International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 4, 2019, pp.-1528-1533.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

EVALUATION OF PLANT-GROWTH PROMOTION POTENTIAL OF ENDOPHYTIC AND RHIZOSPHERIC ACTINOBACTERIAL STRAINS FROM DIFFERENT ORIGINS

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Received: August 22, 2018; Revised: April 16, 2019; Accepted: April 17, 2019; Published: April 30, 2019

Abstract- The phylum *Actinobacteria* stands out in the symbiotic relationship between plant and microorganism, due to its diversity of hydrolases, important in the colonization and, in the production of molecules that help the host, as phytohormones. The aim of this study was to evaluate the endophytic and rhizosphere actinobacteria in terms of their ability to promote plant growth. A total of 9 actinobacterial strains, 4 from sugarcane rhizosphere and 5 endophytes of field mallow and Neem, were tested to produce indole acetic acid (IAA), solubilization of phosphate and zinc, ammonia and hydrocyanic acid production, production of cellulases, antagonism activity against phytopathogen *Fusarium moliniforme* and the capacity to grow under nitrogen absence. Three isolates, NIM3, FA9K1 and FA8K4, showed the best results in the assays and were identified as *Streptomyces* sp. Therewith, the results were promising in the search of actinobacterial inoculants.

Keywords- *Endophyte¸ Plant-growth promotion, Rhizosphere, Streptomyces*

Citation: Igor Daniel Alves Ribeiro, *et al*., (2019) Evaluation of Plant-Growth Promotion Potential of Endophytic and Rhizospheric Actinobacterial Strains from Different Origins. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 4, pp.-1528-1533.

Introduction

Plant tissues are widely inhabited by microorganisms both above and below ground. It is estimated that the total bacterial population only in the phyllosphere, the above-ground portion of plants dominated by leaves, can reach approximately 1026 cells [1]. Generally, the microbial component in this symbiosis is categorized in: epiphytic, those colonizing the surface of plant tissues; endophytes, those that could be theoretically isolated inside plant organs with sterilized surface, which do not cause any symptomatic harm in the host; and rhizosphere, that live in soil under direct influence of roots [2]. Biotechnologically, there is considerable interest in these microorganisms due to their ability to promote plant growth and development either directly, through production and modulation of plant hormones or enhancing nutrient acquisition, or indirectly, by basically protecting from the phytopathogens [3]. Regardless of the involved mechanism, the search for plant-growth promoting (PGP) bacteria and the exploitation of their underlying biological properties are attracting more attention in modern agriculture once they can be used as microbial inoculants. Further the enhancement of crop production, they can also be an environment-friendly alternative to chemical fertilizers [4]. After Proteobacteria, the phylum Actinobacteria constitutes one of the most frequently found members in plant-associated bacterial communities with its population floating depending on the host development stage and environmental conditions [5,6]. The relative predominance of these bacterial phyla in the roots can be explained whereas they have the soil as the natural habitat and, thus, it is in this plant region where it begins the symbiotic relationship [7]. As for other plant symbionts, various actinobacteria switch from an initial status as an epiphytic colonizer to a proper endophyte, thanks to their broad diversity of hydrolytic enzymes, beyond their filamentous lifestyle and the secretion of phytohormones and other signaling related molecules [8,9]. From the perspective of obtaining an inoculant, the characteristic spore formation by these bacteria is a convenient trait for preparing powder formulations which are preferred by its longer lifetime and easiness in the transport and storage [10]. Such as other PGP bacteria, actinobacteria also can influence plant growth through direct mechanisms as the production of essential factors as growth hormones and contributing to the host on nitrogen fixation, phosphate solubilization and iron acquisition. Indirectly, these bacteria assist by controlling and reducing the damaging effects of stresses caused by abiotic or biotic factors, particularly by secretion of inhibitory substances for common plant pathogens and exoenzymes [11]. Even though the first identified endophytic actinobacteria belonged to the genus Frankia, the presence of non-phytopathogenic *Streptomyces* species in the roots of different plants have been already confirmed [12,13] as also their active beneficial role, producing auxins [14], stimulation nutrient acquisition in the plant host [15] or exerting a biocontrol activity against various bacterial and fungal plant pathogens, by generating antibiotics, volatile-organic compounds, extracellular degrading-enzymes or phytotoxins inactivation [16,17]. Even without the economic success of *Rhizobium* and other gram-negative bacteria, there are already commercially available inoculants based on streptomycetes, which are applied to the biocontrol of crop pests and pathogens disseminated by different routes [18]. In this context, the aim of the present study is to screen in vitro PGP properties of endophytic and rhizosphere actinobacteria isolated from different hosts and identify the potential bacterial isolates.

