-	2
L11	*Bradyrhizobium sp.*	Acid	Fast	Butyric	White	43	+	+	10
L13	*Bradyrhizobium kavangense*	Neut	Slow	Gummy	White	45	-	+	3
L14	*Bradyrhizobium sp.*	Neut	Slow	Gummy	White	45	-	+	3
L15	*Bradyrhizobium japonicum*	Alkali	Slow	Gummy	White	45	-	+	3
L16	*Bradyrhizobium japonicum*	Neut	Slow	Butyric	Yellow	45	-	+	3
L17	*Bradyrhizobium sp.*	Alkali	Slow	Viscous	White	41	-	+	3
L18	*Bradyrhizobium sp.*	Neut	Slow	Gummy	White	45	-	+	3
L19	*Bradyrhizobium kavangense*	Neut	Slow	Butyric	White	41	-	+	10
L20	*Bradyrhizobium yuanmingense*	Alkali	Slow	Gummy	White	45	-	+	3
L21	*Bradyrhizobium sp.*	Alkali	Slow	Butyric	White	43	+	+	10
L23	*Bradyrhizobium yuanmingense*	Acid	Slow	Gummy	White	41	-	+	2,5
L24	*Bradyrhizobium yuanmingense*	Neut	Slow	Gummy	White	41	-	+	10
L27	*Bradyrhizobium iriomotense*	Alkali	Slow	Gummy	Yellow	39	+	-	2,5
L29	*Bradyrhizobium kavangense*	Alkali	Slow	Gummy	White	39	-	-	3
BR 3486	*Burkholderia phymatum (BR 3486)*	Acid	Fast	Butyric	Yellow	43	+	+	10
BR 3267	*Bradyrhizobium sp (BR 3267)*	Neut	Slow	Butyric	White	45	-	+	3
BR 3302	*Bradyrhizobium sp (BR 3302)*	Alkali	Slow	Viscous	White	45	-	+	10
BR 3262	*Bradyrhizobium sp (BR 3262)*	Alkali	Slow	Viscous	White	-	+	+	3
BR 3301	*Bradyrhizobium sp (BR 3301)*	Alkali	Slow	Viscous	White	39	+	+	3
BR 2003	*Bradyrhizobium sp (BR 2003)*	Neut	Slow	Butyric	White	-	+	+	3
BR 2801	*Bradyrhizobium elkanii (BR 2801)*	Neut	Slow	Viscous	White	-	+	+	3

Note: † The (+) symbol indicates a positive result while (-) indicates a negative result.
Source: Authors, 2021.

Rhizobia strains showed resistance to several antibiotics and 14 strains (eight isolated strains and six standard strains) were able to produce the amylase enzyme. However, no strain was able to produce xylanase or cellulase (Table 4). Strains that did not show the ability to produce cellulase were selected for cross-feeding tests with actinobacteria.

Table 4: Antibiotic resistance and enzymatic profile of rhizobia strains.

Rhizobium Isolates	Antibiotics															Cellulase	Amylase	Xylanase	Phosphate solubilizing
	CIP 05	CLO 30	GEN 10	PIT 110	SUT 25	TET 30	CFO 30	CAZ 30	CRO 30	AMI 30	AMC 30	AMP 10	ATM 30	CFL 30	CPM 30				
L1	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	-	-	-
L4	-	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	-	-	-
L6	+	+	+	-	+	+	-	+	-	-	-	+	+	-	-	-	+	-	-
L7	+	+	-	+	+	-	-	+	+	-	-	+	+	+	+	-	+	-	-
L9	-	+	-	-	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-
L11	-	+	-	-	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-
L13	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-
L14	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-
L15	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-
L16	-	+	-	-	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-
L17	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-
L18	+	+	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-
L19	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	-	+	-	-
L20	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-
L21	+	+	+	-	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-
L23	+	+	+	-	+	-	+	+	-	-	-	-	+	-	-	-	+	-	-
L24	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
L27	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-
L29	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	+	-	-
BR 3486	-	+	+	+	+	-	-	+	+	-	+	+	+	+	-	-	-	-	-
BR 3267	+	+	+	+	-	+	-	+	+	-	-	+	+	+	+	-	+	-	-
BR 3302	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
BR 3262	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-
BR 3301	+	+	+	-	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-
BR 2003	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-
BR 2801	+	+	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	-

Source: Authors, 2021.

