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Bacillus cereus UFPEDA 1040B as a potential plant growth promoter: an in vitro study

Bacillus cereus UFPEDA 1040B como um potencial promotor de crescimento de plantas: um estudo in vitro

Bacillus cereus UFPEDA 1040B como potencial promotor del crecimiento vegetal: un estudio in vitro

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Abstract

The Brazilian northeastern semi-arid is a region composed of countless microorganisms adapted to extreme conditions, and to ensure their survival, they can produce several biomolecules and develop peculiar mechanisms. The use of microorganisms has been reported as a promising alternative for promoting plant growth through the production of several bioactive molecules, which can replace chemical agents that cause environmental problems. In this study, *Bacillus* sp. Ar 16 (UFPEDA), isolated from the rhizosphere of Aroeira (*Schinus terebinthifolia*) from the Caatinga biome, northeastern semi-arid region, was taxonomically characterized through biochemical tests, Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF) and 16S region rRNA such as *Bacillus cereus* UFPEDA 1060 B (99.86%). In vitro conditions, the *B. cereus* UFPEDA 1060B strain produced exopolysaccharide (EPS), cellulases, and phosphatases and presented maximum phosphate solubilization of 880 µg.mL -1 . Furthermore, the production of 3.96 to 4.06 µg.mL -1 of indole acetic acid (IAA) in a culture medium supplemented with 5 mM of L-tryptophan was evidenced. IAA and other organic acids were extracted and analyzed

by High-Performance Liquid Chromatography (HPLC) at a retention time of 6.2. The studied strain has different mechanisms that can promote plant growth and is also capable of controlling the growth of *Fusarium* spp. and *Colletotrichum* spp. The effectiveness of the results presented by this strain demonstrate its biotechnological potential, which can bring benefits to sustainable agriculture with artifices that favor, in addition to plant growth, the cycling of nutrients.
Keywords: Rhizobacteria; Auxin; Organic acids; Antagonism.

Resumo
O semiárido nordestino é uma região composta por inúmeros microrganismos adaptados a condições extremas e, para garantir sua sobrevivência, podem produzir diversas biomoléculas e desenvolver mecanismos peculiares. A utilização de microrganismos tem sido relatada como uma alternativa promissora para promover o crescimento de plantas através da produção de diversas moléculas bioativas, que podem substituir agentes químicos que causam problemas ambientais. Neste estudo, *Bacillus* sp. Ar 16 (UFPEDA), isolado da rizosfera da Aroeira (*Schinus terebinthifolia*) do bioma Caatinga, semiárido nordestino, foi caracterizado taxonomicamente através de testes bioquímicos, Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF) e região 16S do rRNA como *Bacillus cereus* UFPEDA 1060 B (99,86%). Em condições in vitro, a cepa de *B. cereus* UFPEDA 1060B produziu exopolissacarídeos (EPS), celulases, fosfatases e apresentou solubilização máxima de fosfato de 880 µg.mL-1. Além disso, foi evidenciada a produção de 3,96 a 4,06 µg.mL-1 de ácido indol acético (AIA) em meio de cultura suplementado com 5 mM de L-triptofano. O AIA e outros ácidos orgânicos foram extraídos e analisados por Cromatografia Líquida de Alta Eficiência (CLAE) em um tempo de retenção de 6,2. A linhagem estudada possui diferentes mecanismos que podem promover o crescimento das plantas e também é capaz de controlar o crescimento de *Fusarium* spp. e *Colletotrichum* spp. A eficácia dos resultados apresentados por esta linhagem demonstra seu potencial biotecnológico, que pode trazer benefícios à agricultura sustentável com artifícios que favorecem, além do crescimento das plantas, a ciclagem de nutrientes.
Palavras-chave: Rizobactérias; Auxinas; Ácidos orgânicos; Antagonismo.

Resumen
La región semiárida nororiental está compuesta por numerosos microorganismos adaptados a condiciones extremas y, para asegurar su supervivencia, pueden producir varias biomoléculas y desarrollar mecanismos peculiares. El uso de microorganismos ha sido reportado como una alternativa promisoria para promover el crecimiento de las plantas através de la producción de varias moléculas bioactivas, que pueden reemplazar a los agentes químicos que causan problemas ambientales. En este estudio, Bacillus sp. Ar 16 (UFPEDA), aislado de la rizósfera de Aroeira (Schinus terebinthifolia) en el bioma Caatinga, región semiárida nororiental, se caracterizó taxonómicamente mediante pruebas bioquímicas, espectrometría de masas de tiempo de vuelo de ionización por desorción láser asistida por matriz (MALDI-TOF) y la región 16S del rRNA como Bacillus cereus UFPEDA 1060 B (99,86%). En condiciones in vitro, la cepa de B. cereus UFPEDA 1060 B produjo exopolisacáridos (EPS), celulasas, fosfatasas y presentó una solubilización máxima de fosfato de 880 µg.mL-1. Además, se evidenció la producción de 3.96 a 4.06 µg.mL-1 de ácido indol acético (IAA) en medio de cultivo suplementado con 5 mM de L-triptófano. El IAA y otros ácidos orgánicos se extrajeron y analizaron mediante cromatografía líquida de alta resolución (HPLC) con un tiempo de retención de 6,2. La cepa estudiada tiene diferentes mecanismos que pueden promover el crecimiento de las plantas y también es capaz de controlar el crecimiento de Fusarium spp. y Colletotrichum spp. La efectividad de los resultados presentados por esta cepa demuestra su potencial biotecnológico, que puede traer beneficios a la agricultura sustentable con artificios que favorecen, además del crecimiento de las plantas, el ciclo de nutrientes.
Palabras clave: Rizobacterias; Auxinas; Ácidos orgánicos; Antagonismo.

