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Screening of plant growth-promoting bacteria isolated from sugarcane

Seleção de bactérias promotoras de crescimento de plantas isoladas de cana-de-açúcar

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Highlights

The isolation of *Bacillus* species that have not been isolated from sugarcane before.
First report of *Bacillus wiedmanii* and *Bacillus zhangzhouensis* from sugarcane soil.
First description of *Bacillus wiedmanii* ability to produce IAA and to solubilize P.

Abstract

Plant growth-promoting bacteria (PGPB) are known to establish positive relationships with plants. They act in favoring plant nutrition, production of phytohormones, control of pathogens and enhancement of stress tolerance. Thus, this study aimed to isolate bacteria from soil, rhizosphere, and root endosphere from sugarcane cultivated in the Southeastern of Brazil, to prospect strains with potential for plant growth promotion. The samples were plated in Nutrient Agar medium, and the morphologically distinct colonies were isolated and analyzed about indoleacetic acid production, phosphate solubilization and the growth control of the phytopathogenic fungus *Fusarium verticillioides*. A total of 219 isolates were obtained, of which 86 from soil, 67 from rhizosphere and 66 from sugarcane root endosphere. The strains that presented more than one mechanism of plant growth promotion were identified by the sequencing of 16S gene. Most species belonged to the genus *Bacillus*, which has strains already used in various biological products for the control of diseases in agriculture. Some *Bacillus* species isolated in our study have never been isolated from sugarcane, and others have been studied for the first time as plant growth promoters. The isolated strains constitute an important microbial bank to be explored to compose innovative products for agriculture.

Key words: Plant microbiome. *Bacillus*. Solubilization. Indoleacetic acid. Antagonism.

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Resumo

Bactérias promotoras de crescimento de plantas são conhecidas por estabelecer relações positivas com as plantas. Atuam no favorecimento da nutrição das plantas, produção de fitohormônios, controle de patógenos e aumento da tolerância ao estresse. Desta forma, este estudo teve como objetivo isolar bactérias do solo, rizosfera e endosfera radicular de cana-de-açúcar, cultivada na região Sudeste do Brasil, para prospectar cepas com potencial para promoção de crescimento vegetal. As amostras foram semeadas em meio Ágar Nutriente, e as colônias morfologicamente distintas foram isoladas e analisadas quanto à produção de ácido indolacético, solubilização de fosfato e controle de crescimento do fungo fitopatogênico Fusarium verticillioides. Foram obtidos 219 isolados, sendo 86 do solo, 67 da rizosfera e 66 da endosfera da raiz da cana-de-açúcar. As cepas que apresentaram mais de um mecanismo de promoção do crescimento vegetal foram identificadas pelo sequenciamento do gene 16S. A maioria das espécies pertence ao gênero Bacillus, que possui linhagens utilizadas em diversos produtos biológicos para o controle de doenças na agricultura. Algumas espécies de Bacillus nunca foram isoladas da cana-de-açúcar e outras foram estudadas pela primeira vez como promotoras de crescimento de plantas. As cepas isoladas constituem um importante banco microbiano a ser explorado para a composição de produtos inovadores para a agricultura.

Palavras-chave: Microbioma vegetal. *Bacillus*. Solubilização. Ácido indolacético. Antagonismo.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a culture of economic importance used for feed and food, and as raw material in industry to produce fuels and energy. Brazil is the largest producer of sugarcane in the world, with an estimated production of 568.4 million tons in the 2021/2022 season, which should render 33.9 million tons of sugar and 24.8 billion liters of ethanol (Companhia Nacional de Abastecimento [CONAB], 2021). The sugar and ethanol had a prominent role in the export agenda, and in 2020 the sector had a national share of US$ 9.9 billion, the fourth most representative sector in the country (Angelo et al., 2021).

Due to the demand to increase the productivity, this crop requires the use of large amounts of fertilizers, pesticides, and other chemical inputs, which cause damage to human health and to the environment. Various studies that evaluate the use of microorganisms to replace or decrease the use of toxic inputs in agriculture provide a way to improve crop productivity and increase plant tolerance to stress (De-La-Peña & Loyola-Vargas, 2014). Plants live in association with microbes that can confer positive, negative, or neutral interactions with their host (Schlaeppi & Bulgarelli, 2015). The main target of the technologies based on microorganisms is a more sustainable, economically, and socially fair agriculture (Souza et al., 2016). Microorganisms that promote positive interchanges bring benefits to plants by controlling phytopathogens and/or promoting plant growth through different mechanisms (Orozco-Mosqueda et al., 2018). Bacteria with these characteristics are known as Plant Growth Promoting Bacteria (PGPB). Preliminary studies showed that the use of

PGPB brings promising results concerning sugarcane productivity, with the increase of root and shoot dry mass, besides providing nitrogen accumulation in the soil, favoring crop nutrition (Santos et al., 2019).

