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Amylase-Producing Actinobacteria Facilitate Rhizobia Growth in a Culture Medium with Starch

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Abstract
Plant growth-promoting rhizobacteria (PGPR) are microorganisms that act in the production of metabolites that confer advantages for the growth and development of the plant, receiving in exchange sources of carbon and energy. Two classic examples of PGPR are rhizobia, which act in the bioavailability of nitrogen, and actinobacteria, which perform numerous functions such as production of phytohormones, antibiotics, and solubilization of phosphate and potassium. To establish this microbiota, the plant releases carbohydrate-rich exudates that not only serve as a carbon source for microorganisms, but also determine which microorganisms can colonize its rhizosphere. This study evaluated how amylase-producing actinobacteria are able to allow rhizobia growth without this enzymatic activity when co-inoculated in a culture medium that has starch as the sole carbon source. Since starch is a carbohydrate like those released in exudates, it is inferred that this ability to facilitate growth in vitro would be important in stimulating rhizobia growth in the rhizosphere and, consequently, nitrogen fixation. Ten actinobacteria and seven rhizobia were used, of which we obtained 12.86% of positive results. This cooperation exemplifies another case of substrate cross-feeding, paving the way to further *in vitro* and *in vivo* studies of interbacterial interactions, as well as to the prospect of biofertilizers.

Keywords: Bacterial ecological interactions; Bioinoculant; Cross-Feeding; PGPR; *Streptomyces*.

1. Introduction
Microorganisms play numerous ecological roles in soil, such as changing the spatial distribution and bioavailability of nutrients [1]. Bacteria beneficial to plants are traditionally known as plant growth promoting rhizobacteria (PGPR) and act in the mobilization of nutrients in the soil, production of growth regulators, phytohormones, solubilization of phosphate and potassium, and protection against phytopathogens [2].

Plant growth, productivity, and health rely on the microbial community associated with it, so the plant shapes the composition of the rhizospheric microbiota via rhizosphere feedback. Exudates are rich in carbohydrates, amino acids, organic acids, flavonoids, growth factors, enzymes, among others, which vary from plant to plant due to genetic and environmental factors, and are capable of selecting and stimulating microbial growth [3, 4, 5]. In association with factors such as soil type and pH, exudates determine the nature of the local microbiota [6].

The release of flavonoids from roots attracts bacteria known as rhizobia. These bacteria are the most classic example of PGPR due to its capacity of forming symbiotic nodules in the roots of legumes. There, they perform biological nitrogen fixation, which is a process responsible for making this nutrient bioavailable to the plant [7].

Exudates also attract other PGPRs such as actinobacteria. These bacteria stimulate plant growth, antagonize phytopathogens, improve nutrient availability, and produce phytohormones such as auxins and gibberellins [8]. Outside the rhizospheric environment, actinobacteria also show great biotechnological value for the production of antibiotics, insecticides, herbicides, immunomodulators, and enzymes such as cellulases, xylanases, pectinases, proteases, chitinases and amylases [9].

In this study, we evaluated the ability of actinobacteria with amylolytic activity to facilitate the growth of rhizobia without this enzymatic activity when grown in solid media with starch as sole carbon source. Microorganisms in the rhizosphere are fed by plant exudates and among the carbon sources present in this compound are carbohydrates, such as starch. Therefore, the ability of rhizobacteria such as actinobacteria to allow the growth of PGPR as rhizobia in the presence of this sugar constitutes an important ecological relationship and is the focus of this research.

2. Materials and methods

The microorganisms studied in this work were selected based on their extracellular amylolytic activity from the culture collection of the Laboratory of Environmental Microbiology of the Federal University of Ceará. We used ten strains of actinobacteria with extracellular amylolytic activity statistically different from the others, and 7 strains of rhizobia without this enzymatic activity. The actinobacteria genus was identified by Ribeiro et al. [10], and the species of rhizobia by Silva [11]. This information is shown in Table 1.