1. Introduction

Rhizobacteria are widely used as biofertilizers, since they favor plant development through the production of various growth-regulating substances through phytohormones (auxins, cytokinins, and gibberellins), competition and suppression of phytopathogens, in addition to assisting in the availability of nutrients in the rhizosphere (Babalola, 2010).

The mechanisms promoted by plant growth-promoting rhizobacteria (PGPRs) can be classified as direct and indirect, which act in a positive way, ensuring plant growth. Directly, it is possible to mention the absorption of essential nutrients by biological nitrogen fixation, phosphate solubilization, siderophore production as well as the modulation of hormone levels through the synthesis of 3-indole acetic acid (IAA), gibberellins, cytokinins, oxide nitric and polyamines. Indirectly, it can be attributed to the production of stress-related phytohormones, such as abscisic acid, jasmonic acid, and cadaverine, or ethylene

catabolism, related to the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, even when phytopathogen suppression occurs (Ghyselinck et al., 2013; Da Silva et al., 2018).

The genus Bacillus, belonging to the Bacillaceae family, comprises gram-positive rod-shaped, aerobic or facultative anaerobic bacteria found predominantly in the soil and are extremely important for pharmaceutical, agricultural and industrial processes in the manufacture of antimicrobial pesticides and fertilizers, due to their physiological characteristics and its ability to produce antibiotics, enzymes, and other bioactive compounds. Furthermore, they are easily cultivated in low-cost culture media, and are fast-growing, which facilitates the production of biomass in industrial fermenters (Lanna-Filho et al., 2010). They also stand out for their ability to produce endospores that are extremely resistant to adverse environmental conditions, including low and high temperatures, radiation, desiccation, and chemicals (Baron, 1996; Pérez-García et al., 2011; Madigan et al., 2016).

In addition, microorganisms subjected to selective pressure (Nadeem, et al., 2016) and that manage to survive in regions with high temperature, water deficit, and consequently soil desiccation, such as the characteristics found in the Caatinga biome (Brazilian semi-arid region) (Trovão, et al., 2007), can be sources of important bioactive compounds that can favor the promotion of plant growth (Kavamura et al., 2013; De Moura et al., 2021).

The use of microorganisms adapted to different soil conditions that act as plant growth promoters and biocontrol agents is a viable option for application in agriculture to reduce dependence on the use of pesticides, aiming at a trend toward the development of sustainable agriculture (Campos et al., 2014) suggesting the great biotechnological potential of the strains studied in this work.

Therefore, this work aims to taxonomically characterize an isolate of the rhizosphere of *Schinus terebinthifolia* from Northeastern Caatinga and evaluate its in vitro potential to produce bioactive compounds that can characterize it as a plant growth promoter.

2. Methodology

2.1 Identification

2.1.1 Isolation of *Bacillus* sp. and macroscopic analysis

Bacillus sp. Ar 16 was isolated from soil samples collected from the rhizosphere of Aroeira (*S. terebinthifolia*) during the winter (August 2011) in semi-arid and poorly impacted areas of the Caatinga, near the municipality of Caicó, Rio Grande do Norte/ Brazil (6°35’21”S; 37°15’36”W). Initially, the soil sample was diluted 1:10 (w/v), homogenized, and subjected to thermal shock for 12 minutes at 80 °C, followed by cooling on ice for five minutes. Subsequently, serial dilutions (10^{-2} and 10^{-3}) were carried out in sterilized saline solution (0.9%), seeded in nutrient agar (Meat Extract 3g, Bacteriological Peptone 5g, NaCl 8g, Agar 16g, H_2O 1000 mL, pH 7.2) and incubated at 30 °C for 48 h.

After this period, isolated Bacillus colonies were selected and analyzed under phase-contrast microscopy (1000X) to evaluate the presence or absence of inclusion bodies (protein crystals). The isolate was deposited in the microorganism’s collection of the Department of Antibiotics (UFPEDA) under number 1060B.

2.1.2 Phenotypic identification

The isolate was cultivated in Czapeck medium ($NaNO_3$ 2g, K_2HPO_4 1g, $MgSO_4$ $7H_2O$ 0.5g, KCl 0.5g, $FeSO_4$ $7H_2O$ 0.01g, Sucrose 30g, Agar 16g, H_2O 1000ml, pH 6.6) supplemented with penicillin G (100 µg/mL) and incubated at 30 °C for 24 h.

After this period, the identification was performed through macro- and micromorphological (colony color, shape, and

cellular morphology) and biochemical analyzes according to (Koneman & Allen, 2008). The isolates were subjected to Gram staining and catalase, citrate, lecithinase, amylase, hemolytic activity, oxidation-fermentation, Voges-Proskauer, mannitol, phenylalanine, NaCl tolerance (5%), indole, motility, and antimicrobial susceptibility tests (nalidixic acid, ampicillin cephalothin, chloramphenicol, streptomycin, gentamicin, polymyxin, penicillin G, tetracycline, rifampicin, and vancomycin).

