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Original Research

Biodiversity and Antifungal Activities of Amazonian Actinomycetes Isolated from Rhizospheres of *Inga edulis* Plants

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Abstract

Background: Actinobacteria are major producers of antibacterial and antifungal metabolites and are growing their search for substances of biotechnological interest, especially for use in agriculture, among other applications. The Amazon is potentially rich in actinobacteria; however, almost no research studies exist. Thus, we present a study of the occurrence and antifungal potential of actinobacteria from the rhizosphere of *Inga edulis*, a native South American plant and one that is economically useful in the whole of the Amazon. **Methods**: Among the 64 actinobacteria strains isolated from the rhizosphere of three *Inga edulis* plants, 20 strains were selected and submitted to dual-culture assays against five important phytopathogenic fungi and morphological and 16S rRNA gene analyses. Two strains, LaBMicrA B270 and B280, were also studied for production curves of metabolic extracts and antifungal activities, including their minimum inhibitory concentration (MIC) against phytopathogenic fungi. **Results**: Among the 20 strains, 90% were identified as *Streptomyces* and 10% as *Kitasatospora*. All the strains showed antagonisms against two or more of five phytopathogens: *Corynespora cassiicola*, *Colletotrichum guaranicola*, *Colletotrichum* sp., *Pestalotiopsis* sp., and *Sclerotium coffeicola*. *Streptomyces* spp. strains LaBMicrA B270 and B280 were active against phytopathogens of the guarana plant (*Paullinia cupana*). Furthermore, AcOEt/2-propanol 9:1 extract from the 10-day strain LaBMicrA B280 cultured medium presented activity against all the phytopathogens tested, with a minimum inhibitory concentration of 125 µg/mL. **Conclusions**: The results revealed various actinomycetes in three rhizospheres of *I. edulis* in the Amazon and the high potential of metabolic extracts from some of these bacterial strains against phytopathogenic fungi that destroy numerous crops.

Keywords: rhizobacteria; actinobacteria; biodiversity; agricultural development; phytopathogen biocontrol

1. Introduction

In the last decade, prospections of new isolates of actinomycetes have been carried out in diverse ecosystems on the planet. Many of these actinomycetes have been revealed as sources of promising natural products (NPs) with diverse biological activities or other biotechnological applications [1–4]. These searches for new actinomycetes and their metabolites, among other reasons, result from the scarcity of agricultural resources to stop the spread of multi-resistant phytopathogens and other plant pests that destroy plantations globally. These resistant phytopathogens and pests harm agricultural production and have become more widespread due to the uncontrolled and indiscriminate large-scale use of biocides employed to meet the growing demand for food in the 20th and 21st centuries [5–10].

Widely distributed, actinomycetes have essential ecological activities for the planet and living beings. For example, they help plants through biological nitrogen fixation, organic matter recycling, secretions of phytohormones, antibiotics, and lytic enzymes, and as biocontrol agents of phytopests [11,12]. In symbiotic systems, actinomycetes benefit plants through activities such as the above, and in turn, plants release nutrients, which feed actinomycetes. The activities of actinomycetes in favor of plants make them helpful as biocontrol agents and natural fertilizers [6,9].

The potential of actinomycetes against phytopathogenic fungi has attracted the attention of many researchers seeking new antifungals and more sustainable alternatives for agriculture [7]. In fact, among microorganisms, actinomycetes stand out due to their production of 70% of the antimicrobials available to medicine and

agriculture [13,14]. Notably, the genus *Streptomyces* is the largest producer of the tens of thousands of NPs assigned to the actinomycetes [15–17]. However, other genera, including *Micromonospora* and *Kitasatospora*, have been gaining increasing attention in the search for new NPs [18,19], especially antifungal compounds, such as macrolides, the best-known antimicrobial class [20–22].

Regarding biodiversity, the phylum Actinomycetota has more than 200 genera, among which are *Streptomyces*, the largest, with approximately 700 species [23], and its closely related *Kitasatospora*, the second largest, with 33 species, in the Streptomycetaceae family [18,24,25]. In general, phenotypically, bacteria belonging to these genera are Gram-positive, have aerial mycelia, segments with a cylindrical, spherical, or ovoid shape, and spiral (or partially) or linear spore chains [15,18,25,26]. Their genomes are rich in guanine–cytosine, with sizes varying between 6 and 10 million nucleotide base pairs (bp), and have diversified biosynthetic gene clusters (BGCs) related to the synthesis of NPs: 8 to 83 BGCs for Streptomyces and 22 to 50 for *Kitasatospora* [15,18,25,27,28].

In the context summarized above, we highlight the search for new strains of actinomycetes from the most varied environments, as well as for bioactive metabolites from strains of this bacterial Phylum, aiming to meet, among other demands, the need for new phytopathogenic antifungals of agricultural interest. Although investigation of actinomycetes occurs worldwide, there are almost no studies on those from the Amazon. Thus, here we report part of a study of a sample of actinomycetes isolated from the rhizosphere of three individuals of *Inga edulis* (Fabaceae family), a plant of agroecological importance for the Amazon [29–31]. The *Inga edulis* plants are on the campus of the Federal University of Amazonas (UFAM), the largest urban forest fragment in the Amazon and, at 670 hectares, one of the largest in the world. This forest fragment is well preserved and potentially a representative environment of a part of the countless and almost unexplored Amazonian microbial diversity. In this study, we evaluated the diversity and the antifungal potential of a sample of twenty actinomycetes against fungal phytopathogens of regional and global interest. Among these fungi, we highlight the phytopathogens of the guarana plant (*Paullinia cupana*), one of the best sources of caffeine, and *Sclerotium coffeicola*, a phytopathogen recognized worldwide for affecting hundreds of plants of agricultural importance. However, this study is the first important step toward revealing a small sample of actinomycetes found in rhizospheres of *I. edulis* and investigating their potential applications against phytopathogenic fungi of agricultural interest.