Materials and Methods

Bacterial isolates

A total of 9 actinobacterial isolates were selected for this study and all of them belonged to the bacterial culture collection of the Environmental Microbiology and Biotechnology Lab from the Federal University of Goiás. Four strains (FA8N1.2,

FA8N2, FA8K4 and FA9K1) were isolated from sugarcane rhizosphere and five from two phytotherapic species: *Hyptis suaveolens* (L.) Poit. (MC_{30}, MC_{38} and MC_{45}) and *Azadirachta indica* A. Juss (NIM1 and NIM3). All these plant-associated bacteria were isolated in accordance with well-established methodologies for endophytic [19] and rhizospheric microorganisms [20], in agreement with the adaptations suggested from previous works of the present research group [21-23].

IAA production

For the screening of indole-3-acetic acid (IAA) production, the isolates were antecedently grown on ISP-2 agar (0.4% yeast extract, 1.0% malt extract, 0.4% glucose and 2.0% bacteriological agar) [24] for 7 days at room temperature. Mycelial disks (5 mm in diameter) were cut off from the growth in solid medium and inoculated in 250-mL Erlenmeyer flasks containing 100 mL of yeast extract-tryptone (YT) broth (0.1% yeast extract, 1.0% tryptone and 0.5% NaCl), added with of tryptophan (0.2%) [25,26]. The flasks were kept under orbital agitation (130 rpm) at 30ºC and aliquots were withdrawn from the cultures with 7 and 14 days. The IAA production from the culture's supernatant was evaluated using the colorimetric method with the Salkowski reagent [50 mL of 35% (v/v) perchloric acid and 1 mL of 0.5 M $FeCl_3$ (0.5 M)], according to the adapted method proposed by Gordon & Weber (1951) [27]. 500 µL-supernatant aliquots were incubated with the exact volume of Salkowski reagent for 30 minutes. Following this period, the solution was submitted to reading on spectrophotometer (SmartSpec™ Plus, Bio Rad) at a wavelength of 530 nm. The phytohormone concentration was properly calculated using a standard curve prepared with a commercial IAA solution and expressed in µg/mL.

Phosphate and zinc solubilization

Firstly, the microorganisms were evaluated according to their phosphate and zinc solubilization capacities through the plate-screening methodology. For the zinc solubilization test, it was applied the medium described by Bapiri *et al*. (2012) [28] (1.0% glucose, 0,1% $(NH_4)2SO_4$, 0.02% KCl, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% yeast extract and 1.5% bacteriological agar), supplemented with of zinc oxide (0.1%). For the phosphate solubilization test, it was used the National Botanical Research Institute's phosphate growth medium (NBRIP) (1.0% glucose, 1.0% $Ca_3(PO_4)_2$, 0.5% $MgCl_2.6H_2O$, 0.025% $MgSO_4.7H_2O$, 0.02% KCl, 0.01% $(NH_4)2SO_4$ and 1.5% bacteriological agar) [29] and the Pikovskaya medium (PKV) [0.05% $(NH_4)2SO_4$, 0.01% $MgSO_4{\cdot}7H_2O$, 0.002% NaCl, 0.002% KCl, 0.003% $FeSO_4{\cdot}7H_2O$, 0.003% $MnSO_4{\cdot}H_2O$, 0.5% $Ca_3(PO_4)_2$, 1.0% glucose, 0.05% yeast extract and 1.5% bacteriological agar [30]. Microorganisms were spot-inoculated on the screening medium and the plates were incubated at 30ºC. At 7 and 14 days of microbial growth, it was calculated the solubilization index (SI) according to the ratio between the diameters of halos and respective colonies sizes [31]. The positive strains for phosphate solubilization ability were selected to perform the solubilization assay in liquid medium. Briefly, mycelial disks (5 mm in diameter) of the isolates previously grown on ISP-2 agar were transferred to 250 mL-Erlenmeyer flasks filled with 100 mL of NBRIP broth, followed by incubation under agitation (130 rpm) at 30ºC. Phosphate concentration was determined from culture supernatants recovered after 5, 10 and 14 days applying the colorimetric method with the vanadate-molybdate reagent in accordance with Nosrati *et al*. (2014) [32], with minor alterations. A total of 200 µL of culture supernatants were incubated in the presence of the same volume of the reagent [5.0% $(NH_4)6Mo_7O_{24}$, 0.25% NH_4VO_3, 1:1 (v/v)] and 600 µL of deionized water. The absorbance was measured spectrophotometrically at 420 nm, using a prior established K_2HPO_4 standard curve.