2.1.3 Identification based on 16S rDNA region

Total DNA extraction was performed according to Sambrook & Russell (2001) with adaptations. After cell cultivation for 6- 16 hours in Luria-Bertani broth (LB), at 37 °C and 150 rpm on a shaking table, 1.5 mL of each sample was centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded. The cell mass was resuspended in TEG buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) to which lysozyme (4 mg.L -1) and RNAse solution (20 mg/ml) were added. The samples were incubated in a water bath at 37 °C for 20 minutes. Then, SDS (10% Sodium Dodecyl Sulfate Solution) and proteinase K (150 µg/ml) were added, where they followed again to the water bath for 15 minutes at 65°C.

After this period, they were centrifuged at 10,000 rpm for 5 minutes and the supernatant was transferred to another new and sterilized microtube. Subsequently, 24:1 v/v chlorophyll (chloroform + isoamyl alcohol) was added. The samples were centrifuged at 6,000 rpm for 3 minutes and the resulting aqueous phase was transferred to another microtube. Then, 3 M sodium acetate, pH 5.2, and 0.6 V of isopropanol (P.A) were added, homogenizing the content. Samples were centrifuged for 10 minutes at 10,000 rpm.

The supernatant was discarded, and the pellet was washed with ethanol (70%) and centrifuged for three minutes at 10,000 rpm. The supernatant was discarded and after drying the pellet, TEG buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was incorporated. DNA integrity was evaluated by 0.8% agarose gel electrophoresis using sample buffer and extracted DNA. Subsequently, amplification of the 16S rRNA gene was performed by the polymerase chain reaction (PCR) technique using universal oligonucleotides for Eubacteria 27F (5'-11GAG TTT GAT CCT GGC TCA G-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3'), making use of, for each reaction: DNA (40 ng), buffer (10x), Magnesium chloride ($MgCl_2$) (50 mM), Phosphated Deoxyribonucleotides (dNTPs – 10 mM), Direct (Oligo d) and reverse (Oligo r) oligonucleotides (25pmol/L), sterile distilled water and Taq DNA polymerase (50U/µL, 500U).

The conditions used in the thermocycler for amplification were: Initial denaturation at 94 °C for 5 minutes, 30 cycles of denaturation at 94 °C for 45 minutes, annealing at 52 °C for 45 minutes, extension at 72 °C for 2 minutes, and extension final at 72 °C for 5 minutes. The PCR product was sequenced using the Genetic Analyzer 3500 ABI sequencer from the Human Molecular Genetics Laboratory (LGMH) at the Federal University of Pernambuco, and the resulting sequence was compared to all sequences available in GenBank using BLAST software from the National Information Center on biotechnology (NCBI) (http://www.ncbi.nlm.nih.gov/).

The sequences were aligned with the Clustal software, and a phylogenetic tree was built with Mega 7 using the neighbor-joining method. Tree topology was evaluated by bootstrap analysis (1000 resampling). The sequences obtained were deposited in GenBank.

2.1.4 Proteomic identification

Proteomic identification was performed at the Centro de Tecnologias Estratégicas do Nordeste (CETENE), using a mass spectrometer for ionization of ribosomal and structural proteins from bacteria cultivated on Luria-Bertani agar for 24 hours and incubated at 30 °C, using the technique of Matrix-Assisted Laser Desoption/Ionization Tim-of-Flight Mass Spectrometry (MALDI-TOF MS), according to Lima-Neto et al., (2014), with modifications.

Cell fragments (about 50 µg) were directly transferred from the culture medium to the rings (spots) of the polished metal plate (Polished Steel Target, Bruker Daltonics, Bremen, Germany) of the equipment (MALDI-TOF MS Autoflex III, Bruker Daltonics, Bremen, Germany) in quadruplicate. Immediately, 0.5 µL of 70% formic acid was added over two spots with the bacterial samples and mixed lightly with the biological material.

After total evaporation of the liquid medium, 1 µL of α- cyano-4-hydroxycinnamic acid (CHCA) matrix solution (75 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA) in ethanol/water/acetonitrile was added [1:1:1] with 0.03% trifluoroacetic acid [TFA]) and lightly mixed. For the other two spots, addition of the matrix solution was performed without previous treatment with formic acid. The mass variation between 2000 and 20000 Da was recorded using linear mode with a pulse of 104 ns at a voltage of +20 kV. Final spectra were generated by adding 300 laser shots accumulated per profile and 8 profiles produced per sample, leading to a total of 2,400 laser shots added per spectrum. The peak list was exported to MALDI Biotyper™ 3.0 software (Bruker Daltonics, Bremen, Germany) where final identifications were based on the presence or absence of each peak in the spectrum.

2.2 Selection of plant growth-promoting mechanisms

2.2.1 Production of indole acetic acid (IAA)

The analysis of the production and concentration of indole acetic acid (IAA) was carried out following the methodology described by (Ali et al., 2009; Lins, 2014) with some modifications. The isolate was inoculated in test tubes containing 5 mL of Broth LB and cultured at 37 ˚C for 24 hours. Then, a 10^8 CFU/mL suspension was prepared with saline solution (0.9%) and the optical density (O.D.) was analyzed in a spectrophotometer. Subsequently, 1 µL of it was inoculated into 1 mL of LB broth supplemented with 5 mM L-tryptophan, which were cultured in the dark at 37 °C at 120 rpm. After 24, 48, and 72 hours, all samples were centrifuged in microtubes and Salkowski reagent ($FeCl_3$ 0.02 mol/L and H_2SO_4 10.8 mol/L) was added to the supernatant at a ratio of 1:4 v/v (Marchioro, 2005). Samples were kept in the dark for 30 minutes.

