

Bioprospecting rhizobacteria associated to cacti to water stress resistance and biofilm formation

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Abstract. The aim of this study was to isolate and select bacteria from the cacti rhizosphere naturally occurring in an adverse environment in culture medium under water stress. Samples of rhizospheric cacti soil were collected in two distinct locations with natural occurrence of the Caatinga vegetation of the Alagoas State, Northeast Brazil. With the use of culture-dependent methodologies, the isolation and purification of the bacteria was performed for exopolysaccharide production analyzes by quantification of halos and biofilm formation in a spectrophotometer under optical density (OD) of 560 nm. In total, 42 strains were isolated, with the isolates BCM02, BCM06, BCM10 and BCM13 being the major producers of EPS, followed by bacteria BCM01, BCM05, BCM28 and BCM35 with medium production, and bacteria BCM22 and BCM33 with low production. On the other hand, for the biofilm formation, all the isolates were efficient in the synthesis of the same, being 38% of the bacteria with high formation performance. Thus, strains BCM02 and BCM28 presented the best results in evaluations of water stress tolerance mechanisms, and could later be used for future tests to promote plant growth.

Keywords: Growth promotion bacteria; Exopolysaccharides; Tolerant bacteria.

Received
October 03, 2019

Accepted
December 24, 2019

Released
December 31, 2019



Acesso aberto



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Resumo. Bioprospecção de rizobactérias associadas a cactos para resistência ao estresse hídrico e formação de biofilme. O

objetivo deste estudo foi isolar e selecionar bactérias da rizosfera de cactos que ocorrem naturalmente em um ambiente adverso em meio de cultura sob estresse hídrico. Amostras de solo rizosférico de cactáceas foram coletadas em dois locais distintos, com ocorrência natural da vegetação da Caatinga no Estado de Alagoas, Nordeste do Brasil. Com o uso de metodologias dependentes da cultura, o isolamento e a purificação das bactérias foram realizados para análises de produção de exopolissacarídeos por quantificação de halos e formação de biofilme em um espectrofotômetro sob densidade óptica de 560 nm. No total, foram isoladas 42 linhagens, sendo os isolados BCM02, BCM06, BCM10 e BCM13 os maiores produtores de EPS, seguidos pelas bactérias BCM01, BCM05, BCM28 e BCM35 com produção média e bactérias BCM22 e BCM33 com baixa produção. Por outro lado, para a formação de biofilme, todos os isolados foram eficientes na síntese dos mesmos, sendo 38% das bactérias com alto desempenho de formação. Assim, as linhagens BCM02 e BCM28 apresentaram os melhores resultados nas avaliações dos mecanismos de tolerância ao estresse hídrico, podendo posteriormente ser utilizadas em testes futuros para promover o crescimento das plantas.

Palavra-chave: Bactéria promotora de crescimento; Exopolissacarídeos; Bactéria tolerante.

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Introduction

Water is essential for the maintenance of life and essential for the production of food (Pimentel et al., 2004). However, due to the constant climatic adversities and the misuse of this resource, the Brazilian agricultural sector has suffered serious consequences, limiting the activity in the country.

The United Nations Food and Agriculture Organization (FAO, 2011) estimates that 70% of the world's freshwater (potable water) is used exclusively for agriculture (irrigation), 20% for industrial use and 10% for domestic purposes. In the production of about 1 kg of irrigated rice (*Oryza sativa*), 2,300 L of water are spent; for cereals in general, the average is around 1,500 L.kg⁻¹, for citrus fruits, 1,000 L.kg⁻¹ and for the production of maize (*Zea mays*), are spent about 900 L.kg⁻¹.

In view of the described situation, the concern for alternatives to reduce water consumption by crops, to promote the growth of plants in soils with low water precipitation, as observed in some regions of the Northeast and to recover areas in the process of desertification. Therefore, the use of drought tolerant microorganisms capable of protecting plants and promoting their growth under water stress is an alternative to this problem (Kavamura et al., 2013).

The cacti are plants of the Cactaceae Family, which have around 176 genera and about 2,200 species, which have adapted and developed mechanisms over time that have allowed them to live in dry and warm environments. They are mainly distributed in the Americas and are characterized by a juicy stem filled with spines (Arruda et al., 2005).

However, as these plants adapted to their habitat and evolved to their

survival, the microorganisms associated with these plants also underwent adaptations that gave them tolerance to environments with low water availability, and, on top of that, plant protection against certain environmental adversities (Kavamura et al., 2013).

In addition to cellular protection, it is believed that microorganisms can also offer vegetal protection against desiccation, by maintaining a humid environment conducive to root development, nutrient supply, and hormones, also acting as growth promoters. In addition, the microorganisms can be obtained by means of various mechanisms such as the production of exopolysaccharides (EPS) (Nocker et al., 2012; Montaldo, 2016; Silva et al., 2018) and biofilm formation (Chang et al., 2007; Montaldo, 2016).