Ammonia and HCN production

Ammonia production assay was performed in 96-well microplates, following the method optimized by Cappuccino & Sherman (1996) [33]. Primarily, the actinobacterial isolates were grown on 50 mL of peptone water (1.0 % peptone and 5.0 % NaCl). After 7 days of incubation under agitation (130 rpm), supernatant aliquots of 100 µL and an equal volume of Nessler reagent (10% HgI_2, 7.0% KI, 50% of 32% NaOH aqueous solution) were added to microplates wells, with ammonium sulfate used as a positive control. The characteristic color changing ranging from pale yellow to dark brown was interpreted as positive for ammonia production. To confirm the liberation of cyanide hydrogen (HCN) by the actinobacteria, it was adopted the procedure described by Walpola & Yoon (2013) [34]. Bacteria were inoculated on Tryptic Soy Agar (TSA) 1/10 strength supplemented with glycine (0.44%). Subsequently, it was applied a Whatman no. 1 filter paper impregnated with a solution of picric acid solution (0.5%) and sodium carbonate (2.0%) on the lid of the Petri dishes, which were sealed with parafilm and kept at room temperature for 14 days. A color alteration in the filter paper from yellow to orange-brown was a qualitative indication of HCN production.

Antagonistic activity against *Fusarium moniliforme*

The actinobacterial strains were evaluated in vitro regarding their capacity to inhibit the growth of the phytopathogenic fungi *F. moniliforme*, using the Potato Dextrose Agar (PDA) in the assay. Bacterial isolates were streaked straightly at one extremity on the media surface. Succeeding an incubation at 30ºC for 7 days, mycelial disks (5 mm in diameter) of the phytopathogenic fungi, previously grown on PDA, were placed on the center of the plate. After an additional 7 days of incubation, any level of retardation or blockage of fungi growth was recorded, with the measurement of the diameters of colonies. The negative control consisted of plates without bacterial inoculum. Growth rate inhibition was determined by applying the formula: [C-T/C x 100], with (C) corresponding to the diameter of the fungal colony in the negative control and (T) equivalent to the diameter of pathogen colony in inoculated plates with the actinobacterial isolates [35].

Growth in nitrogen-free medium

A possible ability to fix atmospheric nitrogen (N_2) were assessed by bacterial cultivation in nitrogen-free (NF) medium [36]. All isolates were initially precultivated on 15-mL of ISP-2 broth at 130 rpm, 30ºC for one week. Culture aliquots were then centrifuged at 10.000 rpm for 10 minutes. The supernatant was removed, and bacterial cells were resuspended in 0.85% saline solution. This bacterial suspension was inoculated in cultures tubes containing nitrogen-free bromothymol blue (NFb) semi-solid medium and incubation was carried out at for 7 days at 30ºC with daily monitoring. For those isolates able to grow and promote media alkalization, five successive subcultures were performed in NFb medium under the same conditions.

Cellulase production

The screening for cellulase secretion by the actinobacteria was performed in accordance with Kasana *et al*. (2008) [37], with minor adaptations. Basically, the strains were spot-inoculated on CMC medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone, and 1.7% agar) with subsequent incubated for 7 days at 30ºC. Thereafter, plates were flooded with Lugol's solution and the appearance of a clear zone around the bacterial spot mass was considered a positive result. The enzymatic index (EI) was finally calculated by the ratio between diameters of enzymatic degradation halo and colony sizes of cellulase-producing strains.

Molecular identification and phylogenetic analyses

The most prolific isolates in the in vitro assessment of plant growth promotion were selected for molecular identification based on the amplification and sequencing of the 16S rRNA coding region. Total genomic DNA was extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO) according to the manufacturer's instructions. Amplification by polymerase chain reaction (PCR) of 16S rRNA gene was carried out with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'). The PCR reaction mixture (50 µL) consisted of: 1.5 µL of MgCl2 (50 mM), 1.5 µL of primers (10 µM), 4.0 µL of dNTPs solution (10 mM), 5.0 µL of Taq polymerase buffer 1X, 1.0 µl of Taq polymerase (5U) (Invitrogen), 1.0 µL of DNA (50-100 ng) and 35.5 µL of ultrapure water, with the following applied reaction conditions: initial denaturation at 95ºC for 2 min; 30 cycles of denaturation (1 min at 95ºC), annealing (1 min at 55ºC) and extension (2 min at 72ºC) and a final extension step at 72ºC for 10 minutes.