2. Materials and Methods

2.1 Soil Sample Collections

Samples were collected from the rhizospheres of *I. edulis* plants at three different sites on the UFAM campus (site 1: 3°05′19.4″ S 59°58′00.5″ W; site 2: 3°05′20.8″ S 59°57′57.5″ W; site 3: 3° 5′19.06″ S 59°57′56.26″ W) (**Supplementary Material—SM1**). At each site, three rhizosphere samples were collected at 40 cm from the root collar of the plant and 20 cm below ground in the piliferous region of the roots. To ensure that only rhizosphere samples were collected, pieces of the plant roots were cut, excess soil was removed, and only topsoil that adhered to the surface layer of the root was sampled, taking care not to remove pieces of the surface root itself or pieces of its piliferous structures. Sterilized materials were used in all the sample collections: 50 mL Falcon™ tubes, metal spatulas, and plastic bags to transport the samples. The samples were taken to the Laboratory of Bioassays and Microorganisms of the Amazon (LaBMicrA) - UFAM and stored at –4 °C.

2.2 Obtaining Actinobacterial Strains

Twenty-four hours after the collection, in a biological safety cabinet (Filterflux® Class II A1, Filterflux Comércio de Equipamentos para Laboratório Ltda., Piracicaba, Brazil), 1 g of soil from each site was dissolved in 5 mL of sterilized distilled water containing 0.02% Tween-20. Each suspension was subjected to serial dilution with 0.8% saline. From the 10^{-3}, 10^{-4}, and 10^{-5} dilutions, 50 µL was spread in triplicate in 90 × 15 mm Petri dishes containing International Streptomyces Project 2-oat (ISP2-oat) culture medium (for 1 L: 10 g of oats, 14 g of agar, 10 g of malt, 4 g of yeast extract, and 4 g of glucose) and ISP2-starch culture medium (for 1 L: 10 g of starch, 14 g of agar, 10 g of malt, 4 g of yeast extract, and 4 g of glucose), to which 100 µg/mL of the antifungal nystatin and 100 µg/mL of the antibacterial tetracycline were added. These ISP media are modifications of ISP2 through the addition of oatmeal and starch, respectively. ISP2 is widely used, mainly for actinomycetes cultures, and is from the well-known International *Streptomyces* Project [32]. The culture dishes were incubated at 28 ± 2 °C, and the strains of actinomycetes that germinated were collected daily and purified for 30 days. Actinobacteria strains were cultured for ten days in the same solid media, from which five 0.6 × 0.6 cm^2 pieces containing each strain were preserved in three 1.5 mL cryogenic microtubes (Thermo Fisher Scientific inc., Rochester, NY, USA) containing 20% glycerol. The microtubes were preserved in a freezer at –80 °C.

2.3 Microscopic Study

The isolated and purified actinobacteria were cultured on coverslips inclined to around 45° for ten days or more according to the morphological development of each strain and stained using the Gram staining method (LB Laborclin®). All the stained coverslips were observed under an optical microscope with a 100× objective (Carl Zeiss optical microscope, coupled to Zen AxioCamER - Zen lite 2012 photo documenter) (**Supplementary Material—SM2**). For electron microscopy, the *Streptomyces* spp. LaBMicrA B270 and B280 strains were cultivated in Petri

dishes containing ISP2 culture medium at 28 °C for ten days. Afterward, 0.5 cm diameter discs of the medium containing the cultured strains were analyzed using a scanning electron microscope (SEM) (JSM IT500HR, JEOL Ltd., Tokyo, Japan) (for details, see **Supplementary Material—SM3**) [33]. The images were obtained at voltages of 10 and 15 kV.

2.4 Selection of Strains and Morphological Study

The isolates were cultivated in ISP2 medium and grouped according to their macromorphological characteristics and the pigmentation of the growth medium in the Petri dishes (front and back). After grouping, one strain from each group with a few strains and two from larger groups were selected to continue the studies. After cultivation in the ISP2 culture medium, the selected strains were described morphologically according to the manuals of Shirling and Gottlieb [26]. The strains were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the code AC1746C.

2.5 Molecular Characterization and Phylogenetic Analysis

For DNA extraction, the selected strains of actinobacteria were cultured in 125 mL Erlenmeyer flasks containing 30 mL of potato, dextrose, and yeast extract (PDY) medium [34] at 28 ± 2 °C and 120 rpm for 72 h. The extractions followed the protocol of the Zymo Research Fungal/Bacterial DNA MicroPrep™ kit (Zymo Research, Irvine, CA, USA). The polymerase chain reaction (PCR) was performed using 20 ng of DNA, the PCR kit Illustra™ PuReTaq Ready-to-Go™ PCR beads (GE Healthcare, Chicago, IL, USA) and the primers 27F 5′ AGAGTTTGATCMTGGCTCAG 3′ and 1492R 5′ CGGTTACCTTGTTACGACTT 3′ [35], with a final volume of 25 µL, in a 96-well thermal cycler (Applied Biosystems) [35]. The samples were sequenced using the Sanger method and the BigDye Terminator kit (Applied Biosystems, Waltham, MA, USA).

Identification was initially performed according to the similarity based on the pairwise alignment algorithm of the 16S rRNA region with type species deposited in the EzBioCloud database (https://www.ezbiocloud.net/) [36]. Maximum likelihood analysis was also performed from the alignment of the sequences on the IQ-TREE platform (http://iqtree.cibiv.univie.ac.at/) using 1000 replicates and the evolutionary model TN+F+I+G4. *Streptacidiphilus albus* NBRC100918 was used as an outgroup. The tree topology and support values in the bootstrap were visualized using the iTOL platform (https://itol.embl.de/) and manually edited using CorelDraw software version 2020 software (version 2020, Corel Corporation, Ottawa, Canada).

2.6 Phytopathogens Selected to Evaluate the Antifungal Potential of the Actinobacteria Strains

To evaluate the antifungal potential of the selected actinobacteria strains, five phytopathogens were chosen, which were kindly provided by the Laboratory of Microbiology and Phytopathology of the Department of Agricultural Sciences at UFAM: *Colletotrichum* sp. (habanero-type pepper-ISO01) isolated from leaf tissue lesions of habanero-type pepper (*Capsicum chinense*) plants; *Col. guaranicola* (guarana plant-P01) and *Pestalotiopsis* sp. (guarana plant-3002R2) isolated from leaf tissue lesions of different guarana (*Paullinia cupana*) plants; *Corynespora cassicola* (tomato plant-ISO079) isolated from leaf tissue lesions of tomato (*Solanum lycopersicum*) plants; *Sclerotium coffeicola* (Mango tree-M01) isolated from leaf tissue lesions of mango (*Mangifera indica*) trees. These plants, especially guarana, are economically valuable to the Amazon region since they represent rich sources of caffeine.

