Brazilian Journal of Agriculture **v.96, n.3, p. 526– 537, 2021**
DOI: 10.37856/bja.v96i3.4291

In vitro SCREENING OF SUGARCANE (*Saccharum officinarum* Linnaeus) ASSOCIATED RHIZOBACTERIA TO PLANT GROWTH PROMOTION

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ABSTRACT

The aimed of this study was to test *in vitro* four bacterial isolates (*Bacillus megaterium, B. subtilis, B. pumilus,* and *Enterobacter cloaceae*) with potential to plant growth promotion. The bacterial isolates were subjected to inorganic phosphate solubilization assay in liquid medium, exopolisacarídeos production and production of indole acetic acid (IAA) *in vitro*. The phosphorus solubilizing assay showed that *B. megaterium* is able to solubilizing phosphorus. Exopolysaccharides production test detected that *B. megaterium* and *E. cloaceae* are most efficient, and carbon source and pH were not dependent factors. The IAA production showed efficiency by *E. cloaceae*. These results proposed that bacterial isolates used in this study are applicable to inoculant production program and agricultural applicability.

Keywords: Phosphorus, inoculants, *Bacillus megaterium*, *Enterobacter cloaceae*

TRIAGEM *in vitro* DE RIZOBACTÉRIAS ASSOCIADAS À CANA-DE-AÇÚCAR (*Saccharum officinarum* Linnaeus) PARA PROMOÇÃO DE CRESCIMENTO VEGETAL

RESUMO

O objetivo deste estudo foi testar *in vitro* quatro isolados bacterianos (*Bacillus megaterium, B. subtilis, B. pumilus* e *Enterobacter cloaceae*) com potencial para promoção do crescimento vegetal. Os isolados bacterianos foram submetidos aos ensaios de solubilização de fosfato inorgânico em meio líquido, produção de exopolisacarídeos e produção de ácido indol acético (AIA) *in vitro*. O ensaio de solubilização de fósforo mostrou que *B. megaterium* é capaz de solubilizar fósforo. O teste de produção de exopolissacarídeos detectou que *B. megaterium* e *E. cloaceae* são mais eficientes e a fonte de carbono e o pH não foram fatores dependentes. A

produção de AIA mostrou eficiência por *E. cloaceae*. Esses resultados propõem que os isolados bacterianos usados neste estudo são aplicáveis aos programas de produção de inoculantes e aplicabilidade agrícola.

Palavras-chave: Fósforo, inoculantes, *Bacillus megaterium*, *Enterobacter cloaceae*

INTRODUCTION

Microorganisms compose an important function, as nutrient cycling and the maintenance of ecosystem health (MARSCHNER et al., 2004). It can facilitate the absorption of nutrients by plants (GYANESHWAR et al., 2002), promote plant growth (SILVA et al., 2015; SILVA et al., 2019) and mineralize organic solubilizing phosphates and inorganic phosphate (SILVA et al., 2018; SILVA et al., 2019), allowing the release of phosphorus assimilated by plants, being of great agronomic interest.

Phosphorus (P) is an essential element found in all living beings as part of proteins, nucleic acids, membranes and energy molecules such as ATP, GTP and NADPH, participation of metabolism. Usually, it is the second element-limiting plant growth preceded by nitrogen, but depending on some environmental and biological factors it can be the main growth-limiting nutrient (HINSINGER, 2001). Even though some soils may have high levels of total P, they can still be P-deficient due to low levels of soluble phosphate available to plants (GYANESHWAR et al., 2002).

There are several species of bacteria with phosphate solubilizing capacity, as *Azotobacter* sp., *Pseudomonas* sp., *Enterobacter* sp., *Pantoea agglomerans, Bacillus* sp., *Burkholderia* sp., *Mesorhizobium* sp., *Microbacterium laevaniformans*. These bacteria found in soil can increase the productivity of the plant and is generally recognized as plant growth promoting rhizobacteria (PGPR) (NAUTIYAL, 1999; PANDEY et al., 2006).

Exopolysaccharides (EPS) are defined as extracellular polysaccharides produced by certain micro-organisms which are found attached to the cell surface or are secreted into the medium. Most microorganisms has the ability to synthesize and excrete polysaccharides soluble or insoluble polymers, out of cells, with several functions (SEESURIYACHAN et al., 2012).