The PCR products were purified using the QIAquick PCR Purification Kit® (Qiagen) and were sequenced with the primers 27F, 1541R, 926F (5'-AAACTY AAA KGAATTGAC GG-3'), 530F (5'-TGACTGACTGAGTGCCAGCMGCCGCGG-3'); 519R (5'–GTNTTACNGCGGCKGCTG–3') and 907R (5'–GTNTTACNGCGGCKGCTG–3') [38]. Sequence quality checking and formation of contigs were performed using the CodonCode Aligner software (CodonCode Corporation). The partial 16S rRNA gene sequences were submitted to GenBank search from National Center for Biotechnology Information (NCBI) databases by BLASTn algorithm and most similar sequences were retrieved for phylogenetic purposes. The sequences were then aligned using ClustalW programme in MEGA v. 7.0.212. Trees were generated by the neighbor-joining method with the Jukes-Cantor model and branch supports assessed by 1000 bootstrap replicates.

Statistical analyses

EI and SI data were submitted to one-way analysis of variance (ANOVA) and the obtained means were compared applying the Tukey test, with a significance of 5%. The program ASSISTAT v. 7.7 [39] was used to carry out such analyses.

Results and Discussion

Screening of plant-growth promotion traits of the endophytic and rhizosphere actinobacteria

All tested isolates exhibited positive results for auxin production in the screening test [Fig-1]. IAA concentration ranged from 2.00 to 32.22 µg/mL after 7 days of cultivation and from 22.59 to 70.22 µg/mL after 14 days. For all actinobacterial strains, higher production of the phytohormone was observed following a period of 2 weeks of cultivation.

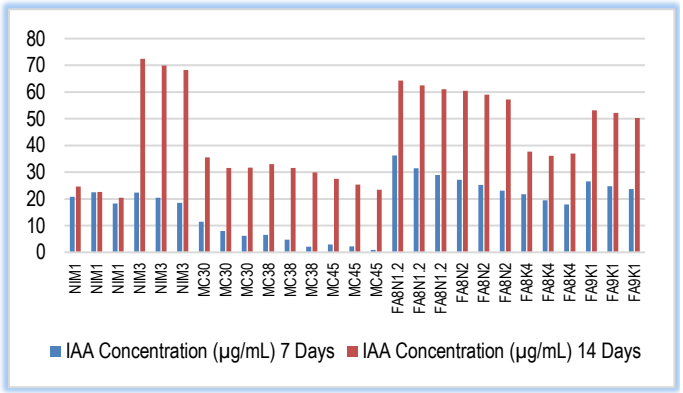

Fig-1 IAA production by the actinobacterial isolates grown on YT medium supplemented with L-tryptophan in two-time periods (7 and 14 days). Error bars indicate the standard deviations of means obtained from triplicates.

Passari *et al*. (2016) [40] evaluated that all the fifteen endophytic actinobacterial strains isolated from *Solanum lycopersicum* were able to produce the phytohormone in L-tryptophan-supplemented medium in similar conditions that was adopted by us, with concentration ranging from 7.4 to 46.3 µg/mL, in counterpoint with what was verified for the isolates in our study, once they readily produced the phytohormone in concentrations higher than 20 µg/mL with 7 days of growth in liquid medium. IAA production has been reported for various pathogenic or non-pathogenic *Streptomyces* strains. However, there is a lack of studies aiming to understand the biochemical origin of this hormone by these bacteria. By associating molecular screening of the *iaaM gene* and HPLC analysis, Lin & Xu (2013) [41] showed the existence of a biosynthetic pathway of indol-3-acetamide (IAM), identified as a key metabolic intermediate for IAA anabolism which has tryptophan as a precursor. The authors concluded that this aromatic amino acid availability in the media is essential for the microorganism to be able to produce the auxin, which has a fundamental role in the mutualistic interaction endophyte-plant. Four actinobacterial strains, FA8N2, FA8K4, FA9K1 and NIM1, were able to solubilize zinc [Table-1]. The SI values ranged from 1.28 to 1.98, considering both time periods (7 and 14 days), with isolate FA9K1 standing out among other isolates (1.97 for 7 days and 1.98 for 14 days). Only three isolates, FA8N1.2, MC_{30} and NIM_3, presented positive results in the phosphate solubilization tests [Table-1]. In both culture media (NBRIP and PKV), there was no significant statistical difference between the SI values for these three strains for the same growth period. The strain MC_{30} exhibited the solubilization halo in NBRIP agar only with 14 days of cultivation. In the PKV medium, the isolate NIM3 did not present visible solubilization halos throughout all the cultivation time. Under these circumstances, the NBRIP medium was chosen for the phosphate solubilization assays in liquid medium.