Table 1: Strains of actinobacteria and Rhizobia from the Semi-arid used in this work.

Actinobacteria		**Rhizobia**	
Strain	Genus	Strain	Species
A108	*Streptomyces*	L1	*Bradyrhizobium elkanii*
A109	*Nocardia*	L4	*Bradyrhizobium elkanii*
A125	*Streptomyces*	L9	*Rhizobium tropici*
A136	*Streptomyces*	L13	*Bradyrhizobium kavangense*
A139	*Streptomyces*	L15	*Bradyrhizobium japonicum*
A143	*Streptomyces*	L24	*Bradyrhizobium yuanmingense*
A144	*Streptosporangium*	L27	*Bradyrhizobium iriomotense*
A145	*Streptomyces*	-	-
A146	*Streptosporangium*	-	-
A148	*Streptomyces*	-	-

Source: Ribeiro *et al*. (2022); Silva (2020).

Co-inoculation was performed according to Silva *et al*. [12] with modifications. The actinobacteria were inoculated by spots in duplicate in a culture medium containing starch as the sole carbon source. The plates were then incubated in a B.O.D. incubator for 7 days at 28°C.

At the end of the actinobacterial incubation period, 1 ml of rhizobia cultured in YM broth (yeast extract-mannitol) was transferred to sterile microtubes in triplicate. The microtubes were centrifuged at 9261 x g for 10 minutes. The supernatant was discarded and the pellet resuspended 3 times in sterile distilled water. 10 µL of purified rhizobia were then transferred to the plates containing the actinobacteria grown in starch medium, approximately 1 cm from the actinobacteria colony. The plates were then incubated in a B.O.D. incubator at 28ºC for another 7 days.

Rhizobium growth characterized a positive result, while the absence of growth characterized a negative result. The compatibility index (CI) of actinobacteria and rhizobia was calculated by the ratio between the number of positive pairs and the number of possible pairs for each strain.

3. Results & Discussion

Among the 70 possible pairs, 9 presented a positive result, which represents 12.86% of positive pairs. Actinobacteria A148 stood out from the others, presenting a compatibility index of 0.429. Rhizobia L1 showed a compatibility index of 0.4, the highest among all rhizobia studied, and was compatible even with actinobacteria A148. Therefore, it is concluded that the pair A148+L1 is the most promising. The results are summarized in Table 2. Some examples of positive pairs are illustrated in Figure 1.

Table 2: *In vitro* co-inoculation between Semiarid actinobacteria and rhizobia in medium with starch.

Rhizobia	**Actinobacteria**										**RCI**
	A108	**A109**	**A125**	**A136**	**A139**	**A143**	**A144**	**A145**	**A146**	**A148**	
L1	-	-	-	-	-	+	-	+	+	+	0,4
L4	-	-	-	-	+	-	-	-	-	+	0,2
L9	-	-	-	-	-	-	-	-	-	-	0
L13	-	-	-	-	-	-	-	-	-	-	0
L15	-	-	-	-	+	-	-	-	-	-	0,1
L24	-	-	-	-	-	-	-	-	-	-	0
L27	-	-	+	-	-	-	-	-	-	+	0,2
ACI	0	0	0,143	0	0,286	0,143	0	0,143	0,143	0,429	

(+) presence of facilitation (positive result). (-) absence of facilitation (negative result). (ACI) actinobacterial compatibility index. (RCI) rhizobia compatibility index. Source: the author.

