Biodiversity and antifungal activities of actinomycetes isolated from the rhizosphere of Inga edulis in an urban forest fragment of the amazon

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Abstract

Actinobacteria are major producers of antibacterial and antifungal metabolites and are targets of increasing research in the search for substances of biotechnological interest, especially for use in agriculture. The Amazon soils is potentially rich in actinobacteria; therefore, we prospected actinobacteria from the rhizospheres of three individuals of Inga edulis found on the campus of the Federal University of Amazonas, the largest urban forest fragment in the Amazon. We isolated 64 strains of actinobacteria, which were distributed in 16 morphogroups. A total of 20 strains representing these 16 groups were subjected to morphological analysis, genotyping using the 16S rRNA gene, and assays against pathogenic fungi of important agricultural plants in the region that also cause annual losses of millions of dollars to world agriculture. Using GenBank-NCBI and EzBioCloud data, 90% of the strains were identified as Streptomyces and 10% as Kitasatospora, indicating at least 16 different species and possible new species. All showed antagonisms for two or more phytopathogen strains of Corysnepora cassiicola, Colletotrichum sp., Colletotrichum guaranicola, Pestalotiopsis sp., and Sclerotium coffeicola. Two stood out for their broad spectrum and better results against phytopathogens of the guarana plant (Paullinia cupana), an important crop for the regional economy and one of the best sources of caffeine in the world, whose powder is used in the food industry and traditional medicine. Complementary analyses ratified the potential of these strains as sources of highly effective antifungal substances. In general, the results exemplify the high diversity and richness of the actinomycetes in the Amazon.

Introduction

In the last decade, prospection of new isolates of actinomycetes that produce natural products (NPs) has been carried out in virtually every ecosystem on the planet. The screening of strains isolated from these environments, mainly by paired culture methods and biological assays with extracts, have revealed many of them to be promising sources of NPs of biotechnological interest (Alkhalifah 2021; Chevrette et al. 2019; Elshafie and Camele 2022; Liu et al. 2019 (a); Pavan et al. 2018; Rego et al. 2019; Xie and Pathom-Aree 2021). These prospections are a result of the scarcity of medicinal and agricultural resources to stop the spread of multidrug-resistant human pathogens and plant pests that ravage plantations in all parts of the planet. These pathogens compromise agricultural production and are due to the large-scale use of biocides that are employed to meet the growing food needs in the 20th and 21st centuries (Butler et al. 2023; Djebaili et al. 2021; Oyedoh et al. 2023; Priyashantha and Tibpromma 2023; Saeed et al.2021; Strobel et al. 2004).

The cosmopolitanism of actinomycetes is strictly related to their essential ecological activities for the planet and other living beings, such as nitrogen fixation, recycling of organic matter, and the secretions of phytohormones, antibiotics, lytic enzymes and biocontrol agents of phytoprages, among others. These activities make them great allies of plants through endophytic-epiphytic relationships and as rhizobacteria (Liu et al. 2019 (a); Liu et al. 2019 (b); Liu et al. 2019 (c)). In fact, actinomycetes maintain symbiotic systems with plants, secreting protective antimicrobials against phytopathogens and hormones that induce plant growth and feeding on nutrients released by plants, which makes them

strong candidates for biocontrol agents and natural fertilizers (Djebaili et al. 2021; Saeed et al.2021). The potential of these microorganisms against fungal phytopathogens, which have attracted the attention of many researchers seeking new antifungals and more sustainable methods for agriculture, should be highlighted (Oyedoh et al. 2023).

Among the microorganisms that produce NPs, actinomycetes stand out for being responsible for the production of 70% of the antimicrobials available to medicine and agriculture (Bérdy 2012; Sharma and Thakur 2020). The genus *Streptomyces* is the best known and largest producer of NPs, with about 7,500 of the more than 10,000 NPs assigned to the more than 200 genera of the phylum *Actinobacteria* (Barka et al. 2016; Bérdy 2005; Law et al. 2019). Other genera, among them *Micromonospora*, *Kitasatospora*, and *Nocardia*, however, are gaining more and more interest in the search for new NPs (Takahashi 2017; Yan et al. 2022), especially the antifungal compounds whose best-known class for this group of microorganisms is that of macrolides (Kim et al. 2012; Nara et al. 2017; Wang et al. 2017).

Regarding biodiversity, the largest currently recognized genera are *Streptomyces,* with approximately 700 species (Komaki 2023), *Micromonospora,* with 110 species (Yan et al. 2022), and *Kitasatospora,* with 23 species (Takahashi 2017). In general, phenotypically, these bacteria are gram-positive, have aerial mycelia or an absence of mycelia, have segments with cylindrical, spherical, and ovoid shapes and spore chains that are spiral (or partially) and linear (Shirling and Gottlieb 1972). Their genomes are rich in guanine-cytosine, with sizes ranging between 8 and 9 million base pairs (BP) of nucleotides, and hold dozens of clusters of biosynthetic genes (BGCs) related to the synthesis of NPs (Van Bergeijk et al. 2020). Whole genome sequencing and DNA-DNA hybridization are the most-used techniques for taxonomic definitions at the species level. In addition to the traditional *16S rRNA* marker, currently, other markers are used to increase reliability in taxonomic definitions in the absence of the total genome sequence, for example, *atpD, gyrB, recA, rpoB* and *trpB* (Komaki 2023).

In this perspective, we investigated the diversity of a sample of actinomycetes isolated from the rhizospheres of three individuals of *Inga edulis* present 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, which potentially houses an incalculable and almost unexplored microbial diversity. The antifungal potential of these actinomycetes against phytopathogens of regional and global interest was also evaluated, among which stand out phytopathogens of the guarana plant (*Paullinia cupana*), one of the largest commercial sources of caffeine, and *Sclerotium coffeicola*, a phytopathogen recognized worldwide for affecting hundreds of plants of agricultural importance. This study is part of a larger project that seeks to collect, preserve, identify, and comprehend the biodiversity and potential applications of microorganisms from the Amazon, particularly from the campus at UFAM.

Materials And Methods

Soil sample collections

Three collections were carried out at different points on the UFAM campus (Point 1 - 3°05'19.4" S 59°58'00.5" W, Point 2 - 3°05'20.8" S 59°57'57.5" W and Point 3 - 3° 5'19.06"S 59°57'56.26"W), in which there were individuals of *I. edulis* that were not the period of seedpod production. At each point, three rhizosphere samples were collected at distances of 40 cm from the collar of the plant and 20 cm below in piliferous regions of the roots. Sterilized materials were used in all sample collections: 50 mL Falcon™ tubes, metal spatulas and plastic bags to transport the samples. The samples were collected and taken to the Laboratory of Bioassays and Microorganisms of the Amazon (LaBMicrA) - UFAM, where they were stored at -20 ºC.

Isolation and purification of actinobacterium strains

All the manipulations of the microorganisms in this study were performed in a biological safety cabin (Filterflux® Class II A1). Twenty-four hours after the collections, 1 g of soil from a sample of each of the points was dissolved in 5 mL of sterilized distilled water containing 0.02% Tween-20. One mL of each suspension was subjected to serial decimal dilution in test tubes containing 9 mL of 0.8% saline. From the 5^{th}, 4^{th} and 3^{rd} dilutions, 50 µL were spread in triplicate in 90 x 15 mm Petri dishes containing the oat-based 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 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 antifungals nystatin and tetracycline were added. This was followed by incubation in a BOD chamber at 28 ± 2 ºC for a period of 30 days, during which the strains of actinomycetes that grew were isolated and purified.