Table-1 *Zinc and phosphate SI values obtained from the actinobacterial isolates*

Isolates	Zinc		Phosphate			
			NBRIP		PKV	
	7 days	14 days	7 days	14 days	7 days	14 days
FA8N1.2	–	–	1,26 [a]	1,29 [a]	1,21 [a]	1,34 [a]
FA8N2	1,43 [b]	1,60 [b]	–	–	–	–
FA8K4	1,17 [b]	1,37 [b]	–	–	–	–
FA9K1	1,97 [a]	1,98 [a]	–	–	–	–
MC_{30}	–	–	–	1,12 [a]	1,19 [a]	1,29 [a]
NIM1	1,28 [b]	1,45 [b]	–	–	–	–
NIM3	–	–	1,19 [a]	1,10 [a]	–	–

(–) indicates isolates with negative results in the in vitro screening assays. Average means in the same table column followed by the same letter do not differ statistically among them at a significance value of 5% by the Tukey test.

The results of the quantification of phosphate solubilization are shown in [Fig-2]. The concentration of soluble phosphate in the culture supernatants ranged from 66.2 to 113.78 µg/mL with 5 days of growth, and from 59.32 to 136.68 in the time interval of 10 days. At the final sampling period (14 days), the phosphate concentration ranged from 63.26 to 153.07 µg/mL. The strain NIM3 was the one with better results in all the three evaluated periods. Only the isolates NIM_3, MC_{30} and FA9K1 were able to grow in the nitrogen-free medium in all subcultivation steps.

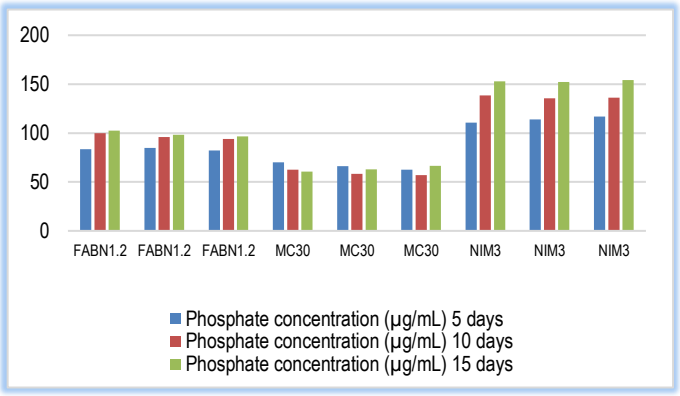

Fig-2 Phosphate solubilization by the actinobacterial isolates grown on NBRIP broth in different time periods (5, 10 and 14 days). Error bars indicate the standard deviations of means obtained from triplicates.

In a study conducted by Oliveira *et al*. (2010) [42], it was isolated a total of 70 endophytic actinobacterial strains from tomato which were screened regarding the IAA production, phosphate solubilization, siderophore secretion and antagonism against phytopathogenic fungi. It was observed that 72.1% of isolates produced the phytohormone, 16.2% solubilized phosphate, 86.6% were able to liberate siderophore and one strain, *Streptomyces* sp. R18(6) presented better results in the inhibition assays against phytopathogens. In another work, Palaniyandi *et al*. (2013) [43] accomplished the isolation and evaluation of PGP of actinobacteria associated with yam rhizosphere and among the 29 isolates, 96% produced IAA and 48% solubilized phosphate, with the supernatant of one isolate, YRA005, showing the capacity to solubilize 120 µg/mL with 6 days of growth. This was similarly found to the endophytic isolate NIM3 in our study, once this strain was able to solubilize almost this same phosphate concentration after 5 days in similar cultivation conditions from this previous work. Nitrogen fixation is one of the most wanted aspects for a microbial inoculant. The ability to fix N_2 in congruence with other PGP properties suggest that the isolates NIM_3 and FA9K1 are the most suitable to the in vivo plant growth promotion assays, which are presently being standardized. Both strains showed the metabolic expression of direct mechanisms of plant growth promotion in the in vitro screening assays and it is well known that