The increasing knowledge about PGPB has led to the production and commercialization of biological products, such as biofertilizers, biostimulants and biopesticides, constituted of selected autochthonous microbial strains isolated from the plant or from soil under plant cultivation (Johns et al., 2016; Bhardwaj et al., 2014; Pérez-Montaño et al., 2014). These strains are adapted to the imposed conditions, and present mechanisms that favor their association with plants, bringing benefits to plant production. In this context, the aim of this study was the isolation, screening, and identification of bacteria from the sugarcane endosphere, rhizosphere and soil, for the prospection of strains with potential for plant growth promotion through indoleacetic acid production (IAA), phosphate solubilization and biological control strategies.

Material and Methods

The samples were collected from sugar cane area of Universidade Federal de São Carlos, Campus of Araras, São Paulo State (latitude 22º18'S, longitude 47º23'W and altitude of 707 m). Rhizosphere and root endophytic bacteria were isolated from five independent sugarcane plants with adhering (rhizosphere) soil at least 2 m away from each other, taken randomly and bulked to obtain a representative composite sample. Root segments were collected at a depth of 5-15 cm from the stem base. The soil samples were collected next to the roots from the same plants. Ten grams of soil were placed in 90 mL of 0.85% NaCl solution. One gram of rhizosphere soil sample was placed in 9 mL of saline solution. Both suspensions were stirred in shaker at 160 rpm for 30 minutes, then the decimal serial dilution of the suspensions was performed up to 10^{-5}.

For root endosphere, 0.62 g of root sample was washed under running water, followed by surface disinfection with immersion of the sample in 70% alcohol solution for 1 minute, sodium hypochlorite 2% for 3 minutes, again 70% alcohol for 30 seconds. Then the roots were rinsed 3 times in sterile distilled water. Subsequently, the roots were macerated for 3 minutes in mortar with saline solution (Mendes et al., 2007), followed by serial decimal dilution up to 10-5 of the suspension obtained from maceration.

Each dilution was plated in Petri dishes containing Nutrient Agar medium (Kasvi®), with the antifungal nystatin to a final concentration of 5 mg/L. Plates were incubated at 30°C and the growth of the colonies was daily monitored. The colonies were evaluated regarding their morphological characteristics such as color, texture, colony border and each morphological type was streaked onto the same culture medium in Petri dishes to obtain pure culture. The isolates were stored in slants and kept under refrigeration.

To screen antagonists, a strain of *Fusarium verticillioides* (causing corn rot) was used. A dual culture assay was carried out with the bacterial culture streaked on a side of a Petri dish and the mycelial disk of the *F. verticillioides* at the opposite side. A scale was created according to the antagonism: (1) the phytopathogen grows up to the limit of the bacteria, (2) the bacterial strain grows

over the phytopathogen, (3) the bacterial strain produces an inhibition halo. To screen IAA producers, a colorimetric technique was performed according to Gordon and Weber (1951), using Salkowski reagent. Positive results were observed when the supernatant solution turned light or dark pink after reaction. The color intensity indicates the IAA concentration (low-light pink, medium-pink, and high concentration-dark pink).

The bacterial strains were also screened for phosphate solubilization on PDYA medium (Potato Dextrose Agar medium added with 10% K_2HPO_4, 0.1% yeast extract, and 10% CaCl2). The solubilization index (IS) was determined by the ratio of total diameter (colony + halo zone) and the colony diameter: low (IS<2), medium (2≤IS<3), and high (IS≥3) (Silva et al., 2014).

The Principal Component Analysis (PCA) was applied to the results. A binary data matrix was considered, in which the lines contained the strains, and the columns presented the data of the scales used to represent the results for each analyzed characteristic, as earlier specified. The Pearson-n coefficient was used in the XLSTAT 2021.2.2 tool (Behbahani et al., 2017).