3.3 Enzyme Profile

Among the analyzed actinobacteria strains, 47 had amylolytic activity (94%), 37 had xylanolytic activity (74%) and 32 had cellulolytic activity (64%). There was no statistical difference in cellulolytic activity (p = 0.360) between the two areas evaluated, while amylolytic (p = 0.000) and xylanolytic (p = 0.005) activities were higher among the strains from the anthropized area (Figure 2). The least expressed enzymes in the strains analyzed were amylolytic and cellulolytic. While xylanolytic presented the highest values of Enzymatic Index (EI), reaching a maximum of 5.8 and statistically standing out from the other activities (p = 0.000).

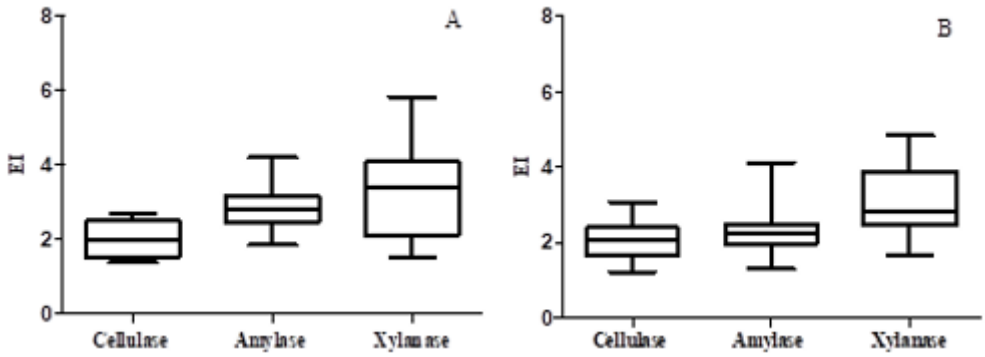

Figure 2: Cellulase, amylase and xylanase enzyme index (EI) of actinobacteria strains isolated from the soil of the semi-arid region - Brazil. Anthropized area (A) and conserved area (B).
Source: Authors, 2021.

Production of cellulase (r = 0.133) and amylase (r = 0.172) enzymes had a low positive Pearson correlation coefficient (p = 0.000) when related to temperature increase, but the xylanase enzyme had a very strong negative correlation coefficient. (r = -0.703, p = 0.000) (Figure 3) when correlated to it. Only three strains were able to show xylanolytic activity at a temperature of 45°C (QX 15 – *Nocardia*, QX 19 – *Streptomyces*, Qx 29 – *Nocardia*) all strains from the conserved area.

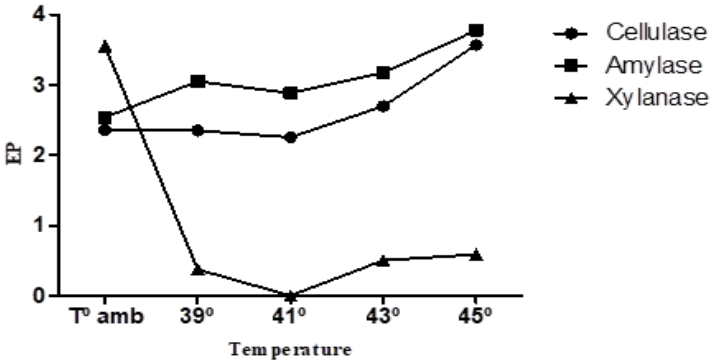

Figure 3: Cellulase, amylase and xylanase enzymatic index (EI) influenced by room temperature, 39ºC, 41ºC, 43ºC and 45ºC, of actinobacterial strains isolated from soils of the semi-arid region of Brazil.
Source: Authors, 2021.

3.4 *In Vitro* Cross-Feeding

None of the rhizobia strains showed the ability to produce cellulase (Table 4), while 32 strains of actinobacteria showed a halo of cellulose degradation. The enzymatic index (EI) was calculated through the ratio between the diameter of the halo and the diameter of the colony, and the result was submitted to an analysis of variance (p = 0.000), where two strains stood out in relation to the others, QX 59 and QX 67.

Strains QX 59 and QX 67 belong to the genus *Streptomyces* and come from the anthropized area. They were selected to be cultivated together with the 19 rhizobia strains that did not show cellulase production. The results obtained revealed 17 positive interactions.

Rhizobia L20 (*B. yuanamingense*) and L27 (*B. iriomontense*) that did not show cross-feeding with strain QX 59 (*Streptomyces*) were able to present this activity with strain QX 67 (*Streptomyces*). Rhizobia L9 (*Rhizobium tropici*), L14 (*Bradyrhizobium sp.*) and L15 (*B. japonicum*) showed cross-feeding only with the strain QX 59 (Figure 5). This lack of cross-feeding can be attributed to the low resistance to antibiotics presented by these rhizobia strains. That can be showed through the in vitro tests where the rhizobia strains were sensitive to 10 out of the 15 antibiotics tested.