Exopolysaccharides are defined as extracellular polysaccharides where innumerable microorganisms have the ability to produce them, bound to the surface of cells or excreted into the medium. This mechanism of tolerance allows adaptation to several types of environmental stresses such as water, saline and temperature variations (Schiavão-Souza et al., 2007) and allows free life to the bacteria, allowing adhesion and colonization on solid surfaces, the involvement of cell membranes against desiccation, and the fixation of nutrients close to the bacteria (Barreto et al., 2011).

Exopolysaccharides also help microorganisms to colonize the roots due to the involvement of biofilms that permanently connect to the root surface. Biofilm, another mechanism of tolerance to water stress, consists of bacterial communities surrounded by substances exuded by the bacteria themselves, being free when released from the community, having the advantage of protecting against several external aggressions, such as lack of nutrients and protection against the loss of water (Boari et al., 2009).

The mechanisms described help the colonization of the root system by microorganisms, giving protection to environmental stresses, thus promoting resistance to plants and providing their growth and productivity in environments with low water availability. In view of the above, the objective was to prospect, isolate and select rhizospheric soil bacteria from Caatinga cacti, with ability to grow in medium with reduced water activity.

Material and methods

Study area and soil sampling

A collect of rhizospheric soil (10 cm of surface depth) were carried out on Piranhas and Olho d'Água do Casado, both located in the backlands of the State of Alagoas, Brazil. Two random points were selected in each municipality, and three cacti were selected for collection. From each plant was collected soil samples, with the subsequent mixing of the same, forming a composed sample of soil per plant. After collection, the samples were stored in appropriate paper bags, for the best preservation of the material.

Bacterial isolation and selection

For the isolation of the rhizospheric soil bacteria, 1 g of soil was transferred to erlenmeyers containing 10 mL of buffer (Araújo et al., 2002). The solution was shaken for 1 hour in a mechanical orbital shaker. After shaking, serial dilutions were made up to 10^{-5} , followed by transfer of 100 μ L of appropriate dilutions, later seeded in Petri dishes containing Tryptone Soy Agar (TSA) culture medium (10%), the number of colony forming units (CFU) being estimated.

For the selection of resistant rhizobacteria, the TSA isolates (10%) were seeded in medium with three different concentrations of mannitol (285 g/L, 520 g/L and 780 g/L), yielding Water activity (A_w) corresponding to

0.957, 0.897 and 0.807, respectively, and incubated at 40 °C. Bacterial isolates were purified by the composite streak technique, stored in TSA culture medium (10%) in a sloped test tube and then stored in 2 mL cryogenic tubes containing 50% glycerol at 18 °C.

Exopolysaccharides production assay

Was incubated 5 µL of the bacterial isolates grown on TSB culture medium (10%) were inoculated into 5 mm sterile filter paper disks in modified culture medium from Guimarães et al. (1999), added with 10% sucrose, in a factorial design with 3 replicates.

The production of EPS was characterized visually by measuring the halo produced with the aid of an electronic caliper, being + (low yield - EPS halo ≤ 10 mm Ø), ++ (middle yield - EPS halo of 10-14 mm Ø) and +++ (optimum yield - EPS halo ≥ 14 mm Ø). The confirmation of the production was performed by the chemical method, mixing the platinum handle impregnated with the colony in 2 mL of ethyl alcohol (Paulo, 2010).

Subsequently, 100 µL of the bacterial isolates grown in TSB culture medium were inoculated into polypropylene tubes with 900 µL of TSB (10%) added to five different concentrations of sorbitol (0.03 M, 0.06 M, 0.30 M, 0.60 M and without sorbitol, corresponding to the control). After incubation at 40 °C for 96 h, the contents of each tube were homogenized and the absorbance in a spectrophotometer at 600 nm was checked for bacterial growth.

The cells were removed and each tube was washed three times with distilled water, followed by addition of 1000 µL of violet crystal solution in each tube. After 15 min of incubation at room

temperature the tubes were washed again three times with distilled water (Montaldo, 2016).

Biofilm formation

The biofilm formation was quantified by the addition of 1000 µL of ethyl alcohol (95%) in each tube to solubilize the violet crystal incorporated into the cell wall. The optical density of the solubilized dye was determined in a spectrophotometer at 560 nm (O'Toole and Kolter, 1998).

Results

The bacterial strains obtained were stored in a freezer, totalizing 42 bacterial strains. In relation to the growth in culture medium, the highest amount of isolates in the medium with mannitol concentration of 285 g/L ($A_w = 0.957$) was obtained, which is the one with the highest water activity, of the three concentrations analyzed. The higher the water activity, the faster the microorganisms will be able to grow.