The bacterial strains that showed two or three mechanisms regardless the scale was identified by sequencing the 16S rDNA gene. Bacterial DNA was extracted with Wizard® Genomic DNA Purification Kit (Promega®). PCR reaction was performed with the universal primers P027F (GAG AGT TTG ATC CTG GCT CAG) and R1378 (CGG TGT GTA CAA GGC CCG GGA ACG). The amplicons were cloned into the pGEM-T vector (Promega®) according to the manufacturer protocol. Sequencing was carried with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer®) and the T7 promoter primer (TAA TAC GAC TCA CTA TAG GG) or the SP6 promoter primer (ATT TAG GTG ACA CTA TAG), according to the manufacturer instructions. DNA was injected into an ABI3730 automatic sequencer (Applied Biosystems®) and the obtained sequences were analyzed using the Blastn tool at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

A total of 219 isolates were obtained, of which 86 from the soil, 67 from the rhizosphere and 66 from the sugarcane root endosphere. Using PCA for the set of mechanisms and their sub-levels for the bacterial strains, a biplot graph was obtained (Figure 1). The F1 and F2 components explained 70.54% of the total variability of the data. The large number of isolates concentrated at the 0;0 point of the biplot refers to those that did not show any mechanism to promote plant growth among the ones evaluated, which represents 58% of the total isolates. The graph shows a cluster of 23 bacteria strains that have only the phosphate solubilization mechanism, 7 from the endosphere, 10 from the soil and 6 from the rhizosphere. For antagonism against the phytopathogen only, there is a grouping of 21 bacteria, 4 from the endosphere, 9 from the soil and 8 from the rhizosphere. For IAA production only, 37 bacteria presented the mechanism, being 17 from the endosphere, 12 from the soil and 8 from the rhizosphere.

The isolates within the circles in Figure 1 presented more than one mechanism. ENDO26 was the only strain that presented three mechanisms at low level; ENDO36

presented IAA production at medium level and antagonism at low level; ENDO2, ENDO33 and ENDO64 presented IAA production and antagonism at medium level; RIZ4 and RIZ40 presented IAA production at medium level and antagonism at high level. ENDO5 was able to produce IAA and solubilize phosphate at low level; SOL21 produced IAA at low level and was able to solubilize phosphate at medium level. RIZ16 and RIZ25 presented low IAA production and high index of solubilization. All strains with two or three mechanisms were able to produce IAA.

The identification of the strains displaying two or three mechanisms is presented in Table 1, exception for the strain RIZ25 which could not be identified successfully. Most strains were identified as belonging to the genus *Bacillus.* The species of this genus are commonly regarded as plant growth promoters (Lyngwi et al., 2016; Akinrinlola et al., 2018; Kashyap et al., 2019). *Bacillus* species stand out for possessing plant growth mechanisms and for being one of the main constituents of commercial biological products, which are successful in agricultural practices due to the resistant endospore, providing maintenance of cell viability during the storage, and after the application of the cells in the field (Cawoy et al., 2011).

In the literature, the most frequent bacterial genera isolated from sugarcane rhizosphere, endosphere and phyllosphere are *Herbaspirillum, Gluconacetobacter, Burkholderia, Pantoea, Enterobacter* and *Pseudomonas* (Rodrigues et al., 2016; Teheran-Sierra et al., 2021). The strains isolated may be a consequence mainly of the culture medium and the enrichment conditions used during the isolation. Singh et al. (2020) isolated bacteria from sugarcane rhizosphere soil to obtain diazotrophic species using a range of culture media including Nutrient Agar medium, which supported the growth of the highest number of isolates. The screening of the isolates began with those capable of controlling phytopathogens, and as a result the authors selected strains of the genus *Bacillus.* These data are similar to our results and show that this group is common in sugarcane, and that its isolation is conditioned, as previously mentioned, to the medium and conditions imposed in the selection process.

The species *Bacillus thuringiensis* isolated from rhizosphere (RIZ16) is already utilized as constituent of biological products to agriculture. *B. thuringiensis* is one of the most known bioinsecticide widely used to pest control. Currently there has been an increase in the interest in the use of this species also as a plant biostimulant (Azizoglu, 2019). The isolate SOL21, identified as *Bacillus wiedmanii*, has not been reported as plant growth promoter yet. This is the first report of isolation of this species from sugarcane soil (Table 1).