Microculture and microscope analysis

The isolated and purified actinobacteria were subjected to microcultures (adapted from Shirling and Gottlieb 1972) and were stained using the Gram method (LB Laborclin®). In summary, the spores of the purified strains were sown in 40 x 15 mm Petri dishes containing their respective isolation media; 18 x 18 mm coverslips were tilted at 45º over the streaks. After 96 hours of incubation at 28 ± 2 ºC in a BOD chamber, the coverslips were removed and attached with plastic tape to 26 x 76 mm slides; then they were subjected to the Gram staining method. All the stained slides were observed under an optical microscope with the 100x objective (Carl Zeiss optical microscope, coupled to Zen AxioCamER - Zen lite 2012 photo documenter).

Conservation, selection, and morphological description of the strains of actinobacteria

For the conservation, the strains of actinobacteria were cultured in a solid medium (as described in Section 2.2) for 10 days. Five fragments of each colony were preserved in triplicates in 1.5 mL microtubes containing 20% glycerol. All the microtubes were stored in a freezer at -80 ºC and are preserved in the LaBMicrA microorganism collection at the UFAM's Analytical Center. As selection criteria and for the definition of the group of biotechnological studies, the macromorphological aspects of isolates grown in ISP2 were observed. The strains were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number

AC1746C. After cultivation in ISP2 culture medium, the selected strains were described morphologically according to the manuals of Shirling and Gottlieb (1972). In the macromorphological aspects, we observed the patterns of the pigmentation of the pseudohyphae, dorsal-dishes and types of edges of the colonies – the colors of the pseudohyphae and dorsal dishes were determined using the ISCC/NBS color system (available at https://www.w3schools.com/colors/colors_nbs.asp).

Phytopathogens selected to evaluate the antifungal potential of the actinobacteria

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

DNA extraction, PCR, sequencing 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 (Souza et al. 2004), at 28 ± 2 °C and 120 rpm, for 72 h. The extractions followed the protocol of the Zymo Research Fungal/Bacterial DNA MicroPrep™kit. The DNAs were quantified in a spectrophotometer (NanoDrop, Thermo Scientific) and their integrities were recorded using electrophoresis with 0.8% agarose gel. The polymerase chain reaction (PCR) was performed using 20 ng of DNA, the PCR kit Illustratm PuReTaq Ready-to-GoTM PCR beads (GE Healthcare) and the primers 27F AGAGTTTGATCMTGGCTCAG and 1492R CGGTTACCTTGTTACGACTT (Haygood & Davidson 1997), with a final volume of 25 µL, in a 96-well thermal cycler (Applied Biosystems) (Ebrahimi-Zarandi et al. 2021). The amplified product was visualized in electrophoresis with 1% agarose gel, stained with GelRed™, using the Invitrogen® 1 Kb Plus DNA Ladder base pair marker, and photo-documented using the L-Pix Touch Loccus® Photo-documentation system. Amplicons were purified with the enzyme ExoSAP-IT (GE Healthcare). The samples were sequenced using the Sanger method and the BigDye Terminator kit (Applied Biosystems).

The products of the partial sequencing of the *16S rRNA* gene were compared with the sequences retrieved from the GenBank databases (NCBI) using the BLASTn alignment (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and EzBioCloud (https://www.ezbiocloud.net/). The alignment of the sequencing products was performed using CLUSTAL W (MEGA 11 (Molecular Evolutionary Genetics Analysis). Subsequently, a phylogenetic tree was constructed using the evolutionary analysis by the maximum likelihood method using the neighbor-joining and BioNJ (Tamura-Nei, 1993) algorithms and the bootstrap test with a maximum of 1,000 replications ((MEGA 11, available

at www.megasoftware.net) (Felsenstein 1985; Tamura et al. 2021). Accession numbers generated by NCBI are available in the Supplementary Material (MS 1).

Screening using the paired cultures method

After being reactivated in ISP2, the actinomycete strains were inoculated linearly, from one end to the other, passing through the center, in new Petri dishes containing ISP2, and incubated at 28 ± 2 ºC in a BOD chamber. Two days previously, the pathogen lines were inoculated in PDA (Souza et al. 2004). On the fifth day of growth of the pathogens and third of the actinomycetes, discs of agar of 5 mm in diameter containing the phytopathogens were removed from the culture in PDA and inoculated at one centimeter from the edges, perpendicularly in relation to the line containing the bacterial inocula, in the dishes with ISP2. At one centimeter from one of these inocula, a cavity was made by removing a 10 mm wide strip of the culture medium from one edge to the other, parallel to the bacterial line, in order to verify the possible production of antifungal volatile substances (Fig.5). Similar control dishes were also made with ISP2, which contained only each phytopathogen at two opposite poles and separated by a 10 mm cavity. All paired cultures were made in triplicate and incubated for 10 days, in order to observe the possible inhibition of pathogens by the actinomycetes. A stacked bar graph was constructed from the means of the inhibition zones of each triplicate using the GraphPad Prism version 8.0.1.

Production curves of the extracts

The strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), which were selected from the bioassay of paired cultures, were cultured for 96 h in Petri dishes with ISP2 and 1.5% microbiological agar, from which two fragments of 5 x 5 mm were inoculated in an Erlenmeyer flask of 250 mL containing 120 mL of ISP2, with a total of 36 flasks. The strains were grown at 120 rpm and 28 ± 2 ºC. During incubation, three Erlenmeyer flasks of each strain were collected at scheduled intervals, totaling 12 samples in triplicate at intervals of 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. At each interval, glucose consumption and pH were measured, and intra- and extracellular extracts were obtained, the masses of which were used for the construction of the production curves of the extracts and biological assays.

To obtain the extracts at each interval, the biomass and cultured medium of each flask were separated via centrifugation (Eppendorf® centrifuge) at 4,000 rpm for 15 min, and then the biomass was washed three times with 10 mL of distilled water. The biomasses were then vortexed and macerated for 24 h with 5 mL of 1:1 MeOH/AcOEt. The cultured medium was partitioned with successive volumes of 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, in order to establish the average values of the triplicate of each point. After this, the three extracts from each point were collected, concentrated, weighed, and stored at 4 ºC until the day of the bioassays.

Assays of the extracts from the points of the curve against phytopathogens

Only the extracts collected from the cultured medium of each of the twelve points of the growth curve of the *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701) strains were tested against the target phytopathogens. To the flasks containing the dried and weighed extracts, 200 µL of DMSO were added and, after being dissolved, 800 µL of autoclaved distilled water were added. From these solutions, stock solutions were made 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 dissolved in 1 mL of water. Phytopathogens were reactivated in Petri dishes containing potato, dextrose, agar and yeast extract (PDAY) ulture medium (Souza et al. 2004) at 28 ± 2 ºC for 96 h, in a BOD chamber. The extract and control assays were performed in triplicate in cell culture plates with 12 wells, containing: test: 0.950 mL PDAY, 50 µL extract + phytopathogen; negative control: 1 mL PDAY + phytopathogen; positive control: 0.950 mL PDAY, 50 µL nystatin solution + phytopathogen. The solutions of the extract or control with the culture medium were homogenized at approximately 40 ºC. For the inoculation of the pathogens in the wells, discs (5 mm in diameter) of the culture in PDAY were used in each of them. The assays were observed for 10 days.