Then, the qualitative reading was carried out by observing the color change and the quantitative reading in OD of 530 nm in a spectrophotometer, then the data were compared with the IAA standard curve and the amount expressed in µg/mL. The experiments were performed in triplicate and *Pantoea agglomerans* UFPEDA-774 was used as a positive control.

2.2.2 Phosphate solubilization

With concentration previously adjusted to 10^8 CFU/mL, aliquots of suspension of saline solution (0.9%) containing 100 µl of *Bacillus* sp. Ar 16 (*B. cereus* 1060B) were inoculated into 10 ml of NBRIP medium (Nautiyal, 1999) and incubated at 28 °C. After 168 hours, 1 ml of the inoculum was centrifuged at 10,000 rpm for five minutes. Then, 29 µl of the supernatant were transferred to microtiter plates containing 114 µL of distilled water and 57 µL of vanado-molybdate reagent (5% ammonium molybdate + 0.25% ammonium vanadate in a ratio of 1:1 v/ v). The colorimetric change of the medium with whitish coloration to yellow in positive samples was seen, then the reading was performed in a spectrophotometer at a wavelength of 420 nm and the data were compared with the standard curve of K_2HPO_4 and the amount expressed in mg/mL (Barton, 1948; Malavolta, Vitti & Oliveira, 1997). The experiments were performed in triplicate, and the strain *P. agglomerans* UFPEDA-774 was used as a positive control.

According to Sindhu & Dadarwal (2001) can be consider the production as <0.05 mg (low solubilization), 0.05-0.1 (average solubilization), 0.101-0.5 (high solubilization), > 0.501 (elevated solubilization).

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2.2.3 Identification and quantification of organic acids by High-Performance Liquid Chromatography (HPLC)

For the detection of indole acetic acid (IAA), the isolates were inoculated in test tubes containing ten milliliters (mL) of Broth LB and cultured at 37 ˚C for 24 hours. Then, a standardized suspension containing 10^8 CFU / mL was prepared with saline solution (0.9%) and the optical density (O.D.) was confirmed through the spectrophotometer. Subsequently, 100 µL of the bacterial suspension was added to 10 mL of LB broth supplemented with 5 mM of L-tryptophan, which were cultured in the dark at 37 °C and 120 rpm for 72 hours (Ali et al., 2009; Lins, 2014).

For the detection of acetic, butyric, citric, and lactic acids, 100 µL aliquots of the suspensions of each strain of *Bacillus* sp. Ar16 were inoculated in 10 mL of NBRIP medium and incubated at 28 °C for 168 hours (Nautiyal, 1999). Subsequently, all samples were filtered, and the determination of organic acids was performed by HPLC, equipped with a quaternary pump, coupled to a degassing system (DGU-20A5r), with an oven to control the temperature of the column, which is set at 28 °C, and an automatic injector (20 µL injection), with a diode array detector (SPD-M20A), working in a range from 190-800 nm.

The software used was LC-Solutions manufactured by Shimadzu Corporation (Kyoto, Japan). The column used in the method was a 300mm x 7.8 mm x 9 µm ion-exchange column (Aminex® HPX-87H, Bio-Rad, USA). Elution was performed isocratically with a mobile phase composed of 5 mM of H_2SO_4, with a flow of 0.6 mL/min. To determine the acid concentrations, a standard curve was constructed relating the individual standard concentrations of each acid to the peak areas generated by the equipment.

2.2.4 Phosphatase production

The production of phosphatases from *Bacillus* sp. Air 16 (UFPEDA 1060B) was observed using the methodology of (Romeiro et al., 2007; Ribeiro & Cardoso, 2012; Lins, 2014). A 0.5% solution of phenolphthalein diphosphate was prepared and sterilized by membrane filtration (0.22 µm). Two milliliters of this solution were added to every 100 ml of Trypticase Soy Agar (TSA) culture medium still liquefied and kept at 50 °C. The culture medium was homogenized and poured into Petri dishes.

Bacterial isolates were inoculated at four points on the surface of the medium, followed by incubation for 24-48 hours at 28 °C. For revelation, drops of ammonium hydroxide (8.4%) were placed on the lid of the Petri dish and the reading was carried out after 15 minutes. The evidence of the production of phosphatases was due to the formation of a pink zone around the colonies.

2.2.5 Antimicrobial activity against phytopathogenic fungi

For the biocontrol tests, phytopathogenic fungi from crops belonging to the Microorganisms Culture Collection (UFPEDA) of the Department of Antibiotics and the Micoteca Culture Collection URM of the Department of Mycology of the Center for Biological Sciences of the Federal University of Pernambuco were used.

Initially, the fungal species *Colletotrichum* sp. (UFPEDA- 2420), *C. atramentarium* (URM-2597), *Fusarium oxysporum* (URM-4117), *F. oxysporum* (URM-5283), *F. solani* (URM- 5903) were cultivated in a Petri dish with Potato Dextrose Agar (PDA), pH adjusted to 6.0, for 4-6 days at 30 °C. Then, a mycelium plug was removed and inoculated in a Petri dish (90 mm in diameter) with a PDA medium under the same conditions as the previous culture.