After the incubation period, the halos formed around the paper discs were measured with the aid of a digital pachymeter, where the mean production of each strain was verified and divided into three groups: low, middle and high production of EPS, where + (low production - EPS halo ≤ 10 mm Ø), ++ (middle production - EPS halo of 10-14 mm Ø) and +++ (optimal production - EPS halo ≥ 14 mm Ø). Figure 1 illustrates the reading of the qualitative tests for the production of EPS by means of the disc method, and it is possible to visualize the EPS production by the lineages, through the formation of a mucoid substance around the disc, where the absence of this substance characterizes non-producing strains.

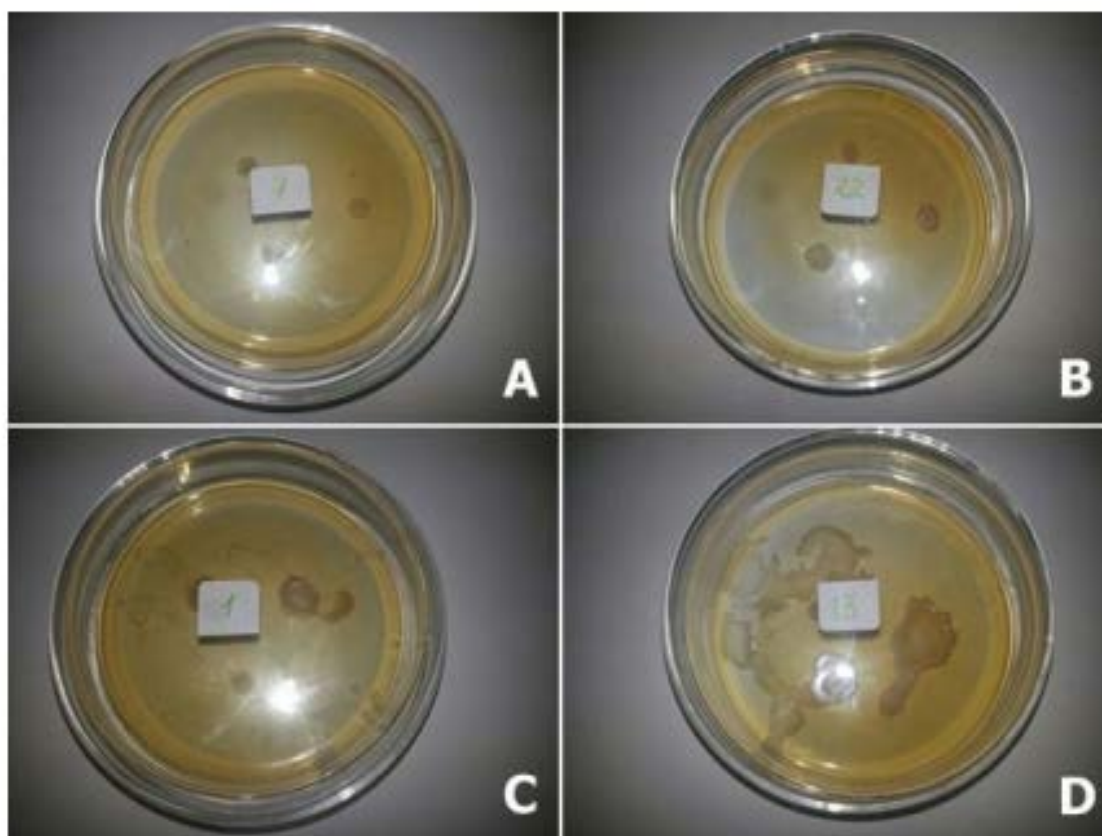


Figure 1. EPS production by rhizobacteria isolated from cacti.

Confirmation of the EPS production was performed by the chemical method. It was observed that of the 11 halo-producing isolates, strain 4 was not characterized as a producer of exopolysaccharides, since even if the isolates produce a halo, the non-EPS producing strains when mixed in ethyl alcohol are suspended, leaving the medium what happened to the bacteria in question. The exopolysaccharides produced by the strains, when mixed in ethyl alcohol, are precipitated, since EPS is insoluble in alcohol.

From the totality of bacterial strains extracted from plant materials collected in Alagoan Caatinga, 23.80% synthesized EPS in medium with restricted water activity (Table 1).

Table 1. Production of exopolysaccharides (EPS) by bacterial strains isolated from rhizospheric cactaceous soil from the Alagoan sertão.

Isolate	EPS production*
BCM01	++
BCM02	+++
BCM05	++
BCM06	+++
BCM10	+++
BCM13	+++
BCM22	+
BCM28	++
BCM23	+
BCM35	++

* (+) low production; (++) micelle production; (+++) high production.

For the biofilm production tests, only 36 isolates were evaluated in order to determine the best parameters for the measurements. The presence of biofilm on the abiotic surface was evidenced by the formation of a violet coloration.