Production of the extracts and minimum inhibitory concentration assay

From the results of the growth curves and bioassays, new extracts of LaBMicrA B270 (OR724732) and B280 (OR724701) strains were prepared, which were cultivated for 10 days using cultivation and extract production methods similar to those described above (Section 2.8.). In the production of the extracts, the only difference was the lyophilization of the cultured medium after partition with AcOEt/2-propanol 9:1. The concentrated extracts and lyophilized material were stored at -18 ºC. To determine the minimum inhibitory concentration (MIC), the AcOEt/2-propanol 9:1 and MeOH/AcOEt 1:1 extracts and the aqueous lyophilized material were successively diluted to the concentrations of: 1,000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL. The extracts were tested with the phytopathogens in a similar way to what is described in the previous item.

Results

Morphological and genotypic diversity

From the three rhizospheres of the *I. edulis* trees, respectively found at the three collection points (P1, P2 and P3) of the UFAM campus (Fig.1), 64 actinomycetes were isolated using oat-based and ISP2 culture media. Among these points, P2 stood out for the largest number of isolates, 30 isolates (46.9%), followed by P1 with 19 isolates (29.7%), and P3 with 15 isolates (23.4%).

Fig.1

The observations of the macromorphology and the pigmentation of the culture media in the Petri dishes (front and back) allowed us to preliminarily group the 64 isolated actinobacteria into 16 morphogroups. The 20 strains selected, one from each smaller group and two from each of the larger group of morphologically similar individuals, and the respective macro- and micromorphological descriptions

according to the criteria established in Section 2.4, were tabulated according to the collection point; these being: seven from P1 (green), ten from P2 (orange) and three from P3 (blue) (Table 1). The photos of the macro and micromorphologies, shown in the photos of the *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701) (Fig. 2), whose antifungal activities stood out among the others (see below), can be observed in the Supplementary Material (SM 1).

(Fig. 2)

The amplicons obtained from the 20 selected strains and subjected to sequencing had extensions of approximately 1400 bp of the *16S rRNA* gene. The sequenced amplicons presented viable sequences with 650-800 nucleotides. The greatest similarities of these sequences with those deposited in the NCBI databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (https://www.ezbiocloud.net/) revealed that 18 strains are of the genus *Streptomyces* (90%) and two of the genus *Kitasatospora* (10%) (Table 1). The following strains presented amplicons with similarities (greater than 99%) to a single strain in one or both databases: *Streptomyces* spp. LaBMicrA B280, B289, and B290 in GenBank-NCBI; *Streptomyces* spp. LaBMicrA B310 and B322 in EzBioCloud; and *Streptomyces* spp. LaBMicrA B267 and B314 in both databases. Among them, only *Streptomyces* sp. LaBMicrA B314 had the same species suggestion, *S. olivaceus* (NBRC 12805, 99.61% in GenBank-NCBI and NRRL B-3009, 99.74% in EzBioCloud). Four other strains, all with similarities over 90% and below 99%, had a single suggested species in both databases: *Streptomyces* spp. LaBMicrA B278 (*S. misionensis*), B304 (*S. pulveraceus*), and B318 (*S. similanensis*) and *Kitasatospora* sp. LaBMicrA B282 (*K. acidiphila*). Three strains showed similarity below 90% in at least one database: *Streptomyces* spp. LaBMicrA B272 (in both), B289 (EzBioCloud) and B299 (GenBank). Among them, the strain *Streptomyces* sp. LaBMicrA B272 stands out as the strongest candidate for a new (or not yet deposited) species, with low similarity with *S. acidicola* K1PN6 (88.60%) and *S. sundarbansensis* MS1/7 (78.35%). Eight strains, six of *Streptomyces* and two of *Kitasatospora*, presented more than one suggestion of species with equal weight: four in both databases, two in GenBank and two in EzBioCloud. More than 50% of the 20 strains present similarity values lower than 98% in both databases (Table 1).

(Table 1).

Phylogeny

In the phylogenetic analysis using the 20 strains of this study and 29 strains of the GenBank-NCBI database, a tree was obtained considering only the values of the nodes from 50% of the bootstrap (Fig.3). The phylogenetic differences between the *Streptomyces* (90%) and *Kitasatospora* (10%) strains only occurred in the root of the tree, with a bootstrap value of less than 50% (not shown) in five monophyletic clades: one formed exclusively by *Kitasatospora* strains, including the two in this study, three with only *Streptomyces* strains, including six in this study, and a larger one, containing almost exclusively *Streptomyces* strains, including the remaining 12 of this study. Four strains (*Streptomyces* LaBMicrA B304, B310, and B325, below 50%, and B318, at the limit of 50%) are the least phylogenetically connected with the GenBank strains. Conversely, four strains (*Kitasatospora* LaBMicrA B282 and *Streptomyces*

LaBMicrA B272, B322, and B289) presented phylogenetic indices of 99-100% with at least one of the strains in this database. All the others are between these two extremes. Of all the strains, *Streptomyces* LaBMicrA B325 is the only one that emerges alone from the root of the tree and does not align with any of its five GenBank species suggestions, all of which have 99% similarity (Table 1).

(Fig.3).

Antifungal activities of the 20 strains against phytopathogens

In the screening using the paired cultures method, all the 20 strains showed antagonism against the phytopathogens *Corysnepora cassiicola* (ISO079) and *Colletotrichum* sp. (ISO01) (Figs. 4 and 5). Thirteen were active against *Pestalotiopsis* sp. (3002R2), the same amount was antagonistic against *Colletotrichum guaranicola* (P01). Only the strains *Streptomyces* sp. LaBMicrA B267, B280, and B301 showed antagonism against *S. coffeicola* (M01) and were also the only ones active against the five phytopathogens.

(Fig.4).

The strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701) stood out for their strong antagonism against *Colletotrichum guaranicola* (P01) and *Pestalotiopsis* sp. (3002R2), respectively, both phytopathogens of the guarana plant (*P. cupana*) (Fig.5). No consistent signs of volatile metabolite activities of any actinobacterium against the phytopathogens were observed.

(Fig.5).

Production curves of the extracts and evolution of antifungal activities of the strains Streptomyces sp. LaBMicrA B270 and B280

The strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), selected due to their promising results in the previous tests, presented production curves of intra- and extracellular extracts that were compatible with the traditional bacterial growth curves (Fig.6). Behavior that was also compatible was the glucose concentration that reached a level close to zero coincident with the beginning of the period of maximum production of the extracts. Interestingly, the behavior of the pH was quite distinct for the two strains: the first reaching a maximum of eight in this period and only falling again in the period of decline, while the second maintained pH 6.5 during all the stages. The decline phases in both curves began between 240 and 288 h of culture (10 and 12 days). The extract yields in the ISP2 medium cultured on the tenth day were respectively 542 and 720 mg/L for *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701).

(Fig.6).

In the assays against the five phytopathogens, the AcOEt/2-propanol 9:1 fractions of the cultured medium of the two strains from each point sampled showed antifungal activities after 36 hours of

growth (Point 3), with the exception that the strain LaBMicrA B270 was confirmed as not being active against *S. coffeicola* (M01). The activities were confirmed for the other sampling points, until the beginning of the decline phases, which were observed in the respective production curves of the extracts.