2.7 Screening Using a Dual Culture Technique

After being reactivated in ISP2, each actinomycete strain was inoculated in a line crossing the center of the Petri dishes containing ISP2 and incubated at 28 ± 2 °C. On the third day, 5 mm diameter agar discs containing each phytopathogen strain previously cultivated for five days in PDA were inoculated at two points in the same test dishes, one centimeter from the edges, and perpendicularly to the line of the actinomycete strain. One centimeter from one of the pathogen inocula and parallel to the actinomycete line, a 10 mm wide strip of culture medium was removed from one edge to the other to avoid the metabolic diffusion through the medium and to check the possible production of volatile antifungal substances. Pathogen control dishes were created in the same way; however, without the actinomyces inocula. Dual culture and control plates were incubated for ten days. Then, the growth of the pathogens in the control and double culture plates was checked. All the assays were carried out in triplicate, and the fungal phytopathogen inhibition zone averages were calculated (**Supplementary Material—SM4**). Edington's formula was applied to this average (I = [(FGC-FGT)/FGC] × 100, where I = percentage of inhibition; FGC = fungal growth in control; FGT = fungal growth in treatment) [37], and the GraphPad Prism program (version 8.0.1, GraphPad Software, Inc., San Diego, CA, USA) was used to construct a heatmap with the percentage of mycelial inhibition.

2.8 Monitoring the Production of Extracts

Streptomyces spp. strains LaBMicrA B270 and B280, selected from the dual culture assays, were cultured for 96 h in ISP2 (1.5% agar medium) in Petri dishes, from which two 5 × 5 mm agar plugs were inoculated in an Erlenmeyer flask of 250 mL containing 120 mL of ISP2, employing a total of 36 flasks. The strains were cultivated at 120 rpm and 28 ± 2 °C. Each cultivated strain was sampled at 12 h, 24 h, 36 h, 48 h, 72 h, 120 h, 168 h, 216 h, 264 h, 312 h, 360 h, and 408 h, each time from three Erlenmeyer flasks. In each sampling, glucose consumption and pH were measured with indicator strips (urinal analysis), and dry masses

of the cell and culture medium extracts were weighed. The culture medium extracts were also subjected to antifungal assays.

The cells and cultured medium from each flask were separated by centrifugation (Eppendorf® centrifuge, Leipzig, Saxony, Germany) at 4000 rpm for 15 min, and then the cells were washed three times with 10 mL of distilled water. The cells were then vortexed and extracted with 5 mL of AcOEt/MeOH 1:1 for 24 h. The cultured medium was partitioned three times, with 40, 30, and 30 mL of AcOEt/2-propanol 9:1, which were then pooled. After evaporation of the solvents, the extracts were weighed, dried in a desiccator with activated silica, and weighed again until they reached a constant weight to establish the average triplicate values of each point. After this, the three extracts from each time point were redissolved, collected, concentrated, and weighed. Then, the culture medium extracts were stored at 4 °C until the day of the bioassays.

2.9 Monitoring the Antifungal Activities

The extracts from the cultured medium of the *Streptomyces* spp. LaBMicrA B270 and B280 strains of the twelve samplings described above were tested against the five target phytopathogens. Extracts were dissolved in 200 µL of dimethyl sulfoxide (DMSO) and mixed with 800 µL of autoclaved distilled water. New solutions were made from these solutions by diluting aliquots corresponding to 20 mg of each dry extract in sterilized distilled water, reaching a final volume of 1 mL. As a positive control, 20 mg of nystatin was suspended in 1 mL water. Assays of the extracts and controls were performed in triplicate in cell culture plates with 12 wells, each well contained: tests: 0.950 mL PDAY (potato, dextrose, agar, and yeast extract), 50 µL extract + phytopathogen; negative controls: 1 mL PDAY + phytopathogen; DMSO controls: 1 mL PDAY + 50 µL 20% aqueous DMSO; positive controls: 0.950 mL PDAY, 50 µL nystatin solution + phytopathogen. The mixtures of the extracts or nystatin with the culture medium, at a final concentration of 1mg/mL, were homogenized at approximately 40 °C. Each phytopathogen was inoculated in the wells using 5 mm diameter discs from its previous culture in Petri dishes containing PDAY culture medium [34] at 28 ± 2 °C for 96 h. After ten days of incubation at 28 ± 2 °C, the activity of each test was considered negative for any growth or positive for no growth.

2.10 Minimum Inhibitory Concentration Assays

After the results of the growth curves and bioassays, strains LaBMicrA B270 and B280 were cultured for ten days, and new extracts were prepared as described in Section 2.8. This time, however, to also be assayed, the aqueous fraction of the cultured medium was lyophilized after partitioning with AcOEt/2-propanol 9:1. The concentrated organic extracts and lyophilized aqueous fractions were stored at –18 °C until the time of the minimum inhibitory concentration (MIC) test against the target phytopathogens. For the MIC test, the organic extracts and aqueous fraction from each actinomycete strain were each mixed at approximately 40 °C in triplicate with PDAY in wells of 12 well-cell culture plates to final concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 µg/mL. Afterward, each phytopathogen was inoculated and tested in the wells in a manner similar to the previous item. Again, the activity for each test was considered negative for any growth or positive for no growth.

3. Results

3.1 Morphological and Genotypic Diversity

This study obtained 64 actinomycetes strains from the rhizospheres of three *I. edulis* individuals in the three sample sites. Site S2 stood out for yielding 30 isolates (46.9%), followed by S1 with 19 isolates (29.7%) and S3 with 15 isolates (23.4%). Following the procedure described in Section 2.4, grouping the 64 strains resulted in 16 morphogroups, and 20 strains were selected for the present study. All 20 strains showed typical morphological characteristics for actinomycetes according to the manuals of Shirling and Gottlieb [26] (**Supplementary Material—SM2**). Molecular identification based on the *16S rRNA* gene revealed that 18 strains belonged to the genus *Streptomyces* (90%), while two strains belonged to the genus *Kitasatospora* (10%) (Table 1).