The isolate ENDO2 was identified as *Serratia marcescens*, commonly found in rice roots, pumpkin flowers, cacti, tea, and medicinal plants. This species is capable to control phytopahogen, produce IAA, siderophore and solubilize phosphate (Devi et al., 2016; Purkayastha et al., 2018). The isolate ENDO33 was identified as *Serratia* sp. (Table 1).

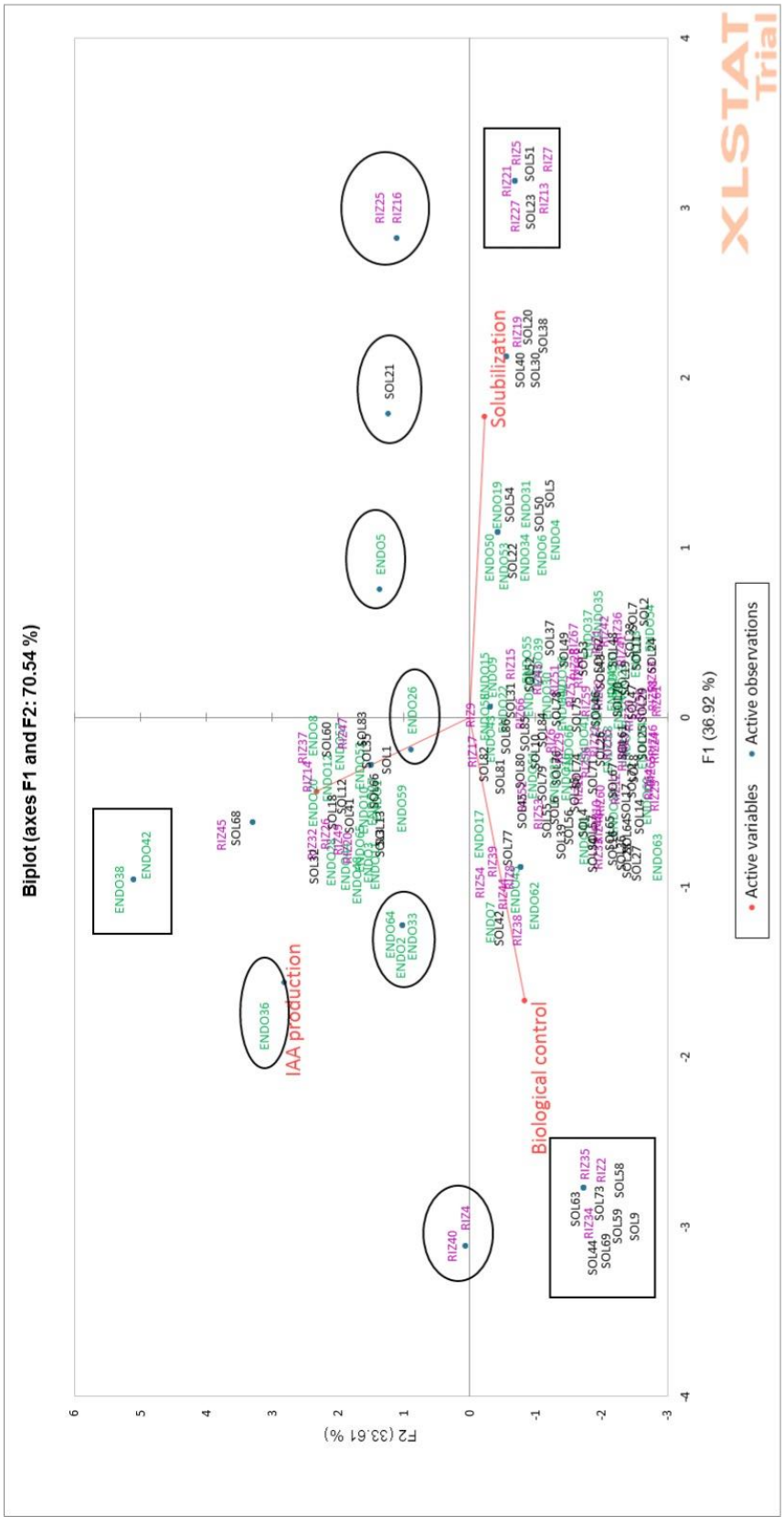

Figure 1. Principal Component Analysis (F1 and F2) of the bacterial strains isolated from the rhizosphere (RIZ, purple), endosphere (ENDO, green) and soil (SOL, black) from sugarcane regarding their results of the mechanisms for plant growth promotion (IAA production, phosphate solubilization and biological control). Strains inside the circles presented two or three mechanisms of plant growth promotion and strains inside the rectangles presented the highest scale for each mechanism of plant growth promotion.