Minimum inhibitory dosage assays

In minimum inhibitory dosage assays of extracts with 10 days of growth of the strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), the best results were obtained with the acetate/isopropanol fractions of the partition of the cultured media (Table 2). Confirming the previous assays, B280 showed activity against all the phytopathogens and B270 did not show activity for *S. coffeicola* (M01). Except in this case, the acetate/isopropanol fractions showed fungicidal activity at the minimum dosages of 250 or 125 µg/mL and, only below that, did they become fungistatic or inactive. In turn, the extracts of the bacterial biomasses in acetate/methanol showed much weaker activity and to only two or three strains. Finally, no activity was observed in the aqueous fractions of the cultured medium after partitioning with acetate/isopropanol.

(Table 2).

Discussion

In this study, a small sample of actinomycetes isolated from the rhizospheres of three individuals of *I. edulis* present in the campus of UFAM, which is the largest urban fragment of the Amazon rainforest, was evaluated for its diversity and antifungal potential. High biodiversity was revealed from the initial macromorphological analysis that resulted in 16 groups in the universe of the 64 strains of actinomycetes collected, which equates to an average of one group for every four strains. Similarly, although the sequences of the *16S rRNA* gene revealed a low diversity of genera, only *Streptomyces* (90%) and *Kitasatospora* (10%), the diversity of species seems to be very high, if one considers the suggestions of the GenBank-NCBI and EzBioCloud databases: seven different species for the eight strains with sequences over 99% similar to at least one of these databases and nine species different from each other and from the previous ones for the 12 strains with similarities below 99%. Thus, only four strains had suggestions of species already suggested for others 20 sequenced strains. Considering that these 20 strains are representatives of the 16 groups of the 64 strains, there is the possibility of at least one different species for every four strains, which should be confirmed in the future by complete sequencing of the genomic DNA.

The phylogenetic analysis not only confirms the high diversity of the strains of this study, among them and also in relation to those deposited in GenBank, but also reveals cases of high phylogenetic distance, mainly represented by the five strains with 50% or less possibility of clustering. Conversely, four other strains had high phylogenetic indices among themselves or with strains from this database, which reveals the high degree of preservation of the *16S rRNA* gene inherited from their common ancestors. Despite the *16S rRNA* marker (27F and 1492R (Haygood & Davidson (1997)) not being the gold standard in the taxonomic identification of this group of microorganisms, the possibility of new species can be

considered due to this gene having the most evolutionarily conserved region among bacteria (Komaki 2023; Takahashi 2017). Thus, this possibility is greater among the more than 50% of the strains with similarities below 98% to those of GenBank and EzBioCloud and especially among the three with similarity below 90% in at least one of these databases.

Added to the rich biodiversity in this sample of actinomycetes is the potential for their application, which is particularly observed in the antifungal assays of the 20 strains against phytopathogens of agricultural importance, namely *Colletotrichum* sp. ISO01, *C. guaranicola* P01, *Pestalotiopsis* sp. 3002R2, *Corynespora cassiicola* ISO079 and *Sclerotium coffeicola* M01. These phytopathogens are reported to cause problems that generate millions of dollars in losses for agriculture. *Corynespora cassiicola* is widely cited as a disease-causing agent in more than 530 plant species of 380 different genera (Ma et al. 2018) and is a mycosis-causing infectious agent in humans working with vegetable crops (Huang et al. 2010). According to O'Connell et al. (2012), the genus *Colletotrichum* is responsible for diseases in more than 3,200 plant species. The genus *Pestalotiopsis* harbors several species of biotechnological interest (Maharachchikumbura et al. 2011; Sun et al. 2014), but some species are reported to be phytopathogens (Deng et al. 2013; Liotti et al. 2018; Marimuthu et al. 2020). *S. coffeicola* has a high impact on agricultural production and is responsible for lesions in Indian mulberry (*Morinda citrifolia* L.) leaves (Gasparotto et al. 2019) and mango trees (*Mangifera* sp.), which are important plants for traditional medicine and in the food and cosmetic industries (Gupta et al. 2022; Ribeiro and Schieber 2010).

The antagonistic results achieved in the *in vitro* assays, which are a prerequisite in methodologies that are currently under development within a more sustainable agricultural model (Oyedoh et al. 2023), confirm the broad capacity of *Streptomyces* species to fight other microorganisms, particularly fungi, and indicate that all 20 strains can be sources of antifungal substances. The production curve assays and concomitant evolution of antifungal activities of periodic extracts from the strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), as well as the minimum inhibitory dosage tests, ratified their potential and suggest highly effective antifungal substances, considering their probable low concentration in these extracts. Chemical studies are being conducted to reveal the active ingredients of the extracts of these strains, which potentially have a broad antifungal spectrum and are strong candidates to combat the phytopathogens *Colletotrichum guaranicola* (P01) and *Pestalotiopsis* sp. (3002R2), which threaten the guarana plant (*P. cupana*), a very important crop in the economy of Amazonian communities. It is one of the best sources of caffeine in the world, and the powdered form is used in the production of soft drinks, as a food supplement and in traditional medicine (Marques et al. 2019). In addition, B280 was also active against *Sclerotium coffeicola* M01, a phytopathogen that is recognized worldwide for affecting hundreds of plants of agricultural importance.

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. Actinomycetes are cited in several studies as being excellent fertilizers, nitrogen fixers, and producers of various lytic enzymes, phytohormones and antimicrobials; as well as siderophores (Liotti et al. 2019; Liu et al. 2019 (a); Liu et al. 2019 (b); Strobel et al. 2004; Oyedoh et al. 2023). *In this context, according to the*

results obtained in the present work, actinomycetes from the rhizosphere of I. edulis represent a viable alternative for sustainable agriculture. Furthermore, the qualities mentioned above for actinomycetes and the results in this work may be related to the success of *I. edulis* as an ecological vector, which is used in South America in mixed crops and forest management that favor plants of economic importance (Nichols and Carpenter 2006; Piato et al. 2021; Piato et al. 2022; Sobanski and Marques 2014; Iglesias et al. 2011). *It is possible that this success is due, in part, to rhizotopic actinomycetes transported via seeds or seedlings from one soil to another and that I. edulis plants, when growing, also spread them through the litter that they commonly generate in abundance (*AbdElgawad et al. 2020; Hardoim et al. 2015; Jayasinghe andParkinson, 2008; Shahzad et al. 2018*). This possibility, however, needs to be investigated.*

Conclusion

In this study, it was revealed that the rhizospheres of three individuals of *I. edulis* harbor a rich diversity of actinomycetes with great potential against phytopathogenic fungi that commonly ravage plantations of numerous crops of global economic importance. Twenty strains showed antifungal activities against at least two phytopathogens, especially the two strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), whose extracts showed fungicidal activities at low concentrations, revealing them to be sources of metabolites with potent antifungal activities. These results are an encouraging example of the richness of actinomycetes that are associated with *I. edulis* in the Amazon, and they should be explored as potential producers of bioactive metabolites, especially against phytopathogenic fungi, and for the development of microbial products to be used in agriculture around the world. Moreover, the possibility of new species was signaled by the genotyping results, and these will be subsequently investigated. This study is thus further evidence of the vast biodiversity in the Amazon and reinforces the need to preserve it.