The lowest sequence similarity was observed for the *Streptomyces* sp. LaBMicrA B278 strain, which showed 94.51% similarity with *S. misionensis* DSM 40306 and *S. cupreus* PSKA01. A similarity of 100% was observed between *Streptomyces* sp. LaBMicrA B372 and *S. acidicola* K1PN6; *Streptomyces* sp. LaBMicrA B310 and *S. pulveraceus* LMG 20322; *Streptomyces* sp. LaBMicrA B317 and *S. jiujiangensis* JXJ 0074; *Streptomyces* sp. LaBMicrA B322 and *S. rochei* NRRL B-2410; *Streptomyces* sp. LaBMicrA B290, *S. murinus* NBRC 12799, and *S. graminearus* NBRC 15420. However, the 16S sequences did not allow us to identify the species of 8 of the 20 strains evaluated in this study, such as S*treptomyces* sp. LaBMicrA B270, which showed the same similarity (98.36%) with four different species (*S. murinus* NBRC 12799, *S. lanatus* NBRC 12787, *S. fractus* MV32, and *S. graminearus* NBRC 15420) (Table 1).

For a better resolution in the identification, a phylogenetic analysis was performed using the 20 strains obtained in this study and the closely related species identified in EzBioCloud through the similarity analysis. The isolates belonging to *Streptomyces* and *Kitasatospora* formed two distinct monophyletic clades, confirming the genus status for the isolates used in this study (Fig. 1). Eleven strains (LaBMicrA B267, LaBMicrA B272, LaBMicrA B278, LaBMicrA B282, LaBMicrA B313, LaBMicrA B314, LaBMicrA B317, LaBMicrA B318, LaBMicrA B2319, LaBMicrA B322 and LaBMicrA B325) were clustered according to the type species that showed the highest similarity in the EzBioCloud analysis (Table 1). As ex-

Table 1. Similarity-based identification of the *16S rRNA* region in actinomycetes from the rhizospheres of *Inga edulis*, located on the Federal University of Amazonas campus, using the EzBioCloud database.

Strains and LaBMicrA code	GenBank accession code	EzBioCloud Taxon Name	Accession number	Similarity (%)
Kitasatospora sp. B282	OR725990	*K. acidiphila* MMS16-CNU292^T	MG189374	100
Kitasatospora sp. B313	OR726028	*K. arboriphila* HKI 0189^T	AY442267	98.70
Streptomyces sp. B267	OR724644	*S. silvae* For3^T	MW479423	99.34
Streptomyces sp. B270	OR724732	*S. murinus* NBRC 12799^T	AB184155	98.36
		S. lanatus NBRC 12787^T	AB184845	
		S. fractus MV32^T	FJ857947	
		S. graminearus NBRC 15420^T	AB184667	
Streptomyces sp. B271	OR724661	*S. murinus* NBRC 12799^T	AB184155	96.66
		S. graminearus NBRC 15420^T	AB184667	
Streptomyces sp. B272	OR725986	*S. acidicola* K1PN6^T	MN319555	100
Streptomyces sp. B278	OR724700	*S. misionensis* DSM 40306^T	FNTD01000004	94.51
		S. cupreus PSKA01^T	SJACMSF010000162	
Streptomyces sp. B280	OR724701	*S. graminearus* NBRC 15420^T	AB184667	98.83
		S. murinus NBRC 12799^T	AB184155	
Streptomyces sp. B301	OR724719	*S. durocortorensis* RHZ10^T	MW582863	96.93
Streptomyces sp. B304	OR725980	*S. pulveraceus* LMG 20322^T	AJ781377	98.70
Streptomyces sp. B310	OR726013	*S. pulveraceus* LMG 20322^T	AJ781377	100
Streptomyces sp. B314	OR726569	*S. olivaceus* NRRL B-3009^T	JOFH01000101	99.74
		S. pactum NBRC 13433^T	AB184398	
Streptomyces sp. B317	OR733339	*S. jiujiangensis* JXJ 0074^T	KF938657	100
Streptomyces sp. B318	OR730983	*S. similanensis* KC-106^T	AB773850	95.45
Streptomyces sp. B319	OR730984	*S. anandii* NRRL B-3590^T	AY999803	97.92
		S. pimonensis^{T}RM75549^T	MW479154	
		S. fumanus NBRC 13042^T	AB184273	
Streptomyces sp. B322	OR730985	*S. rochei* NRRL B-2410^T	MUMD01000370	100
		S. katrae NRRL ISP 5550^T	JZWV01000648	
Streptomyces sp. B325	OR730986	*S. racemochromogenes* NRRL B-5430^T	DQ026656	97.66
		S. polychromogenes NBRC 13072^T	AB184292	
Streptomyces sp. B289	OR733333	*S. griseoflavus* LMG 19344^T	AJ781322	99.59
Streptomyces sp. B290	OR733337	*S. murinus* NBRC 12799^T	AB184155	100
		S. graminearus NBRC 15420^T	AB184667	
Streptomyces sp. B299	OR734917	*S. griseoflavus* LMG 19344^T	AJ781322	99.59

Note: Similarity is based on the pairwise sequence alignment algorithm.

pected, strains LaBMicrA B270, LaBMicrA B271, LaBMicrA B280, and LaBMicrA B290 that show similarity to *S. murinus* and *S. graminearus* (Table 1) form a clade closely related to them. A similar result can be observed for the LaBMicrA B304 and LaBMicrA B310 strains, which show similarity to *S. pulveraceus* LMG 20322 and form a clade with it. Differently, LaBMicrA strains B289 and B299 did not group closely with *S. griseoflavus*, which showed high similarity in the EzBioCloud database.