The exopolysaccharides have the function to protect the bacterial cell against desiccation and phage attack as antibiotics, toxic compounds and protozoa. Another possible function includes

the sequester of essential cations and their involvement in solid surfaces adhesion and biofilm formation (DE VUYST et al., 2001).

Rhizobacteria can act indirectly by suppressing diseases and, directly, for the production or concentration change of phytohormones, nitrogen fixation, phosphorus solubilizing, and other nutrients from the soil, promoting plant growth (MARIANO & KLOEPPER, 2000). The production of hormones, it is known that the rhizobacteria are capable to synthesize substances, such as indole-acetic acid (IAA), which can stimulate in low concentrations, while in the highest concentrations can inhibit the growth of the roots.

Due to soil degradation and nutrient restriction for plants cultivated for agricultural purposes, techniques are developed to meet the nutritional needs and provide the nutrients and plants development. Among the practices used to meet the needs of the soil and plants, there is the use of biological fertilizers or inoculants produced from microorganisms such as fungi or bacteria, with the ability to promote plant growth through several mechanisms.

Observing what was described, this study aimed to prospect phosphorus solubilizing, IAA and, exopolysaccharides production to promote plant growth and use of inoculants production programs.

MATERIAL AND METHODS

Bacterial species

Bacterial species used in this study are isolated from sugarcane rhizosphere cultuvated in comercial field, and identified. All sequences are deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/): *Bacillus subitilis* (accession number KT998653.1), *B. megaterium* (accession number KT998652.1), *B. pumilus* (accession number KT998656.1), and *Enterobacter cloaceae* (accession number KT998648.1).

Quantitative evaluation of phosphorus solubilizing

Quantitative analysis of P-Ca solubilizing was performed according to Nautiyal (1999) which 10ml of test tubes containing NBRIP culture medium, were inoculated in triplicate with 100 µL of bacterial solution (10^8 UFC.mL^{-1} (OD_{550} = 0.1)), the pH had being adjusted to 7.0 before autoclaving. The control consisted of tubes with 10 ml of NBRIP without inoculum. All tubes were incubated for fifteen days at 28 °C under orbital shaking at 180 rpm.

After incubation period, 1000 μL of each sample was transferred to 1.5 ml microtubes which were centrifuged at 10,000 rpm for 5 minutes. Then 145μL of each sample was added 570μL of distilled water and 285μL of ammonium molybdate-vanadate reagent (5% ammonium molybdate and ammonium vanadate (V/V).

To obtain the standard curve, a stock solution of KH_2PO_4 (0.0875%) was prepared (0.1 mg $P.mL^{-1}$), from which were withdrawn aliquots of 1ml to 10ml, which were mixed with 2.5 ml of the molybdate-vanadate reagent ammonium to a final volume of 50ml. Ten minutes after reagent addition, were read in a spectrophotometer at 420nm (MALAVOLTA et al., 1997; SILVA FILHO & VIDOR, 2000). For the negative control spectrophotometer was used without innocuous solution consisting of 145μL of NBRIP medium with the addition of distilled water 570μL and 285μL of the reagent molybdate - ammonium vanadate.

The spectrophotometer was restarted, using negative control consisting of 145μL NBRIP medium without inoculum, 570μL of distilled water and 285μL of molybdate - ammonium vanadate reagent. To obtain the standard curve, a stock solution of KH_2PO_4 (0.0875%) was prepared (0.1 mg $P.ml^{-1}$), from which it was removed aliquots of 1ml to 10ml, which were mixed with 2.5ml of ammonium molybdate-vanadate reagent to a final volume of 50ml. Ten minutes after reagent addition, samples were read in spectrophotometer (model UV-1601 PC, Shimadzu) at 420 nm.

The experiments were performed in triplicate and the positive result was evidenced by the formation of a yellowish color. The results obtained in absorbance (x values) were converted into the concentration of P ($\mu g.ml^{-1}$) (y) through equation $y = (0.3041x^2+0.2566x+0.0213)x1000$. The isolates were classified according to the following ratios: Absence of solubilization (-); low solubility ($<50\mu g.ml^{-1}$); middle solubilization (50-100 $\mu g.ml^{-1}$); high solubilization (101 - $500\mu g.ml^{-1}$) and high solubility ($> 501\mu g.ml^{--1}$).