Declarations

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Competing interests

The authors have no relevant financial interests to disclose

Author contributions

Rafael de Souza Rodrigues: project development and manuscript preparation; Anderson Nogueira Barbosa: data analysis; Sarah Raquel Silveira da Silva Santiago: assistance with morphological identification; Roneres Deniz Barbosa and Jania Lilia da Silva Bentes: isolations and identifications of phytopathogens; Thalita Caroline Lima Alves: biological assays; Jeferson Chagas da Cruz: molecular identification; Gilvan Ferreira da Silva: technical and financial support; Antonia Queiroz Lima de Souza and Afonso Duarte Leão de Souza: technical and financial support, methodology, supervision and preparation of the manuscript.

Data availability

Data are contained in the article or Supplementary Material MS 1.

Ethics approval

The strains were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number AC1746C

Consent to participate

This study did not involve human beings in its experiments.

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Tables

Table 1. Phenotypic and genotypic characteristics of actinomycetes isolated from the rhizospheres of three individuals of *Inga edulis* from the campus of the Federal University of Amazonas.

Isolated strains	Macromophology (colony)	Micromorphology (spore chain)	GenBank/NCBI	EzBioCloud
Streptomyces sp. LaBMicrA B267	Aerial mycelium light gray; deep orange-yellow back; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (99.51%)	*S. silvae* For3 (99.34%)
Streptomyces sp. LaBMicrA B270	Aerial mycelium light gray; back deep yellow-brown; edge rough	Long/flexible partially spiral and cylindrical segments	*S. bullii* C2 (96.41%)	*S. murinus* NBRC 12799 (98.36%)
			S. murinus NRRL B-2286 (96.41%)	
			S. lanatus ISP 5090 (96.41%)	*S. lanatus* NBRC 12787 (98.36%)
			S. fractus MV32 (96.41%)	*S. fractus* MV32 (98.36%)
			S. graminearus NBRC 15420 (96.41%)	*S. graminearus* NBRC 15420 (98.36%)
			S. griseofuscus NBRC 12870 (96.41%)	
Streptomyces sp. LaBMicrA B271	Aerial mycelium light gray-brown; back bright yellow-orange; edge rough	Long/flexible partially spiralled and ovoid segments	*S. graminearus* NBRC 15420 (97.53%)	*S. murinus* NBRC 12799 (96.66%)
			S. murinus NBRC 14802 (97.53%)	
			S. griseofuscus NBRC 12870 (97.53%)	*S. graminearus* NBRC 15420 (96.66%)
			S. murinus NBRC 12799 (97.53%)	
Streptomyces sp. LaBMicrA B272	Aerial mycelium light gray; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, cylindrical and ovoid segments	*S. acidicola* K1PN6 (88.60%)	*S. sundarbansensis* MS1/7 (78.35%)
Streptomyces sp. LaBMicrA B278	Aerial mycelium light gray-brown; back moderate brown; edge rough	Partially long/flexible spirals, with spherical and ovoid clusters and segments	*S. misionensis* JCM 4497 (93.78%)	*S. misionensis* DSM 40306 (94.51%)
Streptomyces sp. LaBMicrA B280	Aerial mycelium yellowish-gray-brown; back deep yellow-orange; edge rough	Long/flexible partially spiralled, cylindrical following	*S. graminearus* NBRC 15420 (99.09%)	*S. graminearus* NBRC 15420 (98.83%)
				S. murinus NBRC 12799 (98.83%)
Kitasatospora sp. LaBMicrA B282	Aerial mycelium white; back deep brownish-yellow; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*K. acidiphila* MMS16-CNU292 (92.45%)	*K. acidiphila* MMS16-CNU292 (90.98%)
Streptomyces sp. LaBMicrA B301	Aerial mycelium bluish-white; back moderate olive-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (96.65%)	S. *durocortorensis* RHZ10 (96.93 %)
Streptomyces sp. LaBMicrA B304	Aerial mycelium light grey-brown; back deep	Long/flexible partially spiralled,	*S. pulveraceus* NBRC 3855 (97.68%)	*S. pulveraceus* LMG 20322 (98.70)

	yellowish-brown; edge rough	spherical and ovoid segments		
Streptomyces sp. LaBMicrA B310	Aerial mycelium light grey-brown; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (98.96%)	*S. durocortorensis* RHZ10 (99.07 %)
Kitasatospora sp. LaBMicrA B313	Aerial mycelium yellowish-gray; back light olive-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*K. arboriphila* HKI 0189 (98.44%) *K. aureofaciens* KACC 20180 (98.44%)	*K. arboriphila* HKI 0189 (98.70%)
Streptomyces sp. LaBMicrA B314	Aerial mycelium white; back deep yellow-orange; edge rough	Partially spiral long/flexible long, spherical and ovoid segments	*S. olivaceus* NBRC 12805 (99.61%)	*S. olivaceus* NRRL B-3009 (99.74%)
Streptomyces sp. LaBMicrA B317	Aerial mycelium pinkish-gray; back deep yellow-orange; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. jiujiangensis* JXJ 0074 (97.11%)	*S. barringtoniae* JA03 (96.79)
Streptomyces sp. LaBMicrA B318	Aerial mycelium white; back deep yellow-orange; edge rough	Long/flexible partially spiral and cylindrical segments	*S. similanensis* KC-106 (95.10%)	*S. similanensis* KC-106 (95.45%)
Streptomyces sp. LaBMicrA B319	Aerial mycelium light bluish-gray; back deep yellowish-brown; edge rough	Long/flexible, short/compact partially spiral and cylindrical segments	*S. acrimycini* NBRC 12736 (97.93%) *S. roietensis* WES2 (97.93%)	*S. anandii* NRRL B-3590 (97.72%) *S. pimonensis* TRM75549 (97.72%)
Streptomyces sp. LaBMicrA B322	Aerial mycelium light grayish-brown; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. enissocaesilis* NRRL B-16365 (96.76%) *S. rochei* NRRL B-1559 (96.76%) *S. vinaceusdrappus* NBRC 13099 (96.76%) *S. plicatus* NBRC 13071 (96.76%) *S. vinaceusdrappus* NRRL 2363 (96.76%)	*S. rochei* NRRL B-2410 (100%)
Streptomyces sp. LaBMicrA B325	Aerial mycelium vivid yellowish-pink; back deep orange; rough edge	Long/flexible partially spiral and cylindrical segments	*S. katrae* NRRL B-3093 (99.19%) *S. racemochromogenes* NBRC 12906 (99.19%) *S. polychromogenes* NRRL B-3032 (99.19%) *S. polychromogenes* NBRC 13072 (99.19%)	*S. katrae* NRRL ISP 5550 (97.66%) *S. racemochromogenes* NRRL B-5430 (97.66%)
Streptomyces sp. LaBMicrA B289	Aerial mycelium white; back deep	Long/flexible partially spiral and	*S. sennicomposti* RCPT1-4 (99.16%)	*S. venetus* CMUAB225 (82.22%)

	orangey-yellow; rough edge	cylindrical segments		
Streptomyces sp. LaBMicrA B290	Aerial mycelium brownish-white; back bright yellow-orange; rough edge	Partially long/flexible spirals, with bunches, spherical and ovoid segment	*S. gramineararus* NBRC 15420 (99.09%)	*S. murinus* NBRC 12799 (99.22%)
				S. gramineararus NBRC 15420 (99.22%)
Streptomyces sp. LaBMicrA B299	Aerial mycelium moderate brown; back bright yellow-orange; rough edge	Long/flexible partially spiralled, spherical and ovoid segments	*S. jiujiangensis* strain JXJ 0074 (87.36%)	*S. panaciradicis* 1MR8 (92.88%)

Note.: The shades of the aerial mycelia and backs were determined using the ISCC/NBS color system. The marker used was the *16S rRNA* gene.