Subsequently, 2 µL of bacterial suspension of *Bacillus* sp. Ar16 (*Bacillus cereus* UFPEDA-1060B) at a concentration of 10^8 CFU/mL were inoculated 25 mm from the center of the plate where a block of mycelium was inserted. The samples, in triplicate, were cultivated for 6 days in an oven at 30 °C. Subsequently, the percentage of fungal growth inhibition was

calculated (Rocha et al., 2017; Chen et al., 2019). The average inhibition obtained was calculated from the average of the three repetitions and applied in the following formula: (control-treatment) / control x 100 and the results were expressed in percentage (%) (Yuan, et al., 2012).

2.2.6 Ammonia production

For analysis of the ammonia production, recent strains of *Bacillus* spp. Ar16 (*B. cereus* UFPEDA 1060B) were adjusted to a concentration of 10^8 CFU/mL and 4 µL of the suspension was inoculated in 4 mL of peptone water and incubated at 28 °C for 48 hours. After incubation, 1 mL of each culture was centrifuged at 10,000 rpm for 10 minutes in microtubes with a capacity of 2 mL, then 50 µl of Nessler's reagent were added to each microtube (Cappuccino & Sherman, 1992; Lins, 2014). The indicative of a brown or yellow color, after the addition of Nessler reagent, demonstrates high ammonia production and lower production, respectively (Dey et al., 2004).

The ammonia levels produced were measured at O.D. of 450 nm in a spectrophotometer and compared with the ammonia standard curve and the amount expressed in µg/mL. The experiments were performed in triplicate and *Pantoea agglomerans* UFPEDA-774 was used as a positive control.

2.2.7 Cellulase production

According to the methodology described by Kasana et al., (2008), 5 µL of a suspension containing 10^8 CFU/mL were inoculated, in quadruplicate, in the carboxymethyl cellulose (CMC) medium composed of 0.2% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05% KCl; 0,2% salt of carboxymethyl cellulose; 0.02% bacteriological peptone; 1.7% agar. The Petri dishes were incubated at 30 °C for 48 hours and to reveal the results, P.A iodine was used in the lid of the Petri dish for 5 minutes. The cellulolytic index (CI) was calculated based on the ratio between the diameter of the halo and the diameter of the colony (Teather & Wood, 1982).

2.2.8 Production of exopolysaccharides (EPS)

The *Bacillus* sp. Ar 16, previously cultivated in Nutrient Agar (NA) medium for 24 hours at 37 °C and with cell concentration adjusted to 10^8 CFU/mL in a spectrophotometer, was inoculated into sterilized paper discs (5 mm in diameter) and placed in the culture medium (2% yeast extract; 1.5% K_2HPO_4; 0.02% $MgSO_4$; 0.0015% $MnSO_4$; 0.0015% $FeSO_4$; 0.003% $CaCl_2$; 0.0015% NaCl; 1.5% agar) modified with addition of 10% of sucrose in Petri dishes, and pH adjusted to 7.5.

Then, they were cultured for 48 hours at 37 °C, and EPS production was analyzed by observing a mucoid halo around each paper disk. The confirmation was performed by adding the mucoid substance present in the halo in 2 mL of absolute ethanol P.A, thus, the formation of a precipitate confirms the presence of exopolysaccharides (Paulo et al. 2012).

According to Paulo et al. (2012), from the calculation of the average of the mucoid halos (mm) obtained after cultivation, based on the ratio between the diameter of the inoculated filter paper and the formed halo, it is possible to consider that: (-) absence of production, halo ≤ 10 mm (+), 10-14 mm (++) and ≥ 14 (+++).

3. Results

3.1 Identification

3.1.1 Isolation and Biochemical and Morphological Identification

The colonies isolated on Nutrient Agar medium showed cream color and rhizoid growth. After microscopic analysis, they were identified as Gram-positive, mobile, and endospore-forming.

The isolate was analyzed by biochemical tests and classified as a non-fermenter of mannitol and a producer of catalase, lecithinase, and amylase. These results identify the isolate as *Bacillus cereus*. Differentiation from the other members of this group was performed by confirming high hemolytic activity and the absence of protein crystal (Table 1). The strain was sensitive to most of the antimicrobials tested, showing resistance only to ampicillin, cephalothin, and penicillin G.

Table 1. Biochemical characteristics of *Bacillus* sp. Ar 16 and *Typical strain* (Biochemical tests). Legend: [1] (Garrity & Stanley, 2001).

