ANTIBACTERIAL ACTIVITY AND VIRULENCE FACTORS INHIBITION BY *Xylaria* sp. (Xylariaceae, Ascomycota): A STUDY OF BIOACTIVE POTENTIAL †

[ACTIVIDAD ANTIBACTERIANA E INHIBICIÓN DE FACTORES DE VIRULENCIA POR *Xylaria* sp. (Xylariaceae, Ascomycota): UN ESTUDIO DEL POTENCIAL BIOACTIVO]

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SUMMARY

Background: The genus *Xylaria* comprises over 300 fungal species distributed worldwide that exhibit adaptability to various ecological roles. Consequently, their potential for the synthesis of bioactive molecules with antibacterial and antivirulence properties has been reported. **Objective:** To characterize the antibacterial and antivirulence properties of *Xylaria* sp. (OG-03) strain against phytopathogenic bacteria (*Pseudomonas syringae*, *Pseudomonas syringae* pv. *tabaci*, *Pseudomonas putida*, and *Chryseobacterium* sp.) and *Chromobacterium violaceum* 553, respectively. **Methodology:** A fungal strain was isolated and characterized morphologically and molecularly, and its evolutionary history was investigated through phylogenetic reconstruction. Mycelial growth was assessed in different culture media with natural substrates, and fungal extracts were obtained to evaluate minimal inhibitory (phytopathogenic bacteria) and antivirulence (biosensor strain) activities. **Results:** Morphological and molecular characterizations of the fungal strain suggested an indeterminate taxonomic classification at the species level within the genus *Xylaria*. The highest mycelial growth was observed in the REA culture medium, and the liquid rice extract promoted ectostomes proliferation. Fungal biomass extracts displayed antibacterial activity against *P. syringae* (MIC 7.81 µg/mL, 88% inhibition), *Pseudomonas syringae* pv. *tabaci* (MIC 1.95 µg/mL, 87% inhibition), *Pseudomonas putida* (MIC 1.95 µg/mL, 79.25% inhibition), and *Chryseobacterium* sp. (MIC 7.81 µg/mL, 85.03% inhibition), respectively. Antivirulence against *C. violaceum* reduced biofilm formation (125 µg/mL, 59% inhibition) and violacein production (62.5 µg/mL, 58% inhibition). **Implications:** *Xylaria* sp. exhibits antibacterial and antivirulence activity against phytopathogenic bacteria. **Conclusions:** The strain studied is suggested to be an undetermined taxon within the genus *Xylaria*. The results of biological assays indicated that the fungus possesses antibiotic properties against phytopathogenic bacteria and can inhibit virulence factors associated with quorum sensing.

Key words: antivirulence; endophytic fungus; minimum inhibitory concentration; phylogenetic reconstruction; phytopathogenic bacteria

RESUMEN

Antecedentes. El género *Xylaria* agrupa más de 300 especies de hongos distribuidas en todo el mundo, y muchas de ellas poseen capacidad adaptativa a diversos roles ecológicos. En virtud de lo anterior, se ha descrito su potencial para sintetizar moléculas bioactivas con actividad antibacteriana y antivirulencia. **Objetivo**. Caracterizar la capacidad

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antibacteriana y de antivirulencia de una cepa de *Xylaria* sp. (OG-03) contra bacterias fitopatógenas (*Pseudomonas syringae*, *Pseudomonas syringae* pv. *tabaci*, *Pseudomonas putida* y *Chryseobacterium* sp.) así como contra *Chromobacterium violaceum* (553), respectivamente. **Metodología.** Se aisló y caracterizó morfológica y molecularmente una cepa del género *Xylaria*. Se estudió su historia evolutiva a través de reconstrucciones filogenéticas. Se evaluó su crecimiento micelial en diferentes medios de cultivo con sustratos naturales, y se obtuvieron extractos fúngicos para evaluar actividades mínimas inhibitorias (bacterias fitopatógenas) y antivirulencia (cepa biosensora). **Resultados.** La caracterización morfológica y molecular de la cepa fúngica sugieren una clasificación taxonómica indeterminada a nivel de especie, perteneciente al género *Xylaria*. El mayor crecimiento micelial se observó en medio de cultivo REA y el extracto de arroz líquido favoreció la proliferación de ectostromas. Los extractos fúngicos de biomasa mostraron actividad antibacteriana contra *P. syringae* (CMI 7.81 µg/mL, 88% de inhibición); *Pseudomonas syringae* pv. *tabaci* (CIM 1.95 µg/mL, 87% de inhibición); *Pseudomonas putida* (CIM 1.95 µg/mL, 79.25% de inhibición) y *Chryseobacterium* sp. (CIM 7.81 µg/mL, 85.03% de inhibición). El efecto antivirulencia sobre *C. violaceum* disminuyó la formación de biopelículas (125 µg/mL, 59% de inhibición) así como la producción de violaceína (62.5 µg/mL, 58% de inhibición). **Implicaciones.** *Xylaria* sp. se encuentra involucrada en fenómenos de actividad antibacteriana y antivirulencia contra bacterias fitopatógenas. **Conclusiones.** Se sugiere que la cepa sujeta a estudio es un taxon indeterminado perteneciente al género *Xylaria*. Los resultados de actividades biológicas indican que el hongo posee actividad antibiótica contra bacterias fitopatógenas, siendo igualmente capaz de inhibir factores de virulencia asociados a la detección del quorum.