Table 2. Minimum inhibitory dosage of extracts of the strains *Streptomyces* sp. LaBMicrA B270 and B280.

Strains tested	Concentrations and activities					Phytopathogens
	1,000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	062 µg/mL	
	Acetate /isopropanol fraction (culture medium)					
Streptomyces sp. LaBMicrA B270	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	±	–	*C. guaranicola* (P01)
	+	+	+	±	–	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	+	±	*C. guaranicola* (P01)
	+	+	+	+	±	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	+	+	+	±	–	*Sclerotium coffeicola* (M01)
Acetate/methanol fraction (cell biomass)						
Streptomyces sp. LaBMicrA B270	–	–	–	–	–	*Colletotrichum* sp. (ISO01)
	–	–	–	–	–	*C. guaranicola* (P01)
	+	+	±	–	–	*Corysnepora cassiicola* (ISO079)
	+	±	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	±	–	–	*Colletotrichum* sp. (ISO01)
	+	±	–	–	–	*C. guaranicola* (P01)
	+	±	–	–	–	*Corysnepora cassiicola* (ISO079)
	–	–	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)

Note: + = fungicidal; ± = Fungistatic – - = no activity

Figures

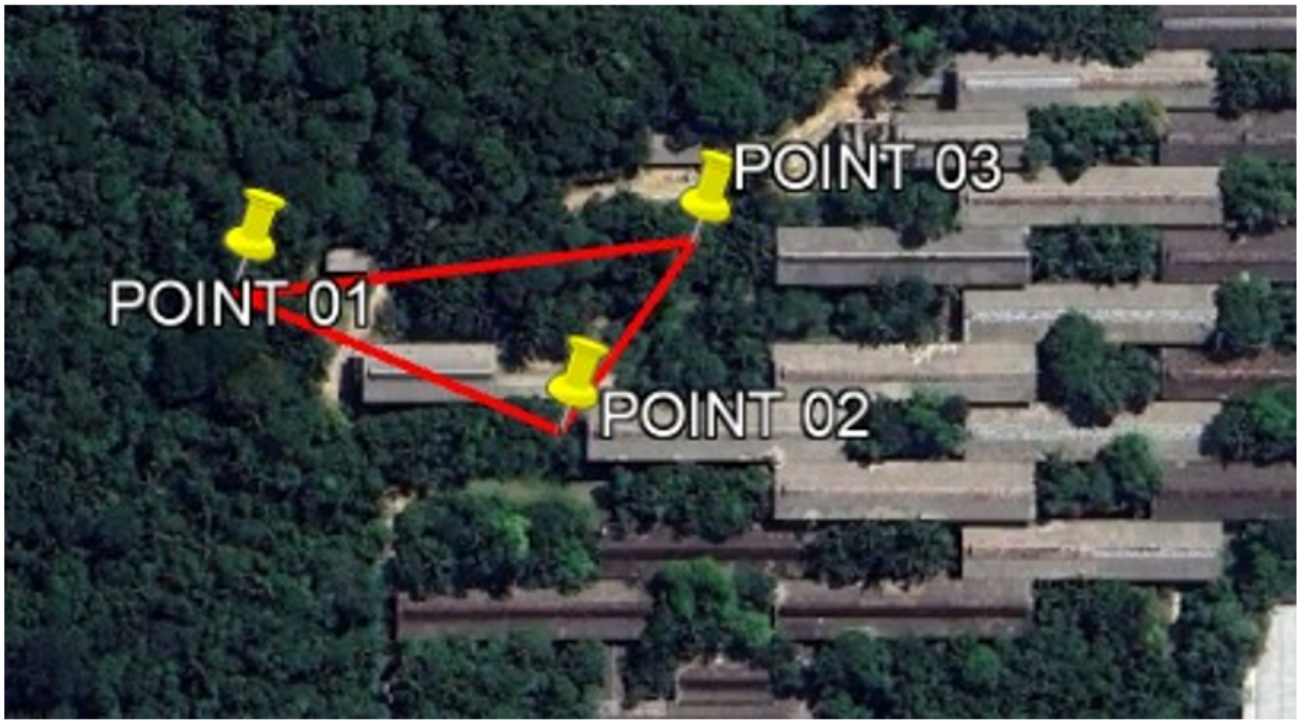

Figure 1

Figure 1

Image of the three collection points on the UFAM campus (source: Google Maps).