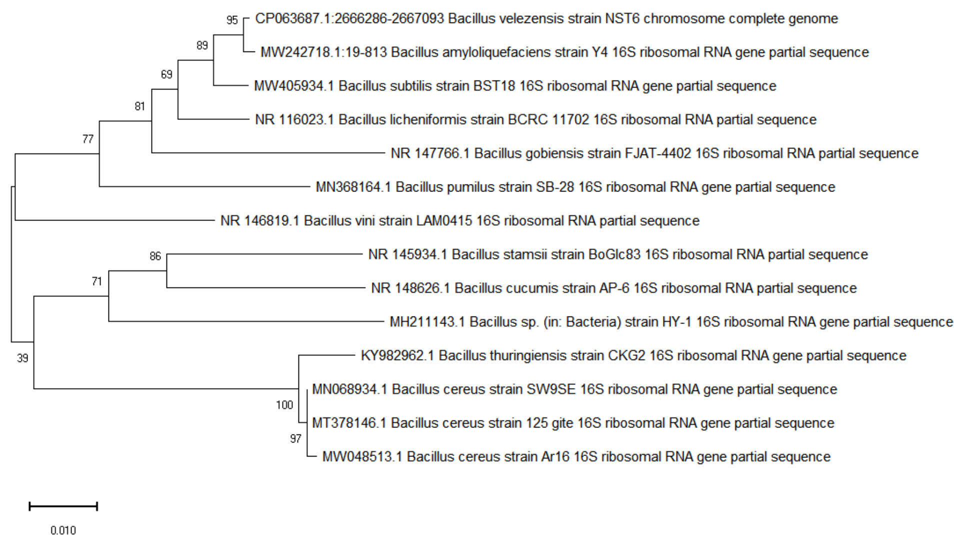
Biochemical tests	*Bacillus* sp. Ar16 (*B. cereus* UFPEDA 1060B)	*Typical strain* *B. cereus* [1]
Catalase	+	+
Citrate	-	+
Lecithinase	+	+
Oxidation-fermentation aerobic and anaerobic	+	+
Temperature optima 37° C	+	+
Spore production	+	+
Mannitol	-	-
Phenylalanine	-	-
Amylase	+	+
Indole	-	-
Motility	+	+
NaCl 5%	+	+
Hemolytic activity	+	+
Voges-Proskauer	+	+

Source: Authors.

3.1.2 Identification based on 16S rDNA region

The result of the phylogenetic analysis of the 16S rRNA gene of the Ar16 isolate demonstrates a 99.86% similarity with other *Bacillus cereus* (Figure 1) present in the database (NCBI). The 1432 bp sequence was sent to GenBank and assigned an accession number -MW048513.

Figure 1. Phylogenetic tree relative to 16S rRNA gene of *Bacillus* sp. Ar16 (UFPEDA-1060B) and reference sequences from NCBI. Tree constructed using neighbor-joining method with 1000 bootstraps.



Source: Authors.

3.1.3 Proteomic identification by MALDI-TOFF

Comparing the peak list to the MALDI Biotyper™ 3.0 software (Bruker Daltonics, Bremen, Germany) according to the protein profile data library with reference spectra, shows the isolate with *scores* of 1.90, relating it to the genus *Bacillus* sp.

2.2 Selection of plant growth-promoting mechanisms

2.2.1 Production of índole acetic acid (IAA)

Qualitatively, it was possible to observe the change in color of the liquid LB medium after cultivation and treatment with Salkoswki's reagent in the three-time periods proposed for the bacterial strains in this study.

Quantitatively, three responses were obtained for the production of IAA. The isolate produced indole acetic acid throughout the analyzed period, with production ranging from 3.96 to 4.06 µg. ml^{-1} in the presence of 5 mM of tryptophan.

2.2.2 Phosphate solubilization

B. cereus UFPEDA 1060B demonstrated significant phosphate solubilization capacity, reaching a value of 880 µg.mL^{-1} in the period analyzed. It was also possible to observe at the end of the cultivation the decrease in the pH of the culture medium used to 4.9 which, initially, was adjusted to 7.0, thus suggesting the possible production of organic acids.

2.2.3 Identification of quantification of organic acids

There was a production of acetic acid by the isolate *B. cereus* UFPEDA 1060B, this study highlighting the positive control (*B. cereus* UFPEDA-108) used with a production of 74.11 mg/L. The synthesis of citric acid was also reported with 5.11, 4.83 mg/mL produced by the strains *B. cereus* UFPEDA 1060B, and the positive control used *B. cereus* UFPEDA 108, respectively. The IAA production reached a higher level, with 2653.32 mg/mL as shown in Table 2.

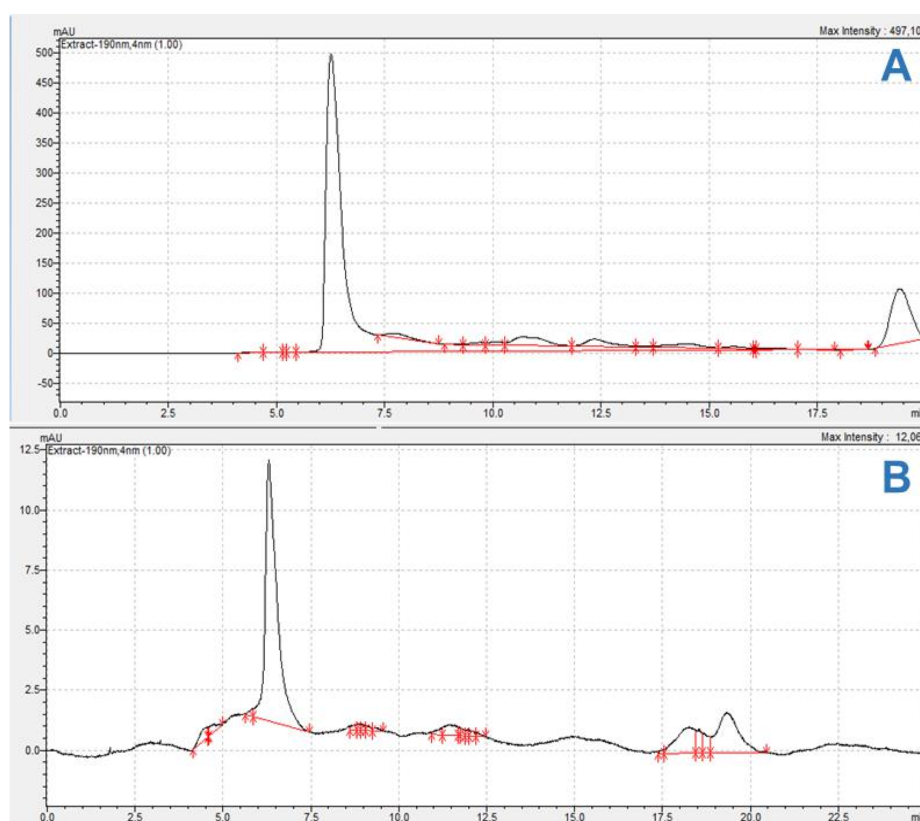
Table 2. Production of organic acids produced by the strains of *Bacillus cereus* UFPEDA-1060B and positive control represented by *B. cereus* UFPEDA-1008, the highest production of indole acetic acid by both stands out.