The biofilm formation was performed in four concentrations of sorbitol (0.03 M, 0.06 M, 0.30 M, 0.60 M) and without sorbitol, simulating different

levels of water stress, however the formation of the same did not differ statistically between the different concentrations, and the isolates were compared for the capacity of formation in medium with reduced water activity. The results are shown in Table 2, where all the isolates were efficient in biofilm formation.

Table 2. Formation of biofilm on abiotic surface by bacterial isolates in TSB medium supplemented with 10% sucrose based on absorbance values obtained OD 560 nm, according to Scott-Knott's test.

Isolate	Biofilm production*	Isolate	Biofilm production*
BMC01	0.93195 b	BMC22	0.80874 b
BMC02	1.54237 a	BMC24	1.94397 a
BMC03	1.76733 a	BMC25	0.37360 b
BMC04	0.91772 b	BMC26	1.97850 a
BMC05	0.66498 b	BMC28	1.86382 a
BMC06	1.23846 b	BMC29	2.47920 a
BMC07	0.45400 b	BMC30	0.58641 b
BMC08	0.77147 b	BMC31	0.92327 b
BMC09	0.44752 b	BMC33	1.16353 b
BMC10	0.83642 b	BMC34	0.76059 b
BMC11	1.36859 a	BMC35	1.18309 b
BMC12	0.47149 b	BMC36	0.84238 b
BMC13	0.88171 b	BMC37	0.56398 b
BMC14	0.64888 b	BMC38	0.37188 b
BMC15	0.32421 b	BMC39	2.23971 a
BMC16	1.04795 b	BMC40	1.77706 a
BMC17	0.87133 b	BMC41	1.76699 a
BMC18	1.08905 b	BMC42	2.49460 a

*Means followed by the same letter do not differ statistically from one another by the Scott-Knott test ($p \leq 0.05$).

Discussion

The results differ from those found by Montaldo (2016) in bacteria associated with sugarcane (*Saccharum officinarum*), whose majority of the isolates obtained had low production of exopolysaccharides (54%), followed by medium production strains (42%) and high production (4%), which presupposes that the amount produced of this substance differs according to the associated plant. As sugarcane is a very widespread agricultural crop, with great

expressiveness in Brazil, it receives all the necessary conditions for its normal development, such as fertilization, pest and disease control and irrigation, and as generally the supply of water in the fields of production is constant, it does not require strategies and physiological changes to resist water scarcity, therefore, the bacteria associated with sugarcane studied by Montaldo (2016), had in their great majority, low production, because the own environment where these microorganisms are inserted has normal water

availability, and does not need to synthesize defense mechanisms to environmental stresses, unlike cacti, whose scarce environment promotes the adaptation of all living organisms.

Ashraf et al. (2005) evaluating exopolysaccharide-producing bacteria, observed that they promoted mechanisms of tolerance to saline stress in inoculated wheat plants, improving the physical-chemical characteristics of the soil. Therefore, the synthesis of exopolysaccharides can be an efficient tool to combat this stress, since according to FAO (2000), salinity affects around 397 million hectares worldwide, out of a total of 230 million irrigated hectares, 45 million are salinized, limiting agricultural activity and drastically increasing desertified or in process areas.

Therefore, the halotolerant bacteria associated to xerophytes are capable of producing EPS, directly helping to protect the plant against several types of stresses, besides favoring root development and improving soil quality (Silva et al., 2016).

From the total number of strains evaluated, 11 obtained high biofilm formation, which presupposes the high efficiency with respect to the synthesis of the mechanism and the tolerance of the bacteria, which will aid in promoting the growth of the colonized plants, since the biofilm formation appears to be a widely disseminated feature among bacteria, given the positive formation among the isolates evaluated. The formation of biofilms can be an intimate relation between plant and microorganism, and that it product not only of bacterial exopolysaccharides but also of compounds that are excreted and/or produced by the plant that stimulate the formation of biofilms according to the species, as well as the environment (Silva et al., 2018).

This mechanism, together with exopolysaccharides, assists in root colonization, protecting plant cells from environmental stresses, contributing to

the absorption of nutrients present in the soil solution and the synthesis of essential hormones for the growth, development and establishment of vegetables in the environment, ensuring the growth, development and quality of Brazilian agricultural production.

Conclusions

Of all bacterial isolates evaluated, the strains BCM02 and BCM28 were the ones that obtained the best performance in the assessments of water stress tolerance, since they were efficient in the synthesis of both evaluated mechanisms, and could be used later growth promotion tests in plants, such as the aid in the synthesis of plant hormones such as indole-acetic acid (AIA), the solubilization of inorganic phosphates, nitrogen fixation in a nitrogen-free environment, among others.

Acknowledgment

CNPq and Universidade Federal de Alagoas for financial and institutional support.

Conflict of interests

Authors declare that they have no conflict of interests.

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