endophytic and rhizosphere actinobacteria that stand out concerning these properties stimulate extensively plant development [44]. It is important to highlight that the number of nitrogen-fixing bacteria may have been underestimated. Gtari *et al*. (2012) [45] argued that even for *non-Frankia* actinobacteria, the initial screening for nitrogen fixation should consider their slow growth, what was addressed in the present study considering the evaluated incubation time, as well the molecular detection of coding genes for the protein domains for nitrogenase (*nif*), since members of this phylum could present unique and not yet described nitrogen fixation mechanisms not easily detectable by classical phenotypic approaches. Three isolates (MC_{30}, NIM_1 and NIM_3) showed positive results for the ammonia production in microplate screening assay and none strain were able to generate HCN according to the applied method [Table-2]. The isolates NIM1 and FA9K1 antagonized the growth of *F. moniliforme* by the plate-screening assay, with growth rate inhibition of 24.31% and 27.84%, respectively. There is no significant statistical difference with respect to rate inhibition means.

Table-2 *Screening for PGP traits (ammonia and HCN production, antagonism against F. moniliforme and cellulase production) in the actinobacterial isolates*

Isolate	Ammonia	HCN	Inhibition of *F. moliniforme* (%)	Cellulases (EI)*
FA8N1.2	–	–	–	3,33 ab
FA8N2	–	–	–	1,51 d
FA8K4	–	–	–	3,79 a
FA9K1	–	–	24.31 a	3,21 ab
MC_{30}	+	–	–	2,45 c
MC_{38}	–	–	–	1,32 d
MC_{45}	-	–	–	3,37 ab
NIM_1	+	–	27.84 a	2,67 bc
NIM_3	+	–	–	2,30 c

* EI: enzymatic index; (+): positive results; (–): negative results. Average means in the same table column followed by the same letter do not differ statistically among them at a significance value of 5% by the Tukey test.

Plants consume a multitude of macro and micronutrients throughout their growth and development. Against this background, ammonia production along with solubilization of K^+, Fe^{3+}, PO_4^{2-} e Zn^{2+} by endophytes aid in a minor energetic cost to these hosts to keep the basal levels of these micronutrients, specially under stressful conditions. These properties also are commercially interesting since would drastically reduce the use of non-renewable and expensive chemical fertilizers [46,47]. When analyzing the potential of soil and endophytic actinobacteria as plant growth promoters, Kaur *et al*. (2013) [48] verified the ammonia production for 13 from the 60 strains and this direct mechanism is associated with the exoenzyme secretion and antifungal secondary metabolites (indirect mechanisms) by these streptomycetes for the preparation of an ideal inoculant [49]. Chen *et al*. (2016) [50] tested two endophytic *Streptomyces* strains against the phytopathogen *Sclerotinia sclerotiorum* by in vivo assays in growth chambers and in experiments in the greenhouse environment, using lettuce (*Lactuca sativa* var. capitata) as the plant model. The authors noticed in different proportions a distinguishable reduction of phytopathogen infection incidence with 7 days of incubation, what enabled the sporulation of streptomycetes isolates and the aerial mycelium formation and, consequently, secondary metabolites production with the biocontrol activity. It was also confirmed root colonization of the lettuce variety, what could be related to the primary need of colonization by the symbiont for later suppression of phytopathogen development. Singh & Gaur (2017) [51] observed that endophytic *Streptomyces* strains were capable to induce the expression of enzymes associated with oxidative stress and phenolic compounds related to the antagonistic activity against the phytopathogen *Sclerotium rolfsii*, what was confirmed by electron microscopy and in vivo chickpea development assays. In our study, the presumable mechanism by which the isolate NIM1 was able to inhibit *F. moniliforme* may have been both production of secondary metabolites and extracellular enzymes once none of the strains generated HCN, and this gas release consists in one of the indirect mechanisms of plant growth promotion, particularly for biocontrol of infections caused by airborne fungi [52]. All isolates presented cellulolytic activity in the plate-screening assay. The EI values ranged from 1.32 to 3.79, with the higher EI being found for the isolate FA8K4 [Table-2]. Actinobacteria, mainly those from the *Streptomyces* genus, are notoriously known by the production of all enzymes from the cellulolytic complex [53]. Hata *et al*. (2015) [54] clustered endophytic *Streptomyces* strains based on certain traits as hydrolytic enzymes secretion, direct antibiosis against phytopathogens and auxin production and analyzed the most promisor strains for inoculant development, once they aggregate all required in vitro features expected for an endophyte isolate with this aim. In the present work, the rhizosphere actinobacterial morphotypes stood out as for the cellulolytic activity, what could be explained by the need for release of these hydrolases to allow the rhizosphere colonization. The isolate NIM3 achieved the best performance in vitro assays for auxin production and phosphate solubilization, besides being able to grow in the NBRIP medium. This endophytic strain was then selected for molecular identification. Furthermore, it was included the isolate FA8K4, which presented the higher EI value for cellulolytic activity, and the isolate FA9K1, that was able to solubilize zinc, grow in the nitrogen-free medium and inhibit *F. moniliforme*. The results of the BLASTn search based on 16S rRNA gene sequences of these three actinobacterial strains and their closest relatives are presented in [Table-3]. Partial 16S rRNA gene sequences of NIM3, FA8K4 and FA9K1 strains were deposited on GenBank database under the respective accession numbers KY794411, KY794418 and KY793906 [Table-3]. According to 16S rRNA gene sequencing, all three selected isolates were identified to the genus *Streptomyces* with the phylogenetic tree shown in the [Fig-3]. The strain FA8K4 demonstrated a similarity of 99% with *Streptomyces lannesis*, what was supported by the high bootstrap value (82%) in the clade where the samples clustered in the phylogenetic tree. Unfortunately, although the isolates FA9K1 and NIM3 are related to other *Streptomyces* strains, there was not possible to achieve a more delineated phylogenetic inference between rRNA sequences from these sequences and other retrieved from the database reflected by the low bootstrap values, what could indicate that these strains may correspond to not yet described species.