Table 1
Identification of the bacterial strains isolated from the rhizosphere (RIZ), endosphere (ENDO) and soil (SOL) from sugarcane based on 16S gene sequences associated with the mechanisms presented in the present study and compared to the literature reports

Strain	Similarity (%)	Fragment size (pb)	Closest species	Mechanism[1]	Mechanism already observed	References
ENDO2	100%	588	*Serratia marcescens*	IAA; antagonism	Stimulation of phytohormone production; phosphate solubilization; AIA and siderophore production; hydrolytic enzymes	Devi et al. (2016) Purkayastha et al. (2018)
ENDO5	100%	565	*Lysinibacillus sphaericus*	IAA; P solubilization	Insecticide; ammonia, IAA, ACC deaminase and siderophore production; P and K solubilization; antagonism against Alternaria alternata, Curvularia lunata, Aspergillus sp., Sclerotinia sp., Bipolaris spicifera, Trichophyton sp., Rhizoctonia solani (rice endosphere and maize rhizosphere)	Naureen et al. (2017) Shabanamol et al. (2017) Shabanamol et al. (2018)
ENDO26	100%	1085	*Bacillus subtilis*	IAA; P solubilization; antagonism	Phosphate and zinc solubilization; ACC deaminase, siderophore, ammonia and AIA production; antagonism against Fusarium sp.	Khedher et al. (2020) Chandra et al. (2021) Wu et al. (2021)
ENDO33	99%	588	*Serratia sp*	IAA; antagonism	Pigmented and non-pigmented Serratia species are able to produce a range of bioactive secondary metabolites of interest to industry and agriculture, such as biosurfactants, glucosamine derivatives, siderophores, bacteriocins, and serratin	Clements et al. (2021)
ENDO36	99%	222	*Bacillus methylotrophicus*	IAA; antagonism	Phytostimulant; IAA and gibberelic acid production; antagonism against Ralstonia solanacearum (rice endosphere and pepper rhizosphere)	Dunlap et al. (2015) Im et al. (2020)
ENDO64	100%	469	*Bacillus zhangzhouensis*	IAA; antagonism	Amonia and IAA production; P solubilization (endosphere of legume root and nodules)	Bhutani et al. (2021)

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RIZ4	100%	627	*Bacillus licheniformis*	IAA; antagonism	Antagonism against Phytophthora capsici, Fusarium oxysporum, Monilinia fructicola, Sporisorium scitamineum; siderophore, IAA, ACC deaminase and ammonia production; P solubilization (cucumber rhizosphere, tomato rhizosphere, sugarcane rhizosphere)	Prashanth and Mathivanan (2010) Sukkasem et al. (2018) Li et al. (2020) Ji et al. (2020) Singh et al. (2020)
RIZ16	100%	587	*Bacillus thuringiensis*	IAA; P solubilization	Insecticide; IAA, siderophore and ACC deaminase production; P solubilization (legume root endosphere, sugarcane rhizosphere)	Raddadi et al. (2008) Singh et al. (2020)
RIZ40	100%	473	*Bacillus sp.*	IAA; antagonism	A number of species and isolates of Bacillus have plant growth mechanisms such as nitrogen fixation, biological control, P solubilization, among others.	Singh et al. (2020)
SOL21	100%	516	*Bacillus wiedmannii*	IAA; P solubilization	No report about the abilities to promote plant growth	-

[1] IAA production, phosphate (P) solubilization and antagonism against *F. verticillioides.*

Conclusion

Considering the results, we can conclude that the genus *Bacillus* is an important component of the sugarcane microbiome. Our study successfully constituted a bacterial collection of strains with potential application in biological products for agriculture, besides the indication of species not reported yet to be plant growth promoters or isolated from sugarcane. All strains must be further evaluated to compose innovative products to agriculture.

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Authors' contributions

GBA: performed research, analyzed data, wrote the paper; MAT: analyzed data, review the paper; CDT: analyzed data, review the paper; SRCA: analyzed data, wrote the paper; MMRM: conceived and designed study, analyzed data and wrote the paper. All authors have read and agreed with the final version of the manuscript.

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