Regarding soil fertility, the conserved area has a higher concentration of nutrients for plants and organisms, which is expressed by a greater sum of bases (SB) and cation exchange capacity (T), and its limitation is in the acidity (higher H + Al). Low pH can reduce the availability of nutrients in the conserved area, causing the impression that the anthropized area is more fertile. This impression occurs by the correction of pH and preparation of the soil in the anthropized area.

Formation of actinobacteria aerial hyphae depends on the characteristics of the species, nutritional conditions or environmental factors. There is a difference in actinobacteria color expression between areas, but chromogenesis alone is not sufficient for classification of the species. Parameters, such as spore chain morphology and spore and colony shape, are necessary for the taxonomy of this bacteria genera (Thampi & Bhai, 2017).

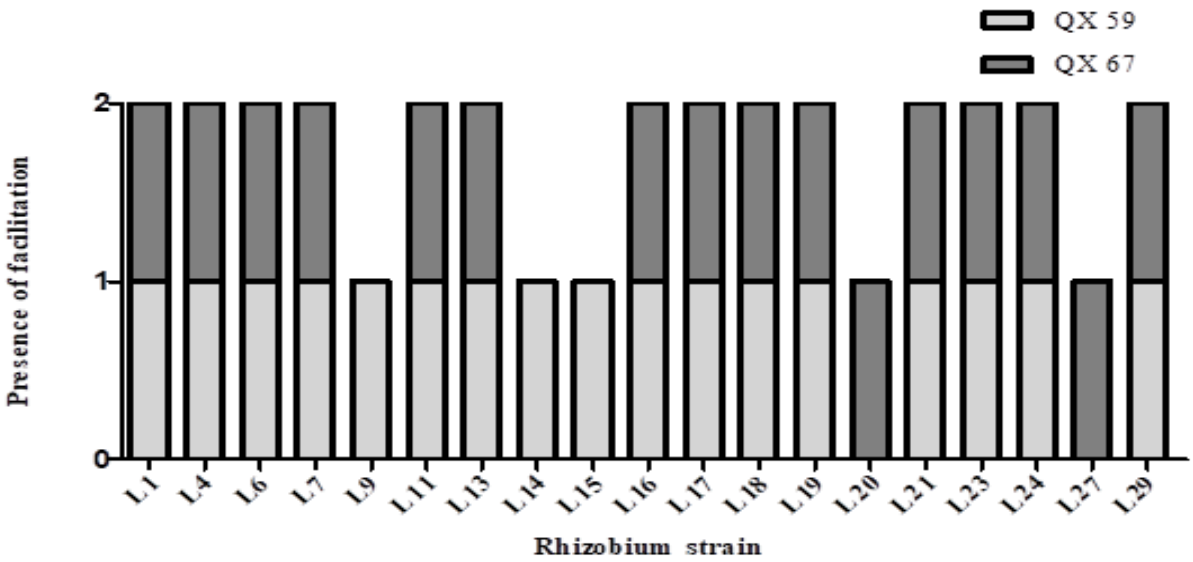

Figure 4: Cross-feeding between nineteen strains of rhizobia and two strains of actinobacteria isolated from the semi-arid region of Brazil.
Source: Authors, 2021.

Results found in this study are as expected for arid environments, such as those observed in the desert in the Tibetan plateau (Ding et al., 2013). In their study, the bacteria strains showed white, gray, yellow and brown colors, with a predominance of the genera *Streptomyces*, *Micromonospora* and *Streptosporangium*. In Brazilian semi-arid soils, there are reports of the presence of the genera *Streptomyces* and *Nocardia* (Silva et al., 2019).

Locally isolated strains are able to withstand environmental stresses that could otherwise limit their productivity. Isolated rhizobia from Egypt by Yanni et al., (2016) were used for the production of biofertilizers and provided a significant increase in seed yield and agronomic efficiency of common bean (*Phaseolus vulgaris*) under field conditions. Ali et al., (2019) isolated rhizobia strains of soy nodules in Bangladesh and found that local strains showed efficacy and resistance to conditions of physical stress, pH, salinity and temperature.

The selection of antibiotic-resistant rhizobia strains results in greater competitive ability. Anand et al., (2012) showed that soybean inoculation with antibiotic and phage resistant *Bradyrhizobium* showed high nitrogen fixation capacity, providing increased soybean production in soils from India.

Antagonism between rhizobia and actinobacteria has been studied for some time (Jha et al., 2020 and a preliminary study was needed to assess the resistance of the strains to antibiotics. The strain QX 67 (*Streptomyces*) from the anthropized area, presented high enzymatic indices in the three activities among the analyzed enzymes. Mostly of the strains where the highest enzymatic index values were observed come from the anthropized area. This can be explained more by the release of enzymes in the soil, which is related to metabolic requirements and available nutrients, than the diversity of organisms.