Table-3 Identification of the selected actinobacterial isolates based on 16S rRNA gene sequencing.

Isolate	Closest species	Identity	Accession number of the closest species	Accession numbers of the isolates' 16S rRNA gene sequences
FA8K4	*Streptomyces lannensis*	99%	NR_113181.1	KY794418
FA9K1	*Streptomyces* sp.	99%	NR_041099.1	KY793906
NIM3	*Streptomyces* sp.	99%	NR_041066.1	KY794411

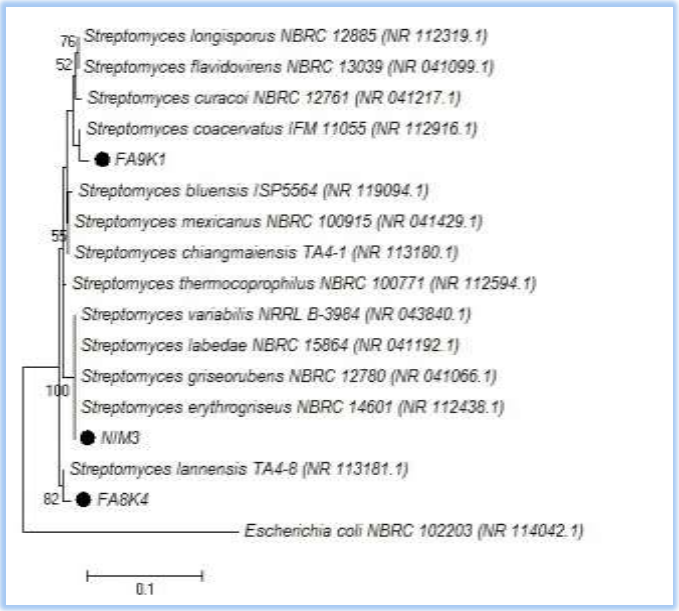

Fig-3 Phylogenetic tree based on 16S rRNA gene sequences from the actinobacterial isolates from different plant species and closest *Streptomyces* strains obtained from the NCBI database. Phylogeny was inferred adopting the neighbor-joining distance method according to the Jukes-Cantor model and number of bootstrap values in 1000 replicates. *Escherichia coli* NBRC 102203 (NR 114042.1) was used as the outgroup representative.