	Organic Acids (mg/mL)		
Strains	IAA	Acetic acid	Citric acid
Bacillus cereus UFPEDA - 1060B	2653.32	31.95	5.21
Bacillus cereus UFPEDA- 108	2480.37	74.10	4.83

Source: Authors.

Peak areas generated in the chromatogram using the commercial indole acetic acid standard were generated at retention time 6.2 and, thus, it is also possible to observe at the same retention time the peak area generated by *B. cereus* 1060B (Figure 2).

Figure 2. Peaks presented in the chromatogram from the HPLC analysis detecting the production of indole acetic acid in "A" which corresponds to *B. cereus* UFPEDA-1060B compared to the commercial IAA standard injected in "B". Note that both have peak area generated with a retention time of 6.2.



Source: Authors.

2.2.4 Phosphatase production

In this present study, *B. cereus* 1060B also demonstrated the ability to produce phosphatase enzymes in a Petri dish assay, indicating another mechanism for phosphate solubilization.

The Petri dish culture assay demonstrated the ability of *B. cereus* UFPEDA-1060B to produce phosphatases after 48 hours of incubation, after the development of pink staining.

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2.2.5 Antimicrobial activity against phytopathogenic fungi

B. cereus demonstrated the ability to inhibit the fungal mycelium in 60% of the fungal strains analyzed, highlighting the percentage of inhibition of 88.2% for *F. oxysporum* URM 5283, 75% against *Colletotrichum* sp. UFPEDA 2420 and 12.5% for *C. atramentarium* URM 2597.

2.2.6 Ammonia production (NH_3)

Nitrogen fixation can be observed, where the concentrations of NH_3 150.38 and 150.47 $\mu g.mL^{-1}$ were measured corresponding to *B. cereus* UFPEDA-1060B and *P. agglomerans* UFPEDA-774, respectively, after 48 hours of incubation.

2.2.7 Cellulase production

Results for cellulolytic hydrolysis were observed for the strain of this study, which showed aptitude with a CI of 11.33 mm, suggesting the high capacity of enzyme production that degrade cellulose by it. As for the positive control used, *Bacillus subtilis* UFPEDA-434, the CI was 4 mm.

2.2.8 Exopolysaccharides production

Bacillus cereus UFPEDA-1060B demonstrated a high capacity for synthesizing extracellular polysaccharides, presenting a 14 mm mucoid halo. The formation of a precipitate in ethanol P.A indicated confirmation of its production (Figure 4).

Figure 4. A. Mucoid substance representing exopolysaccharide formation after incubation time. B. Highlight for the precipitate presented in absolute alcohol confirming the production of EPS by *B. cereus* 1060B.

Source: Authors.

4. Discussion

Members of the genus Bacillus, representatives of PGPRs, have been reported in the literature for the inhibition of ethylene synthesis, production of siderophores, antibiotics, nitrogen fixation, and mechanisms for phosphate solubilization, among other substances associated with plant growth (Vafadar et al., 2014). Thus, Bacillus strains are described as producers of aromatic compounds, plant hormones, and various enzymes that are related to the metabolism of phenylpropanoids, in addition to lipopeptides and polysaccharides (Ek-Ramos et al., 2019).

Beneficial effects of these activities have been reported in economically important crops such as potatoes and onions (Vafadar et al., 2014). As emphasized by (Reinhold-Hurek & Hurek, 2011; Frank et al., 2017), among Gram-positive bacteria, *Bacillus* spp. has stood out in the production of metabolites in an abundant way.

Numerous formulations using this genus have been prepared and marketed, since, for biological control activities, Bacillus are good candidates, acting as antagonists. The antagonism is succeeded by the production of numerous polypeptides with antimicrobial action, as it is possible to exemplify: mersacidin, mycosubilitin, iturine, bacilomycin, phengycine, bacillicin, and surfactin (Zouari et al., 2016; Carrer Filho et al., 2015). These are characterized by low toxicity and high biodegradability, as well as considerable inhibitory activity on a range of microorganisms such as viruses, bacteria, fungi, mosquitoes, and oomycetes. To intensify the action between the different polypeptides, the synergistic action between them can lead to this result (Romero et al., 2007).

The use of microorganisms adapted to extreme soil conditions, as is the case of the Caatinga Biome, is presented as a viable option for application in agriculture to reduce dependence on the use of pesticides, aiming at a trend towards the development of sustainable agriculture, as they can act as plant growth producers and biocontrol agents (Campos et al., 2014). Sustainable agriculture advocates the restricted use of chemicals and dependence on biological pest control and organic fertilizers as essential means to maintain ecosystem health and crop productivity (Prasad et al., 2015).