3.2 Antifungal Activities of the 20 Strains against Phytopathogens

In the selection using the dual culture assay method, all 20 strains showed antagonism against the phytopathogens *Corynespora cassiicola* (ISO079) and *Colletotrichum* sp. (ISO01) (Fig. 2). Thirteen were active against *Pestalotiopsis* sp. (3002R2), the same number was antagonistic against *Colletotrichum guaranicola* (P01). Strains *Streptomyces* sp. LaBMicrA B267, B280, and B301 were the only ones active against *S. coffeicola* (M01) and the five phytopathogens. *Streptomyces* spp. LaBMicrA B270 and B280 (Fig. 3A) were noteworthy for their strong antagonism against *Pestalotiopsis* sp. (3002R2) and *Col. guaranicola* (P01) (Fig. 3B,C). No consistent activity was observed against the phytopathogens of the volatile metabolites of the actinomycetes.

3.3 Production Curves of the Strains LaBMicrA B270 and B280 Extracts and their Antifungal Activities over Time

Due to their promising results in the previous dual culture tests, the LaBMicrA B270 and B280 strains were selected to study the production curves of the cell and culture medium extracts and the activities of the same extracts against the test phytopathogens over the whole growth time. As can be observed, the curves of the cell and culture medium extracts were similar to the classic bacterial growth curves (Fig. 4). Likewise, glucose concentration reached a level close to zero when the period of maximum extract pro-

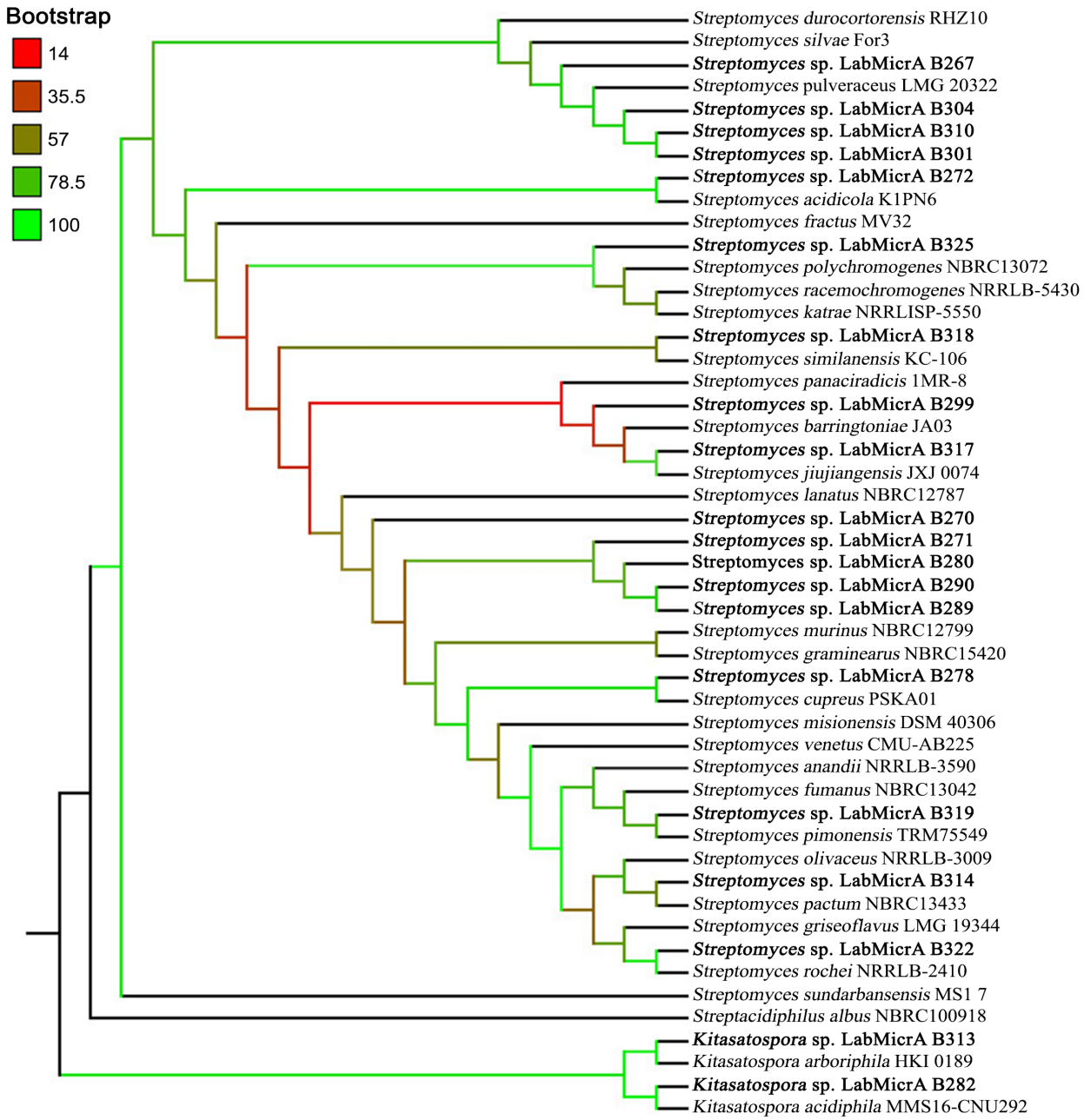

Fig. 1. Phylogenetic tree of partial sequences of the 16S rRNA region using the maximum likelihood analysis of actinomycetes from the rhizospheres of three individuals of *Inga edulis* at the campus of the Federal University of Amazonas (UFAM) with type species in the EzBioCloud database.

duction began, signaling the end of the exponential phase and beginning of the stationary phase. Interestingly, the behavior of the pH was quite distinct for the two strains; the first reached a maximum of 8.0 in the maximum extract production period and only decreased in the period of decline, while the second maintained a pH of 6.5 during all the phases. The decrease in the extract masses, typical of the decline phases, began in both curves after 272 h of culturing (11th day). One day before, on the 10th day, the extract yields in the ISP2 medium cultured were 542 and 720 mg/L for *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), respectively.

To confirm the dual culture assays and verify the necessary growth time for the antifungal activities observed in the assays, AcOEt/2-propanol 9:1 extracts of the culture medium of the LaBMicrA B270 and B280 strains were tested from each time point against the same phytopathogens. As in the dual culture assays, extracts from all time points, between 36 hours of growth (time point 3) and the beginning of the decline phases, showed antifungal activities against all phytopathogens. As expected from the dual culture assays, the LaBMicrA B270 extracts again did not show activity against *S. coffeicola* (M01).