Quantitative evaluation of exopolysaccharides

The evaluations were conducted according to Silva (2009), 5μL of bacterial solution grown in medium Tryptone Soy Broth (TSB) (10%) adjusted to 10^9 mL^{-1} cells (O.D. = 600nm), it was inoculated on filter paper discs sterilized with 5 mm Ø and deposited on the surface of the culture medium modified by Gyaneshwar et al. (2002), added with 10% fructose, glucose and sucrose at pH 5.5 and 7.5.

DOI: 10.37856/bja.v96i3.4291

The cultures were incubated at 30 °C for 48h and production of EPS was observed based on the formation of a mucoid layer around the filter paper discs inoculated with the bacteria. Production of EPS was characterized by visually measuring produced EPS halo, being assigned ,+low EPS production, halo ≤ 10mmØ, ++ average EPS halo production between 10 and 14mmØ, +++ great production EPS, halo ≥ 14mmØ. The Confirmation of the production of EPS was performed by the chemical method. The mucoid layer was removed with a platinum loop, and mixed in two mL of absolute ethanol, the formation of a precipitate confirmed the presence of EPS (HINSINGER, 2001).

Quantitative production of indole acetic acid

Test tubes with 10 ml of TSB medium (10%) supplemented with 5 mM *L*-tryptophan are inoculated in triplicate with 100 µL of bacterial inoculum [10^8 UFC.mL^{-1} (OD 550nm = 0.1)], calorimetric method described by (15). The culture medium conditioned at 28 °C in the dark under constant agitation for 24 hours. Afterwards, samples were centrifuged at 10,000 rpm for 10 minutes to obtain the supernatant.

The amount of IAA per mL was estimated by mixing 750µL of Salkowski reagent (7.9 equiv. L^{-1} H_2SO_4 and 12 g $FeCl_3$) at 750µL of supernatant, incubated for 30 minutes in the dark, followed density optical reading 530nm in spectrophotometer (model SP – 22 Shimatzu) (SEESURIYACHAN et al., 2012). The positive result was shown by formation of the pink color. The concentration of the culture medium IAA (y) was determined by comparison with a standard curve using commercial IAA, using the equation $y = 34.507x^2 + 43.802x + 0.843$, where x equals the obtained absorbance values.

Isolates were grouped according to Venieraki et al. (2011) which sets the following parameters for production of IAA: low yield (<1µg.ml^{-1}); average production (1-10µg.ml^{-1}); high output (11-50µg.ml^{-1}) and high yield (>51µg.ml^{-1}).

Experimental design and Statistical analisys

For the phosphate solubilization tests, pH change, indole-acetic acid production, and EPS production, a completely randomized design was used, organized in a factorial arrangement. The assumptions for performing the analisys of variance (ANOVA) were verified, evaluating the normality and homogeneity of the variances using the Lilliefors and Cochran tests, respectively. Non-homogeneous data were transformed and subjected to analysis of variance by applying the F

test (P<0.05) and the means compared by the Scott-Knott agglomerative test (P<0.05) which aims to separate the means of treatments in distinct groups, by minimizing the variation within and maximizing the variation between groups, generating results of greater objectivity and clarity. When necessary, polynomial regression analysis was applied. The Assistat 7.6 beta statistical software was used (SILVA; AZEVEDO, 2009).

RESULTS AND DISCUSSION

Inorganic phosphorus solubilizing assay

By means of the data obtained in phosphate solubilization assay the isolated *B. megaterium* showed the highest solubility index differing from all the others (Figure 1), with solubilization peak 15 hours after inoculation.