In addition, they have characteristics that facilitate their cultivation and study, such as the facility of cultivation in low-cost culture media, having fast growth, and allowing for the production of biomass in industrial fermenters (Lanna Filho, et al., 2010). They also stand out for their capacity to produce endospores that have relevant resistance to adverse environmental conditions, including low and high temperatures, radiation, desiccation, and chemicals (Banerjee et al., 2010; Pérez-García, 2011; Madigan et al., 2016) Regarding the direct and indirect mechanisms presented by the PGPRs, the expression of some genes involved in the biosynthesis of tryptophan (trp) and IAA (iaaH, iaaM, for example) as well as environmental factors can influence the levels of IAA production (Spaepen et al., 2007; Batista, 2017).

Thus, the metabolism and population density of bacteria are associated with bacterial growth, being the stationary phase of the growth curve favorable for the production of IAA. Thus, molecular signaling occurs by the bacteria, providing their adhesion and adaptation to the roots and plant signaling through the generation of exudates that help in colonization (Spaepen et al., 2007).

The production of auxins has already been reported in several studies, as discussed (Kaur et al., 2016; Ferreira et al., 2019) by members of the genus *Bacillus* sp. and *Brevibacillus* sp. (Mumtaz et al., 2017). Likewise, the production of auxins by members of the Bacillus genus is also mentioned, affirming that their production levels can also be influenced by the concentrations of the amino acid L-tryptophan available in the bacterial culture medium, which in larger quantities can produce remarkable levels of auxin. Furthermore, the conditions of the culture medium and availability of nutrients can also interfere (Sindhu & Dadarwal, 2001).

Another important mechanism, such as the production of volatile compounds, notably the production of ammonia, has already been verified in Bacillus as claimed (Sindhu & Dadarwal, 2001; Banerjee et al., 2010; Radhakrishnan et al., 2017), constituting an indirect method of plant growth control, as it interferes in the metabolic cycles of pathogens (Nain et al., 2012) to bring positive results for agriculture (Aloo et al., 2019).

The production of enzymes can contribute to soil nutrient cycling (Sindhu & Dadarwal, 2001; Hameeda et al., 2006), such as cellulases. *Bacillus* spp. and fungi are the microorganisms that produce cellulases in a broad way and with high economic value. However, because they have a fast growth cycle, bacteria present a differential characteristic for the industrial production of cellulases, in addition to being found in different habitats (Vimal et al., 2016). They are described as participating in the hydrolysis of some groups of enzymes, namely: endoglucanases, beta-glucosidase, cellobiohydrolase, or exoglucanase (Dionisio et al., 2016). Different methods have been reported for using soluble cellulose derivatives to be readily degraded, such as carboxymethylcellulose (CMC). Thus, in the research of bacteria and fungi, agar plates containing CMC as

substrate are often used (Johnsen & Krause, 2014).

Another benefit of using rhizospheric microorganisms is the ability to produce extracellular exopolysaccharides (EPS), which are high molecular weight extracellular polymers. They are synthesized during microbial metabolic activities and secreted into the environment (Silambarasan et al., 2019), constituting an outlet for physiological stress, which participates in the development of the biofilm (Marvasi et al., 2010).

The production and maintenance of biofilm facilitate the binding of microorganisms to solid surfaces (Madigan et al., 2016), including plants, constituting an advantage for their colonization. Furthermore, extracellular polymers produced by microorganisms can bind to cations, such as Na +, decreasing the absorption of this molecule by plants, thus avoiding salt stress (Geddie & Sutherland, 1993).

Members of the exopolysaccharide-producing Bacillus genus survived to high sodium concentrations when inoculated together with cucumber cultures to improve their salinity tolerance (Nadeem et al., 2016). The isolate *Bacillus cereus* UFPEDA-1060B was also evaluated for phosphate solubilization in NBRIP medium, with phosphate source dicalcium phosphate ($CaHPO_4$).

According to Nautiyal (1999), phosphate solubilization detection tests when performed in a solid medium may not always present a positive result, with the formation of a halo, but in a liquid medium, such microorganisms can solubilize several insoluble inorganic phosphates with reliable results reducing various inorganic acids in NBRIP culture medium, which is reported to be more efficient. Inorganic phosphate solubilization occurs with the synthesis of organic acids. For this reason, there is a decline in pH, which may present an increase in the chelation of cations bound to phosphorus (P) as well as the formation of soluble complexes with metallic ions that release P (Sharma et al., 2013).

The recognition of microorganisms that convert chemical elements by releasing organic acids or phosphatases is of paramount importance (Moreira & Araújo, 2013). When these become available to the plants, the microorganisms they produce are notable examples of plant growth-promoting agents, thus converting insoluble into soluble phosphate compounds via mineralization of organic phosphorus (Nahas, 2002).

5. Conclusion

The results of the present study showed the properties of the *Bacillus cereus* UFPEDA 1060B strain in the production of the main mechanisms for a microorganism to be considered a plant growth promoter. The strain was able to carry out the phosphate solubilization and produce organic acids, and exopolysaccharides, among others.

In view of this, the mechanisms produced by *Bacillus cereus* UFPEDA 1060B can be used for various purposes from an agricultural point of view, from sustainable products whose use does not cause damage to the environment. Therefore, the strain can be evaluated in further tests to understand these activities through in vivo inoculation in plant seeds.

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