Among the characterized plant growth-promoting actinobacteria, the genus *Streptomyces* has been more frequently reported, independently the origin, as endophytes [42,40,51] or rhizobacteria [43,55]. Kruasuwan & Thamchaipeinet (2016) [56] isolated bacterial endophytes from sugarcane roots and after the in vitro screening of PGP characteristics found a potential isolate, *Streptomyces* sp. GKU 895. This strain solubilized phosphate, produced IAA, 1-aminocyclopropane1-carboxylate (ACC)-deaminase and siderophores. Additionally, it also expressed antagonism towards *Bacillus cereus* ATCC 11778 and two sugarcane root pathogens, *Colletotrichum falcatum* and *F. moniliforme*, beyond confirmed enhancement of this cultivar development in pot setup assays. This potency was confirmed after its genome was completely sequenced and coding genes for ACC-deaminase, and those related to IAA biosynthesis, phosphate solubilization, root colonization as also some involved in secondary metabolism [57]. Recently, Dias *et al*. (2017) [58] demonstrated that six rhizobacterial strains of *Streptomyces* exhibited a series of desirable traits for their use as plant growth-promoting bacteria, including the production of siderophores and ACC deaminase, phosphate solubilization and generation of volatile organic compounds (VOC). The authors confirmed this potential differentially for these strains through in vivo assays with tomato as model, verifying positive responses for all evaluated growth parameters when compared to the control as well a decrease of incidence of soft rot disease, besides a clear stimulation of plant defense-related enzymes, particularly for one actinobacterial strain which they suggested could be used as an elicitor for resistance against the tested phytopathogen. In the actual work, the rhizoactinobacteria did not exhibit several traits compared to the *Streptomyces* strains from these two former cited studies, although this does not mean that we should ignore the hidden potential of these isolates. One evident limitation from the present study was the lack of other important phenotypic assays, like those for ACC-deaminase and siderophore production, and for other biocontrol and host, colonization related- exoenzymes, as chitinase and glucanase. The molecular detection of genes from the *nif* operon is also one of our primary future perspectives, once it consists of a more accurate confirmation of the nitrogen-fixing property instead of successive subcultivation steps in the nitrogen-free medium. Moreover, we had some obstacles to standardize in vivo assays with rice as the initial model, probably due to the slower growth of these bacteria and, therefore, it is necessary to better adapt our pre-established protocol.

Conclusion

The selected plant-associated actinobacterial strains demonstrated a broad set of PGP traits of interest. Three isolates, one endophyte and two isolated from rhizosphere, stood out differentially according to the tested activities and were identified based on the 16S rRNA gene sequencing as *Streptomyces* sp. Broadly, the obtained results are encouraging to the continuity of investigations, mainly molecular screening for genes associated with these traits and the greenhouse experiments with various plant species. In the context of the search of an effective microbial inoculant aggregating all advantages that a streptomycete strain naturally possess, these actinobacterial isolates may comprehend good candidates.

Application of research: The present study could be employed for the development of stable and with reliable actinobacterial inoculants.

Research category: Agricultural Microbiology

Abbreviations: analysis of variance (ANOVA), carboxymethylcellulose (CMC), cyanide hydrogen (HCN), Enzymatic Index (EI), indole-3-acetic acid (IAA), National Botanical Research Institute's phosphate growth medium (NBRIP), National Center for Biotechnology Information (NCBI), nitrogen free (NF), nitrogen-free bromothymol blue (NFb), Pikovskaya medium (PKV), plant growth promoting (PGP), potato dextrose agar (PDA), Solubilization Index (SI), Trytic Soy Agar (TSA), yeast extract-tryptone (YT).

Acknowledgment / Funding resource: The authors would like to thank the collection of rhizospheric soil, Centro de Tecnologia Canavieira (CTC) for donating the *F. moniliforme* strain used in antagonism assays. AAR and MVFA were recipients of Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) scholarships.

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Author Contributions: IDAR conceived the study, standardized assay conditions and produced the main work results, along with BFRO. BFRO wrote the manuscript. AAR supervised the molecular identification and was responsible for phylogenetic analyses and 16S rRNA gene sequences submission to NCBI. MVFA isolated the actinobacterial strains from sugarcane rhizosphere. JDGV supervised the study. All authors read and reviewed the final manuscript.

Study area / Sample Collection: Endophytic and rhizosphere bacterial strains / Jalles Machado, Goiasa-Goiatuba Álcool Ltda, Goiatuba, Goianésia, GO, Brazil

Cultivar / Variety name: Sugarcane, *Hyptis suaveolens* (L.) Poit., *Azadirachta indica* A. Juss

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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