Palabras clave: antivirulencia; bacterias fitopatógenas; concentración mínima inhibitoria; hongos endófitos; reconstrucción filogenética.

INTRODUCTION

Xylaria Hill ex Schrank, a prominent genus within the Xylariaceae family, is widely distributed and encompasses numerous Xylariales species (Helaly *et al*., 2018). As of 2022, the Index Fungorum database records over 800 epithets with more than 300 species reported globally (Ma *et al*., 2022). At least 109 species have been described in Mexico, with those inhabiting tropical deciduous forests accounting for 18% of the country's total records (Osorio-Navarro *et al*., 2022; Raymundo *et al*., 2014, 2017). *Xylaria* species are distinguished by the shape of their stroma, which protrudes into forests and is particularly noticeable in *Xylaria polymorpha* (Pers.) Grev, commonly known as "dead man's fingers." The genus thrives on decaying wood and demonstrates a broad distribution capacity, surviving as both endophytes and saprophytes.

Certain fungi within the genus *Xylaria* inhabit fruits, fallen seeds, leaves, petioles, and termite nests (Ma *et al*., 2022). Notably, they synthesize compounds with antibacterial activity (Indarmawan *et al*., 2016; Santiago *et al*., 2021) and have been reported to induce allelopathic effects on turnip (*Raphanus sativus* L.) and wheat (*Triticum aestivum* L.), which are attributed to the production of cytochalasins (Wen-Bo *et al*., 2019). These effects are potentially linked to their capacity to act as endophytes in various plant species to produce compounds with diverse biological activities. In turn, this may be associated with an inhibitory effect on multiple virulence factors related to bacterial infections (Zhao *et al*., 2020).

The aforementioned phenomenon occurs when bacterial cells communicate through autoinductive molecules (signaling pathways), a process also known as "quorum sensing," which is directly related to microbial population density. A virulence factor inhibitor interferes with this communication by targeting specific elements such as the synthesis of autoinducer molecules and receptors involved in bacterial transcription processes (Zhao *et al*., 2020). Consequently, inhibition of this cell communication mechanism by fungi such as Xylariales offers a novel biotechnological alternative. This approach has the potential to combat various infection processes caused by bacterial populations, primarily in the field of phytopathology and other emerging disciplines within the natural sciences, including public health (Indarmawan *et al*., 2016).

Considering the aforementioned information, this study provides initial insights into the ability of a *Xylaria* strain to inhibit various virulence factors mediated by quorum sensing. Furthermore, this is the first report of the antibacterial activity of fungal extracts against two phytopathogenic strains (genera *Pseudomonas* and *Chryseobacterium*) utilized in this research.

MATERIALS AND METHODS

Isolation of the fungal strain

Ten grams of healthy oregano (*Origanum vulgare* L.) leaves were collected from an agroecological garden in Xalapa (19°31'52.46" N, 96°54'57.2" W), Veracruz, Mexico. The plant material was stored in sterile airtight plastic bags and transported to the laboratory. The leaves were washed with distilled water, followed by a 10% diluted detergent, and rinsed to remove any impurities. Leaf surfaces were sterilized using the

protocol described by Kathawut *et al*. (2020): samples were immersed in 70% ethanol for 1 min, washed with 2.5% NaClO solution for 4 min, immersed in 70% ethanol for 30 s, and finally rinsed with distilled water. Sterile leaves were cut into 1 × 1 cm pieces and placed in Petri dishes containing potato dextrose agar (PDA) (DIBICO®) supplemented with chloramphenicol (5 µg/mL). The dishes were incubated for four weeks at 25 ± 2 °C. Hyphal tips from several previously purified isolates were reseeded to obtain pure culture. The fungal isolate exhibiting the characteristics of interest was labeled OG-03.