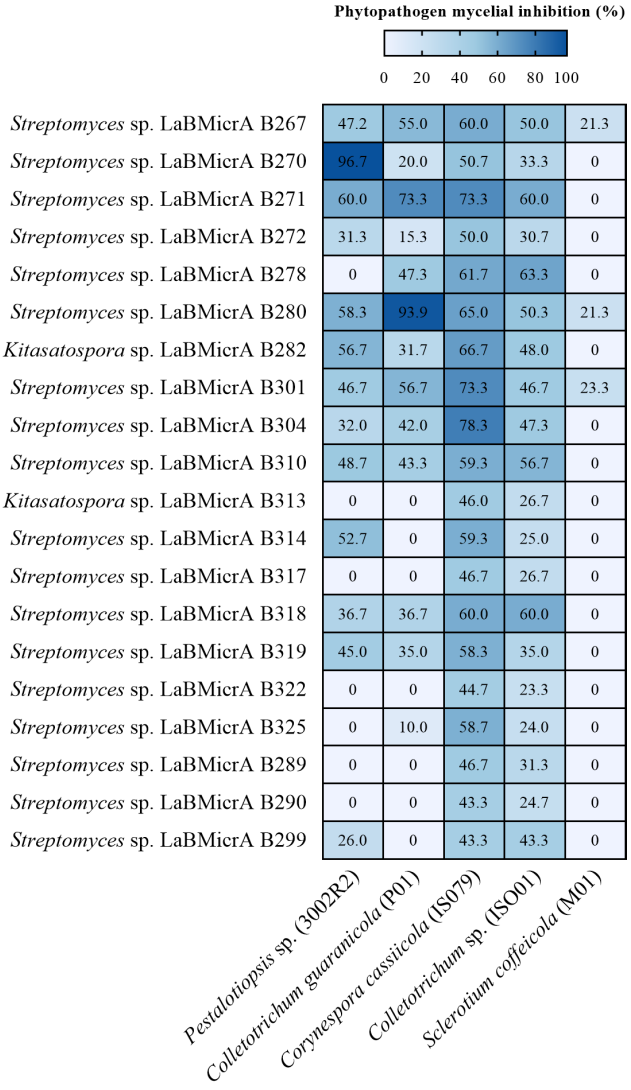

Fig. 2. Heatmap showing the average percentage values of inhibition zones of mycelial growth of phytopathogens by actinomycetes (GraphPad Prism version 8.0.1).

3.4 Minimum Inhibitory Concentration Assays

After observing the results of the periodic assays described above, extracts from the LaBMicrA B270 and B280 strains with ten days of growth were chosen for the minimum inhibitory concentration assays. This time, intracellular extracts were also assayed to determine whether the cells possessed the same antifungal activities. The best results were obtained with the AcOEt/2-propanol 9:1 fractions of the partition of the cultured media (Table 2). Confirming the previous assays, the LaBMicrA B280 strain showed activity against all phytopathogens, and the LaBMicrA B270 strain did not only show activity for *S. coffeicola* (M01). Except for this case, the AcOEt/2-propanol 9:1 extracts did not show growth at the minimum doses of 250 or 125 µg/mL, and only very little growth or normal growth was observed below these doses. However, the extracts of the bacterial biomasses in AcOEt/MeOH 1:1 showed much weaker antifungal activity and only for two or three strains. Finally, no activity was observed in the aqueous fractions of the cultured medium after partitioning with AcOEt/2-propanol 9:1, demonstrating the effectiveness of this extraction.

4. Discussion

Many examples show that in any ecosystem, the interaction between a plant and microorganisms can be beneficial, neutral, or harmful, determining, among other factors, the adaptability of the plant (to a greater or lesser degree) to the environment in which it grows [3,28]. *I. edulis*, a plant with a positive inductive effect for the agricultural production of vegetables of economic interest, for example, *Coffea canephora* var. *robusta* (robusta coffee), *Theobroma cacao*, and *Terminalia amazonia*, also have good receptivity to beneficial microorganisms [29–31,38–40]. In this study, 64 actinomycete strains were isolated from the rhizospheres of three individuals of *I. edulis* present on the campus of UFAM, the largest urban forest fragment of the Amazon rainforest. The isolated strains were clustered in 16 macromorphologically different groups, and 20 strains were selected and evaluated for biodiversity and their antifungal potential. Phylogenetic analysis using 16S sequences grouped most of the twenty strains by similarity with type strains from the EzBioCloud database. This suggests that the genetic similarity obtained by paired alignment may be a good indicator for the initial classification of these strains with the type species of greater similarity. However, this analysis under no circumstances indicates delimitation at the species level. Currently, the delimitation of *Streptomyces* species, as well as other bacterial species, has been carried out by dDDH (digital DNA-DNA hybridization) and ANI (average nucleotide identity) analyses using the entire genome [41,42].

Different examples in the literature, in which strains with similarity greater than 98% in the 16S region belong to other species, ratify that even high similarity in this bacterial region does not guarantee species identity. Indeed, this was identified in the recent description of the *Streptomyces herbicida* strain NEAU-HV9, which presented sequence similarity to *S. panaciradicis* 1MR-8 (98.90%), *S. sasae* JR-39 (98.89%), and *S. barringtoniae* JA03T (98.69%) [43]. A similar result was observed for the LaBMicrA B270 and B280 strains, which, although showing similarities of 98.36% and 98.86% in the 16S region with *S. murinus* NBRC 12,799 and *S. graminearus* NBRC 15,420, respectively, belong to a new species of *Streptomyces* according to complete genome data (data not shown). Examples similar to this may indicate that many strains obtained in this study may not be fully represented in the currently known species, revealing the need for more in-depth studies using multilocus phylogenetic or phylogenomic analysis of the complete genome to characterize these strains accurately.