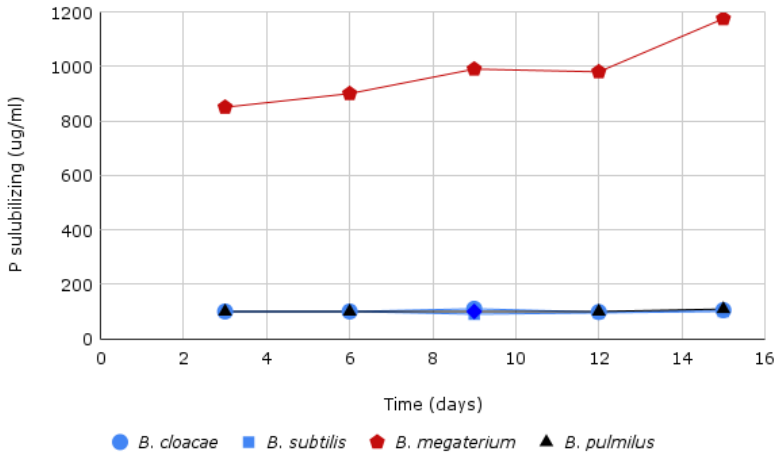

Figure 1. Solubilization of phosphate on NBRIP culture medium, in fifteen days of cultivation for bacterial isolates. Value of each point represents an average of nine replications. Rio Largo, Alagoas State, Brazil (2021).

Phosphate solubilizing bacteria has been reported like plant growth promoting agent. This mechanisms are described by authors, such as Matos et al. (2017) showing that phosphatase activity was detected in 65% of the isolates, being *Aneurinibacillus* sp., and *Lysinibacillus* sp. isolates presented with the best solubilization indexes.

To Suleman et al. (2018), the P higher solubilizing is related to glucose presence in the culture medium. The authors also relate to other mechanisms related to production solubilizing

DOI: 10.37856/bja.v96i3.4291

phosphate as inorganic acids, exopolysaccharides production, H_2S, and the phytases production and acid phosphatases production (H_2CO_3).

The phosphate solubilization by bacteria dissolve the insoluble phosphate for the production of organic acids in the medium in which the microorganism develops, whose action has been attributed to its chelating properties, enabling the formation of stable complexes with Ca^{2+} ions, Fe^{3+} and Al^{3+} (VANIEKARI et al., 2010). With this synthesis, the pH of the soil is reduced, and subsequently organic phosphorus mineralized by means of acid phosphatases (KHAN et al., 2009).

Khan et al. (2009) related bacteria of the genera *Bacillus* and *Enterobacter* like phosphorus solubilizing and its increased in crop production. Qureshi et al. (2012) show that *Bacillus* sp. associated with phosphorus solubilizing increased the yield enhancing cotton growth promotion.

Exopolysaccharides production

All isolates produced EPS, varying depending on the carbon source used (Table 1). The highest yield of EPS was observed in medium containing sucrose, which detected no significant differences between fructose and glucose, not being also a significant interaction between the carbon source and the pH, indicating that there was no dependency between the two factors.

Table 1. Production of EPS at 28 °C in culture medium containing three sources of carbon and two pH values by visually characterized as halo. Rio Largo, Alagoas State, Brazil (2021).

Isolate	Halo Ø (mm)					
	Fructose		Glucose		Saccharose	
	pH					
	5.5	7.5	5.5	7.5	5.5	7.5
B. megaterium	3.40 bB*	4.80bB	8.1aA	4.85bB	10.50bA	12.65aA
E. cloaceae	13.70 aA	3.75 bB	10.95 aA	8.10 aB	12.20 bA	5.85 bB
B. subitilis	9.35 aB	3.95 bC	4.75 bC	4.85 bC	21.00 aA	9.95 aB
B. pulmilus	9.80 aA	8.05 aA	10.60 aA	4.40 bB	12.85 bA	3.65 bB

*Means followed by different letters do not differ statistically among others by Tukey Test ($p \leq 0.05$). Capital letter in the lines, and lower letters in the columns.

The highest yield of EPS was observed in medium containing sucrose, not being detected significant differences between fructose and glucose, not being also a significant interaction between the carbon source and the pH (indicating that there was no dependency between the two).

The frequency of isolated in EPS production medium with sucrose that showed in the carbon sources and temperature influence the synthesis of EPS. found that glucose was the most efficient source in addition to the concentrations used also influenced, with the highest concentration. Past studies (HINSINGER, 2001) from rhizospheric bacteria *Cereus jamacaru* also observed increased production of EPS in medium containing sucrose at pH 7.5. According to Silva et al. (2019) these characteristics provide resistence ability to growth in extreme environmental conditions.