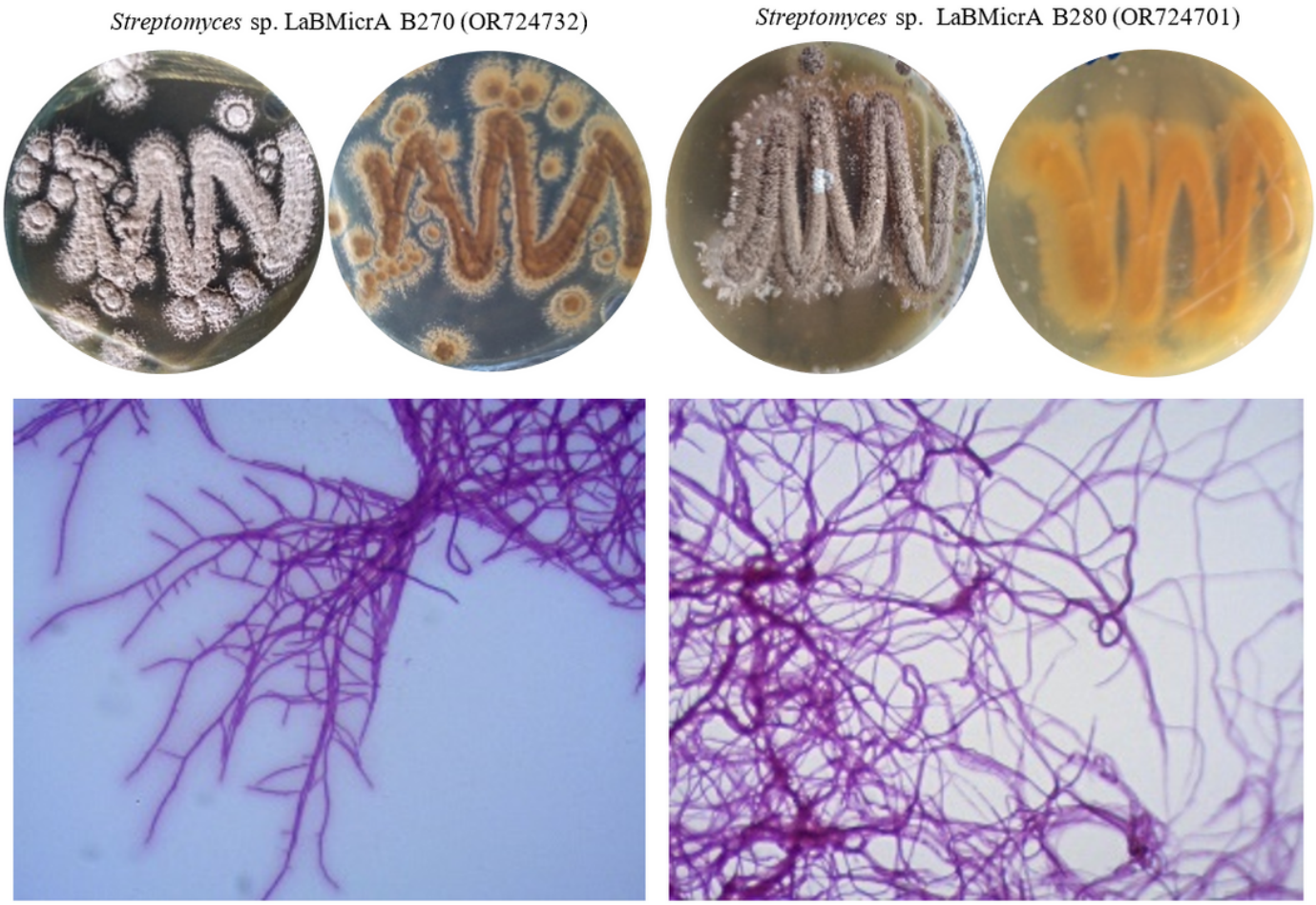

Figure 2

Figure 2

Macro- and micromorphological characteristics of strains with outstanding antifungal activities. Microcultures visualized under an optical microscope (Carl Zeiss) coupled to the Zen Photo/Image program (AxioCamER-Zen lite 2012), using a 100x objective and 0.5 µm scale. Below: mycelial ornamentations; above: front and back of colonies on the twentieth day of cultivation.

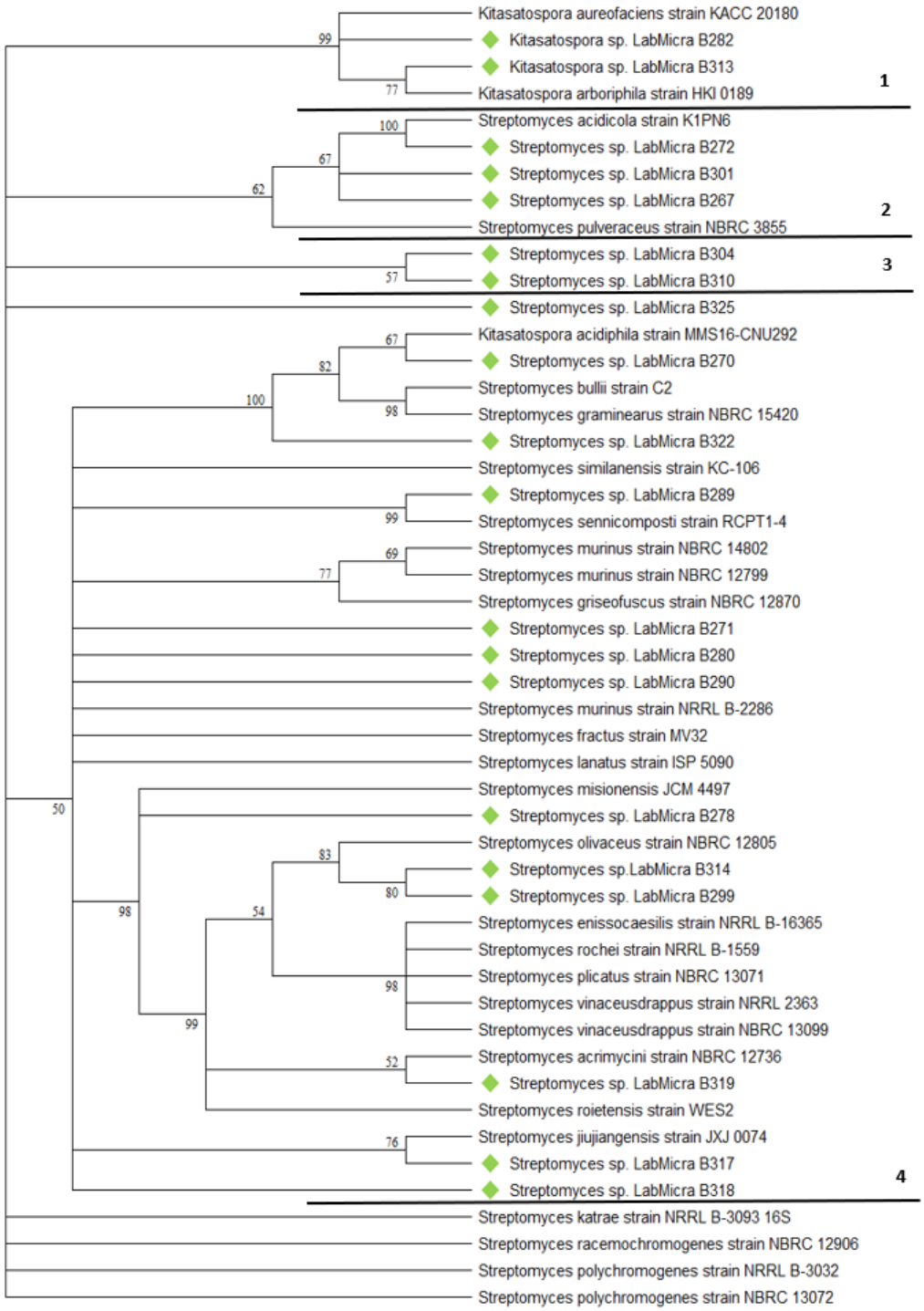

Figure 3

Figure 3

Phylogenetic tree of partial sequences of the *16S rRNA* gene (650-800 nt) of actinobacteria from the rhizospheres of three individuals of *Inga edulis* (green rhombus) and strains of the GenBank-NCBI database. Bootstrap values ≥ 50% only, with a maximum of 1,000 randomizations and 0.05 substitutions per nucleotide position (bootstrap method).