The ability of microbial communities to maintain functional diversity through disturbance, stress or ecological succession may be more important for ecosystem productivity than taxonomic diversity. This is reflected in cellulolytic, amylolytic and xylanolytic activities that are strongly influenced by nutrient deprivation (Chroni et al., 2009)

Physicochemical characteristics of the soil can influence the production of xylanase by fungi, bacteria and actinobacteria (Ramanjaneyulu et al., 2017). Higher concentrations of these bacteria occurred in a soil with pH 5.1. This is consistent with the results found in this study, where the conserved area has a pH more acidic and showed a lower concentration of xylanase enzyme-producing in the actinobacteria strains.

The origin of the actinobacteria influences their ability to produce functional enzymes under high stress conditions. Strains originating from warmer environments, such as arid and semi-arid, have an ability to produce enzymes even when subjected to high temperatures (Nithya et al., 2017). Chroni et al. (2009) observed that there is an increase in the halo indicative of cellulose degradation with the increase in temperature. Minotto et al. (2014) observed the same halo increase for cellulase and amylase enzymes. According to Nithya et al., (2017) enzyme production will depend mainly on the strain, composition of the medium, cultivation methods, growth, nutritional requirements, pH, temperature and incubation time.

The optimum temperature for the enzymatic production of actinobacteria may vary depending on the origin of the bacteria. Chaudhary and Prabhu, (2016) described 55ºC as the optimum temperature for the production of amylase and cellulase. While works with actinobacteria from marine environments, reported the optimal temperature for amylase production to range from 25 to 30ºC, with a decrease in production above this range (Krishnakumar et al., 2015).

Although there are reports in the literature on actinobacteria that exhibit xylanolytic activity at temperatures up to 80°C (Rahmani et al., 2018), the strains studied were affected by temperature changes. Only five strains showed xylanolytic activity at a temperature of 39ºC. Sanjivkumar et al., (2017), actinobacteria strains produced xylanase up to 40º C, showing decreasing activity in higher temperatures.

Actinobacteria strains produce many extracellular metabolites. *Streptomyces* can suppress phytopathogens and act as plant growth regulators (Saif et al., 2014). These bacteria can also increase seed germination and number of nodules by inhibiting competing bacteria present in the soil. These characteristics, and better performance at high temperatures, make actinobacteria ideal candidates to be used as microbial inoculants for agricultural use and in the recovery of degraded areas.

The compatibility index of the two strains of actinobacteria, QX 59 and QX 67, was 0.89 and 0.84 respectively. There was no difference in the ability to cross-feeding with the rhizobia strains. However, this confirms the hypothesis that these microorganisms have the ability to in vitro cross-feeding with noduliferous diazotrophic bacteria.

The use of microbial consortia is an emerging field that allows microorganisms to perform complex functions that are impossible for a single organism and provides solutions to environmental stress factors. Coinoculation of two different bacterial species exerts stronger effects on plant growth than a single-species inoculation. This suggests that the synergistic functions of multiple strains are more effective in plant-bacteria interactions (Santiago et al., 2017).

The use of coinoculation between actinobacteria and rhizobia is still new, but it has been documented with promising results. Soe and Yamakawa, (2013), coinoculated *Bradyrhizobium yuanmingense* and *Streptomyces griseoflavus* and the results revealed an increase in nodulation, nitrogen fixation and seed yield in different soybean varieties. Le et al., (2016) reported that *Sinorhizobium meliloti* coinoculated with *Streptomyces* in alfalfa plants increased the dry weight of the plant by up to 30%.

The study of antagonistic interactions between actinobacteria and rhizobia has been widely reported (Lima et al., 2017). However, there are few reports of a synergistic relationship between these microorganisms. Actinobacteria and rhizobia species from the same environment interact with each other and this over time promote coexistence, with both antagonistic and mutualistic interactions (Silva et al., 2019).

4 FINAL CONSIDERATIONS

The level of anthropization did not influence the phenotypic, taxonomic and enzymatic profile of actinobacteria, showing that the strains are able to survive the anthropic effects occurring in the environment. Human action for soil preparation and cultivation did not reduce the viability and capacity of these strains, indicating potential for their use in agricultural areas.

Strains QX 59 and QX 67 stood out as they presented the highest values of enzyme index and were selected for the cross-feeding tests. They were identified as *Streptomyces* and are from the anthropized area.

Rhizobia strains showed resistance to antibiotics allowing a high occurrence of in vitro cross-feeding with actinobacteria strains.

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