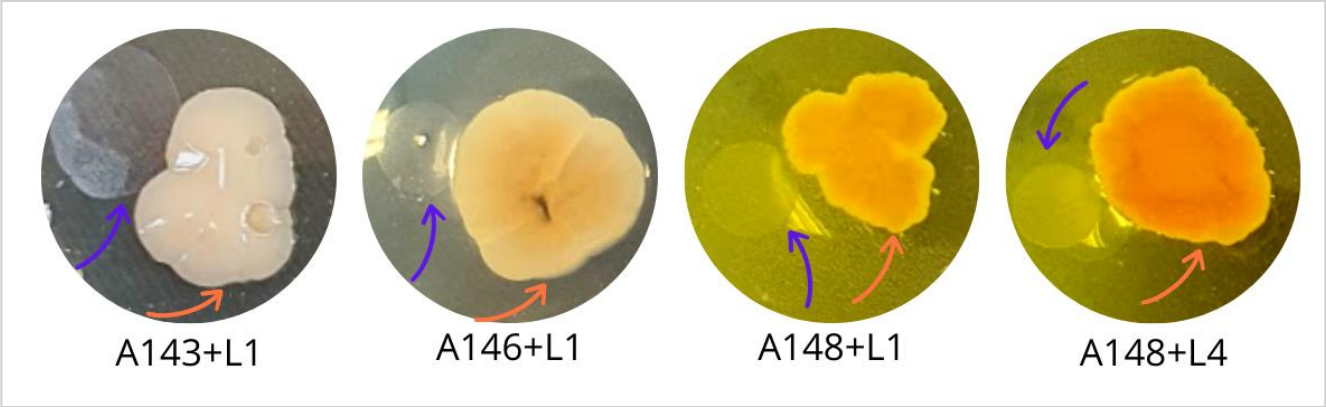

Fig. 1: Examples of positive pairs from *in vitro* coinoculation. The blue arrows indicate rhizobia colonies. Red arrows indicate actinobacterial colonies. The images of A148 actinobacterium present a yellow background, as the intense production of pigments by the strain colored the entire medium. Source: the author.

Even though bacteria commonly compete for nutrients with each other, more complex relationships can be established. One of them is substrate cross-feeding, an interaction in which a strain feeds on molecules produced by the metabolic apparatus of another strain. These molecules can usually be exploited by both strains. An example would be the partial degradation of oligofructose or inulin by *Bacteroides thetaiotaomicron* in coculture with bifidobacteria. Single-chain sugars resulting from this extracellular degradation serve as a carbon source for both strains [13].

There is also evidence that the production of LdhA lactate dehydrogenase by *Pseudomonas aeruginosa*, which reduces pyruvate to D-lactate under anaerobic conditions, enables cross-feeding in biofilms in cystic fibrosis patients. This enzyme would be responsible for producing D-lactate in the anaerobic zone of the biofilm, which would be used as a substrate in the aerobic zone. This cooperation is one of the reasons why it is so complicated to treat patients with this type of lung infection [14]. We co-inoculated amylolytic actinobacteria and non-amylolytic rhizobia in culture medium containing starch as the sole carbon source. Extracellular degradation of starch by actinobacteria, mainly by the A148 strain, allowed the growth of rhizobia in this culture medium in a new example of substrate cross-feeding.

According to Zheng et al. [15], the presence of α-amylase strongly influences the adaptation strategies of bacterial metabolism and stimulates the production of metabolic signals such as acetic acid. This acid induces numerous changes in the physicochemical properties of the bacterial surface, such as hydrophobicity and surface charge, which directly affects the interactions that these bacteria are able to establish. This may explain why only 12.86% of our pairs were positive. As all strains of actinobacteria in this study are amylase producers and the rhizobia are not, the production of this enzyme by the actinobacteria may have influenced the production of other metabolites that inhibited the rhizobia growth. This makes the presence of positive results even more surprising, which can be explained by the production of secondary metabolites by the actinobacteria itself [16] or by the biochemical apparatus of rhizobia [17].

4. Conclusions

The actinobacteria allowing the growth of non-amylase-producing rhizobia in the medium containing starch is an interaction that exhibits great biotechnological potential for the creation of new biofertilizers, as well as for a better understanding of the ecological relationships that occur in the soil of the Brazilian semiarid region.

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