The EPS production by the microorganism-plant interaction, may form a biofilm, limiting the diffusion of secreted compounds by the roots and also by bacteria, which enables better adherence and colonization of areas in which the nutrients accumulate and contribute in the fixation nutrients and minerals, water retention and protection of the plant against environmental stresses such as desiccation, salinity and temperature variation. It has also been found that accumulation or formation of this substance tends to increase from the moment in which the salt stress or other stress is expressed most pronounced in the environment (BARRETO et al., 2011;. LIU et al., 2013; CERQUEIRA et al., 2015).

This cellular protection afforded by EPS against adverse environmental conditions, was also detected by Karygianni et al. (2020), modulating the microbial community. Bacteria (*Bacillus* sp., *A. hydrophila* and *B. insolitus*) are EPS-producing plants sensitive to salinity could alleviate salinity stress. The EPS works for type of cations of Na, which would reduce the Na^+ content available for uptake by plants (ASHRAF et al., 2004).

Qureshi et al. (2012) the studied the mechanism under the influence of salt stress, they reported that increased EPS production at higher levels of salinity, favors the formation of biofilm and protects the plant by maintaining a water layer around the cells, contributing significantly to the improvement of soil fertility and plant growth.

Knowing the production of EPS increase crop production, there is an increased aggregation of the soil around the roots of the inoculated wheat plants grown in saline soil, positively affecting their chemical-physical characteristics. To compensate for the stress imposed by salinity, exopolysaccharides production is a significant strategy to assist in salt metabolism in tolerant bacteria (ASHRAF & FOOLAD, 2005).

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IAA production

The presence of IAA was evidenced by the formation of a red color. All isolates were able to synthesize IAA precursor in the presence of *L*-Tryptophan (Table 2). Highest quantities at the end of 72 hours, the isolates were checked for *E. cloaceae* and *B. megaterium*.

Table 2. Production of IAA by bacterial isolate on TSA supplemented with tryptophan (O.D. 600 nm in 72 hour). Rio Largo, Alagoas State, Brazil (2021).

Isolate	µgmL-¹
E. cloaceae	51.62 a*
B. megaterium	29.51 b
B. subtilis	23.71 b
B. pumilus	21.09 b

*Means followed by same letter do not have statistical difference by Tukey test ($p \leq 0.05$).

Pedraza et al. (2004) found that amounts of excreted by IAA isolated depend on the species or even strain that is being studied, as well as the conditions in which the organisms are grown, such as the presence or absence of IAA precursor in the culture medium (tryptophan), oxygenation, pH and growth phase in which they are isolated.

The results are in line obtained by other authors. Ashraf et al. (2011) observed the potential of indole acetic acid production in the rhizosphere bacteria associated with sugarcane, getting maximum output of IAA 4.49 $\mu g.ml^{-1}$. Already Sgroy et al. (2009) studied endophytic bacteria associated with *Prosopis strombulifera*, had the highest values for IAA production of 2.2 $\mu g.ml^{-1}$. Rocha et al. (2011) with values of 0.8-12 $\mu g.ml^{-1}$, Oliveira (2009) with values of 13.72-19.62 $\mu g.ml^{-1}$, Verma et al (2001) with values of 0.16-0.70 $\mu g.ml^{-1}$, and Silva et al. (2019), with values of 11.24 $\mu g.ml^{-1}$.

Indole-acetic acid does not function as a hormone in bacterial cells however have ability to produce the same may have evolved as it is important in plant–bacteria relationship. IAA levels produced by bacteria rely on bacterial growth, metabolic activity and expression of genes encoding enzymes for IAA biosynthesis (LAMBRECHT et al., 2000), therefore the nutritional deficiency is one of the factors capable of inhibiting the production of this hormone.

Bacterial IAA is a secondary metabolite being produced in the stationary phase of bacterial growth, however, depends on the duration of each species. Besides the stationary phase knowledge

for reading the auxin growth curve gives the relationship between the bacterial density and the production of the hormone.

CONCLUSIONS

The isolated *B. megaterium* and *E. cloacae* were the most efficient in tests developed in this study, with promising agents for inoculant production program for use in agriculture to plant growth promotion and crop production.

ACKNOWLEDGEMENTS

To Coordination for the Improvement of Higher Education Personnel (CAPES Foundation) for financial support, and Agricultural Microbiology Laboratory team.

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Received in: November, 29, 2021.
Accepted in: December, 18, 2021