In relation to their antifungal potential, the 20 strains were also interesting in the tests against the phytopathogens of agricultural importance, *Colletotrichum* sp. ISO01, *Col. guaranicola* P01, *Pestalotiopsis* sp. 3002R2, *Corynespora*

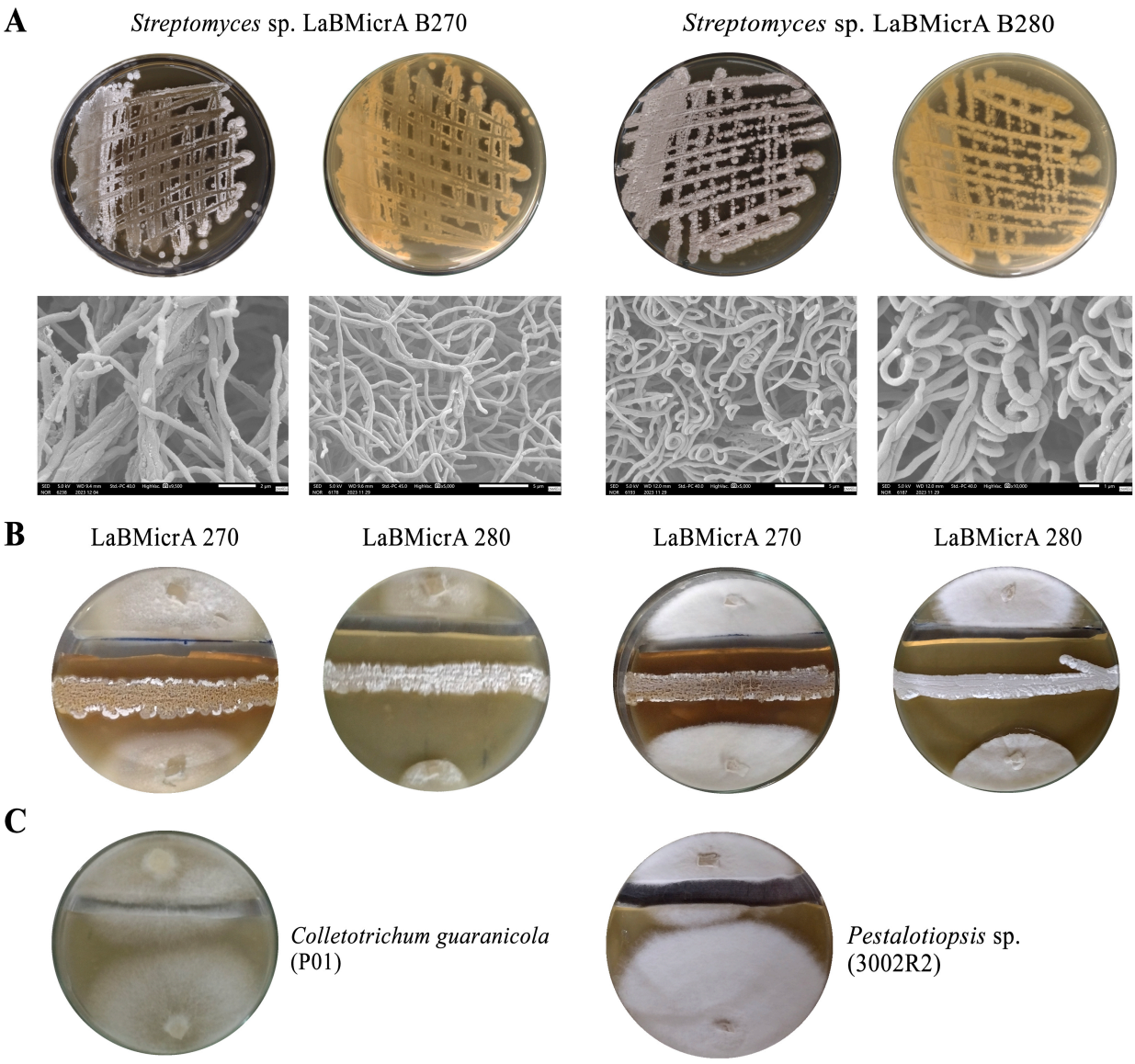

Fig. 3. Morphological details and assay plates of *Streptomyces* spp. LaBMicrA B270 and B280 strains against phytopathogens. (A) Macro and micromorphological characteristics of these strains after 10 days of growth in the International Streptomyces Project 2 (ISP2) medium. Above: front and back of the Petri dishes; below: mycelial ornamentations—scanning electron microscope (SEM) images. (Scales: B270: 2 and 5 µm; B280: 5 and 1 µm). (B) Dual culture assays of the LaBMicrA B270 and B280 strains with the phytopathogens Colletotrichum guaranicola (P01) (right) and *Pestalotiopsis* sp. (3002R2) (left), after 10 days of growth in ISP2 medium. (C) The control containing only the phytopathogens after the tenth day of culture.

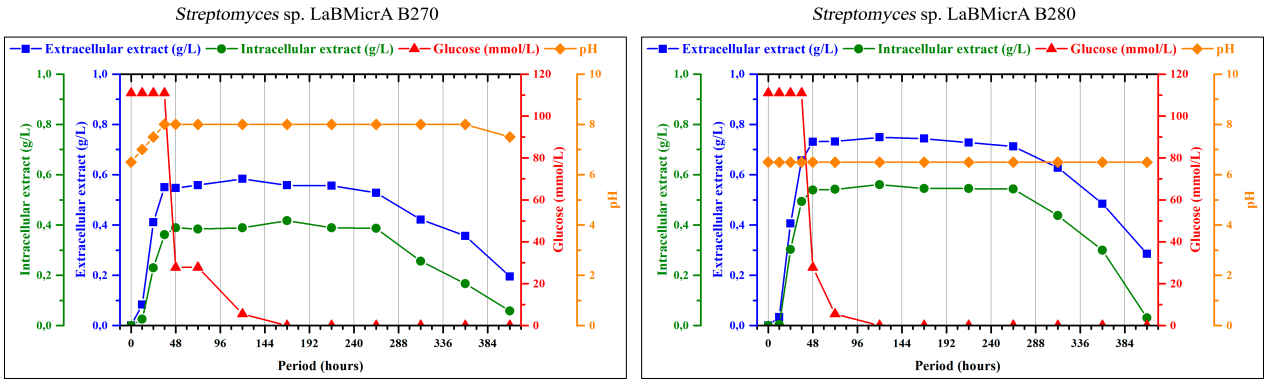

Fig. 4. Yield curves of metabolic extracts of the strains selected for biological studies.