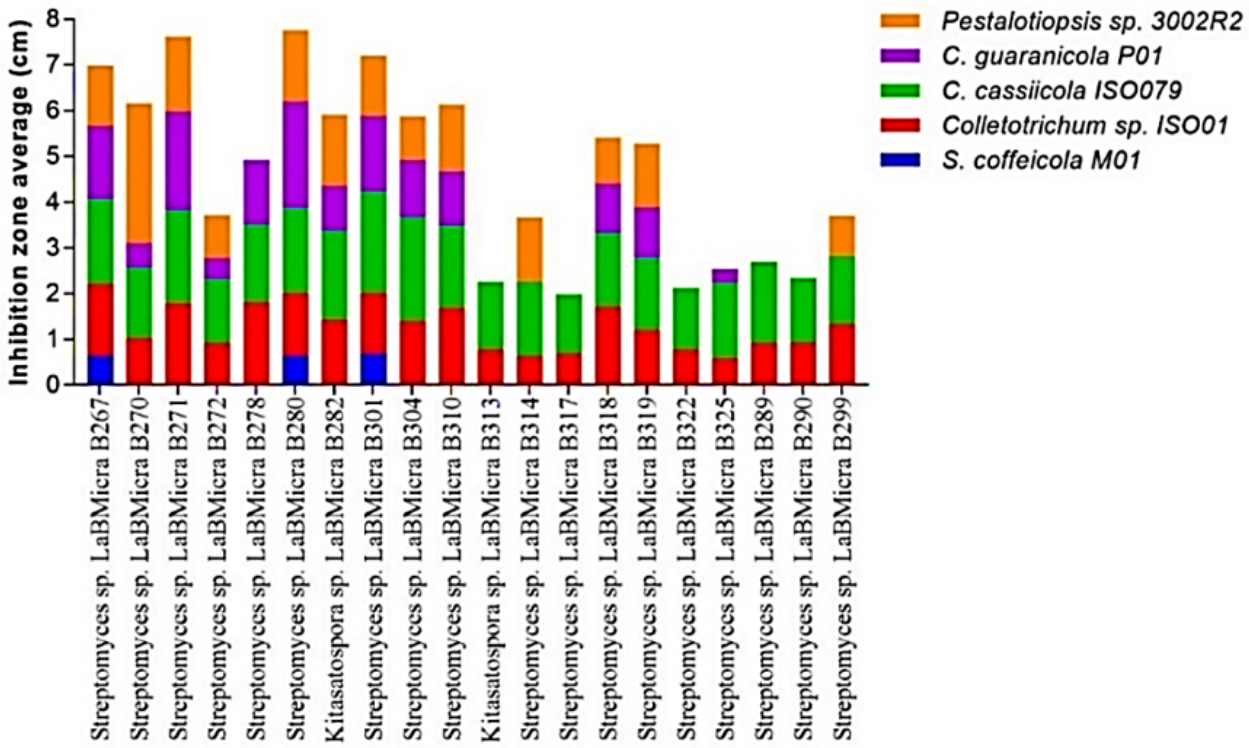

Figure 4

Figure 4

Stacked bar graph proportional to the mean values of the inhibition zones of the respective phytopathogens, considered as the distances between the cultures (Fig. 5; GraphPad Prism version 8.0.1).

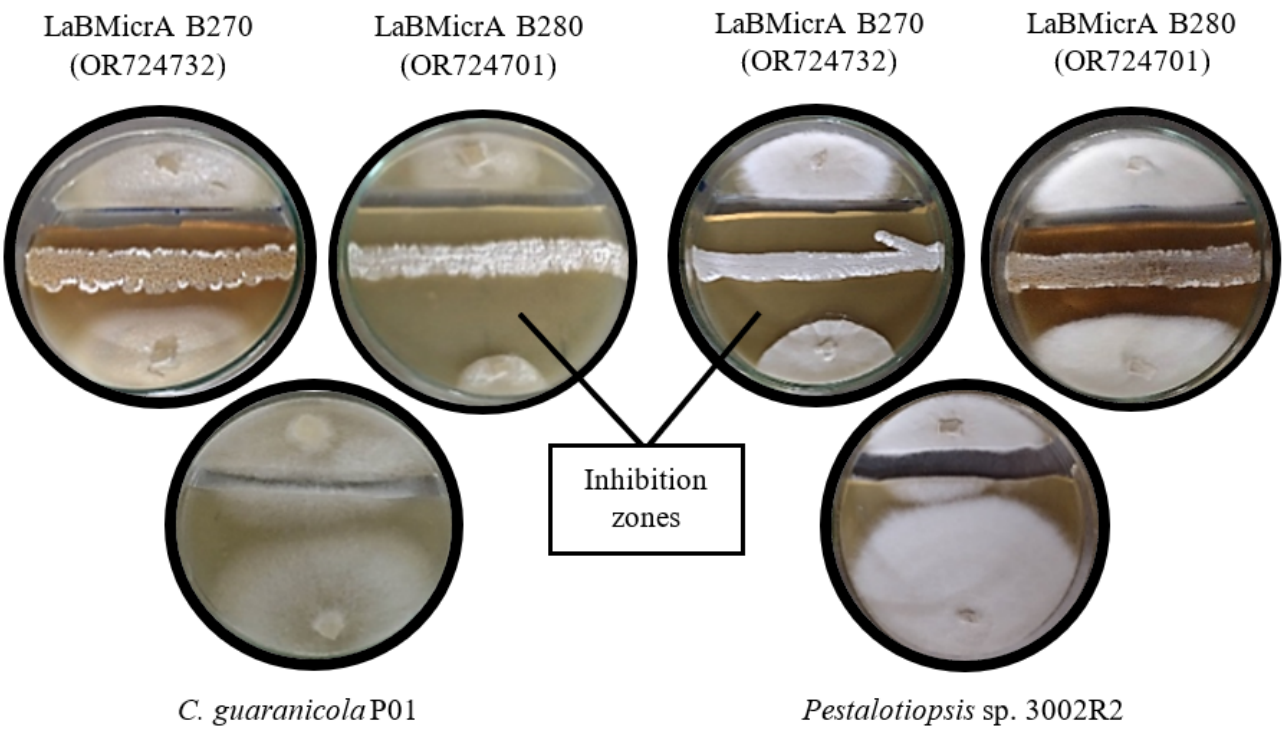

Figure 5

Figure 5

Paired cultures of the strains *Streptomyces* sp. LaBMicrA B270 (OR724732) and B280 (OR724701) with the phytopathogens *Colletotrichum guaranicola* (P01) and *Pestalotiopsis* sp. (3002R2). Above: the pairing on the seventh day, with the actinomycetes in line at the center of the plates. Below: the control containing only the phytopathogens after the tenth day of culture.

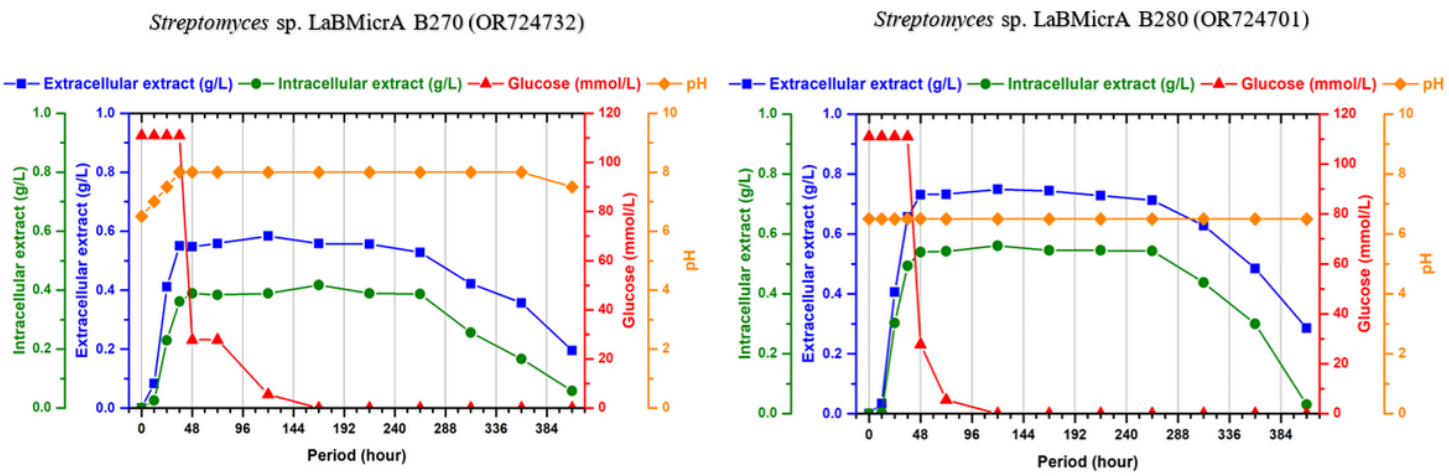

Figure 6

Figure 6

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

Supplementary Files

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- SupplementarymaterialMS1.docx