Table 2. Minimum inhibitory concentration of 10-day-old extracts of the *Streptomyces* spp. LaBMicrA B270 and B280 strains.

Strains tested	Concentrations and activities					Phytopathogens
	1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	62 µg/mL	
	AcOEt/2-propanol 9:1 extract (culture medium)					
Streptomyces sp. LaBMicrA B270	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	±	–	*Col. guaranicola* (P01)
	+	+	+	±	–	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	+	±	*Col. guaranicola* (P01)
	+	+	+	+	±	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	+	+	+	±	–	*Sclerotium coffeicola* (M01)
	AcOEt/MeOH 1:1 extract (cell biomass)					
Streptomyces sp. LaBMicrA B270	–	–	–	–	–	*Colletotrichum* sp. (ISO01)
	–	–	–	–	–	*Col. guaranicola* (P01)
	+	+	±	–	–	*Corysnepora cassiicola* (ISO079)
	+	±	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	±	–	–	*Colletotrichum* sp. (ISO01)
	+	±	–	–	–	*Col. guaranicola* (P01)
	+	±	–	–	–	*Corysnepora cassiicola* (ISO079)
	–	–	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)

Note: + = no growth; ± = very little growth; – = growth.

cassiicola ISO079, and *S. coffeicola* M01. These phytopathogens cause problems that generate millions of dollars in agriculture losses. *Corynespora cassiicola* is widely cited as a disease-causing agent in more than 530 plant species of 380 genera [44] and is a mycosis-causing infectious agent in humans working with vegetable crops [45]. The genus *Colletotrichum* is responsible for diseases in more than 3200 plant species [46]. The genus *Pestalotiopsis* harbors several species of biotechnological interest [47,48], although some species are reportedly phytopathogens [49–51]. *S. coffeicola* has a high impact on agricultural production and is responsible for lesions in Indian mulberry (*Morinda citrifolia* L.) leaves [52] and mango trees (*Mangifera* sp.), important plants for traditional medicine and in the food and cosmetic industries [53,54].

The antagonistic results in the *in vitro* assays confirm the broad capacity of the *Streptomyces* species to combat other microorganisms, particularly fungi, and indicate that the 20 strains may be sources of antifungal substances. The antifungal activities of periodically sampled extracts from the *Streptomyces* spp. LaBMicrA B270 and B280 strains and the minimum inhibitory concentration tests ratified their potential and suggested highly effective antifungal substances, considering their probable low concentration in these extracts. Both strains are strong candidates to combat the phytopathogens *Colletotrichum guaranicola* (P01) and *Pestalotiopsis* sp. (3002R2), which threaten the guarana (*P. cupana*) plant, a very important crop in the economy of Amazonian communities. Guarana seeds are among the best sources of caffeine in the world and are used to make soft drinks, as a food supplement, and in traditional medicine [55]. Furthermore, LaBMicrA B280 was also active against *S. coffeicola* M01, a phytopathogen affecting hundreds of plants of agricultural importance worldwide.

The development of less harmful, more ecologically correct, and more sustainable methods for agriculture depends on the availability of microorganisms that promote biocontrol and fertilization. Many examples highlight that *Streptomyces* and other actinomycetes are useful for this, combating phytopathogens [56–59], improving fruit production, and promoting their fertilization [4,7,10,11,60]. In this context, according to the results obtained in the present work, actinomycetes from the rhizosphere of *I. edulis* can represent a viable alternative for sustainable agriculture. Seedlings or seeds may transport them from one soil to another and by litter from *I. edulis* plants, commonly generated in abundance [61–64]. These possibilities, however, need to be investigated.

5. Conclusions

This unprecedented study revealed that the rhizospheres of *I. edulis* can harbor a wide variety of actinomycetes, the species diversity of which has yet to be in-

vestigated. This is what we were able to infer by comparing the *16S rRNA* genes of the 20 actinomycetes strains from the rhizosphere of three individuals of this plant with the EzBioCloud database, whose result indicates that these 20 strains are distributed in distinct groups within the genera *Streptomyces* and *Kitasatospora*. We also revealed the great potential of these 20 strains against the phytopathogenic fungal strains of *Colletotrichum* sp., *Col. guaranicola*, *Pestalotiopsis* sp., *Corynespora cassiicola*, and *S. coffeicola*, which commonly destroy numerous crop plantations of global and regional economic importance: The two *Streptomyces* spp. strains LaBMicrA B270 and B280 should also be highlighted since, after 10 days of growth, their 9:1 AcOEt/2-propanol extracts from the culture medium completely inhibited the growth of four, except *S. coffeicola* (B270) or all five fungi (B280), respectively, at minimum doses of 250 or 125 µg/mL, revealing that they possess metabolites with potent antifungal activities. Coherently, the periodic study showed that the cultured media extracts from both actinomycetes had antifungal activities between the end of the growth phase (third day) and the beginning of the decline phase.

Naturally, the present study needs to be expanded and broadened to provide a better understanding and use of the biotechnological potential of these strains. However, it does reinforce the hypothesis that Amazonian microorganisms, particularly actinomycetes, although less investigated and perceived than plants, are not less important as sources of invaluable potential for solving many modern problems, particularly agricultural ones. More studies are required to increase understanding of the potential of these strains for use in agriculture and medicine. The active ingredients of the extracts and the activation of unexpressed BGCs related to the synthesis of NPs will be the objects of research to discover new bioactive metabolites. Moreover, the possibility of new species will also be investigated by the complete sequencing of the actinomycetes genome. We expect similar studies to stimulate other regional groups to explore actinomycetes, among other microorganisms, from the vast microbial biodiversity in the Amazon and reinforce the need to preserve it.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

RSR: contributed to conception and design, acquisition of data, analysis and interpretation of data, and drafting the manuscript; RDB and JLSB: isolations and identifications of phytopathogens; ANB and TCLA: biological assays; SRSSS and JCC: molecular identification; ASV and GFS: molecular identification and technical support; AQLS and ADLS: technical support, methodology, supervision, and preparation of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

According to Brazil's regulations and policies, this study does not involve human or animal subjects; therefore, ethical committee approval is not necessary.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbe1604039.

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