Molecular characterization of the fungal strain

The isolated strain underwent preliminary identification using morphological keys (Becerril-Navarrete *et al*., 2018), and was further molecularly characterized. First, superficial stromal fragments of the strain were disinfected with NaClO. To induce mycelial growth, the slices were placed in Petri dishes containing PDA for one week. Following the protocol of Lin *et al*. (2001), genomic DNA was extracted from young mycelia using a methanol-chloroform mixture (1:1, v/v) as the solvent. Sequencing of transcripts generated by conventional PCR of conserved genomic regions (ITS1-ITS2 and 5.8 S rRNA) and elongation factors (EF-1α) was performed using a sequencing-by-synthesis platform (NextSeq 550 Illumina). A composite maximum likelihood model (Stolz *et al*., 2019) was employed to calculate the position and mean of each amplicon. The homology of each amplicon was compared with nucleotide collections (nr/nt) from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), selecting accessions with the highest identity percentages and read coverage as references. To further analyze the data, multiple sequence comparison using log-normal expectation (MUSCLE) was performed (Madeira *et al*., 2022). Consensus regions (≈90%) consisted of ITS1-ITS2 and 5.8 S rRNA fragments, and a short elongation factor sequence to a lesser extent.

Molecular taxonomic evolution study (phylogenetic reconstruction)

The statistical methods of nearest neighbor-joining (NJ) and maximum likelihood (MLE) were employed to reconstruct the referenced cladograms (Saitou and Nei, 1987), with 500 and 1,000 replicates, respectively (Felsenstein, 1985). The evolutionary distances were determined using the composite maximum likelihood method (Tamura *et al*., 2004). MEGA7 version 7.0 software (Kumar *et al*., 2016) was used for each evolutionary analysis. BLASTn analysis was then performed against the reference nucleotide sequences to generate optimal reconstructions. The dataset includes over 70 accessions of fungal species previously deposited in the NCBI repository. Additionally, evolutionary divergence between species was calculated by comparing the maximum likelihood value for the given topology under the Tamura-Nei model (Tamura *et al*., 2021).

Obtaining fungal extracts

To obtain fungal extracts, three 250 mL Erlenmeyer flasks were inoculated with 50 mL of PDA medium. After observing mycelial growth at 25 ± 2 °C for four weeks, a spore suspension was prepared in Tween 80 solution and distributed into twelve 250 mL Erlenmeyer flasks containing 125 mL of PDB broth (MCD LAB). These flasks were incubated at room temperature and 150 rpm for 12 days, followed by 12 days in a static state at 28 ± 2 °C (Navarro *et al*., 2022). The biomass was then filtered from the broth by vacuum filtration with sterile Whatman filter paper grade 1 (11 mm), separating the two phases. To extract fungal metabolites, the biomass and broth samples were first frozen and then dehydrated in a lyophilizer. The dried materials were macerated separately with a chloroform-methanol solution (1:1, v/v) for 24 h, sonicated, and concentrated in a rotary evaporator at 40 °C (Bhardwaj *et al*., 2015; Lagunes *et al*., 2015).

Development of fungal culture media with natural substrates

The culture media previously described by Koley and Mahapatra (2015) were prepared without the presence of salts. The prepared media and their preparation methods were as follows: (1) rice agar (REA): rice (50 g/L) was boiled in distilled water and agar (14 g/L) was added; (2) oat-agar (OM):20 g/L of ground oat flakes were used until a homogeneous powder was obtained, to which distilled water and agar (14 g/L) were added; (3) corn flour agar (CM):50 g/L of MASECA® brand corn flour, 12 g/L of dextrose, and 14 g/L of agar were used. The media were sterilized at 120 psi for 15 min, the initial pH was adjusted to 7.0, and chloramphenicol (5 µg/mL) was added to prevent possible contamination. Sterile PDA was used as a control, and all cultures were incubated in triplicates for 10 days. Additionally, a liquid culture of the fungus was performed in a 500 mL Erlenmeyer flask supplemented with rice substrate (100 g) in 100 mL of sterile water, following the methodology described by Long *et al*. (2023) with some modifications. They were then incubated for 60 days at 25 ± 2 °C under alternating light and dark conditions (30 days each).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of the biomass and broth extracts were determined against

phytopathogenic bacteria, including *P. syringae* A20 and *P. syringae* pv. *tabaci*, *Pseudomonas putida* M11, and *Chryseobacterium* sp. B1-5. These strains were part of a collection belonging to the Center for Applied Mycology Research of the University of Veracruz, whose institute made a donation. The assays were carried out using microdilutions in 96-well round-bottom plates (Sarstedt Inc.), following the methodology described by Talukdar *et al*. (2021). Extracts were diluted to concentrations of 0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, and 125 µg/mL, and the final volume of fungal extract was calculated for each case. The wells were filled with 2 µL of the corresponding bacterial suspension (1.5 x 10^8 CFU/mL) to reach a final volume of 200 µL per well. Dimethyl sulfoxide (DMSO) 10% was used as a negative control, and bacterial growth determination without extract served as a positive control. The density of microorganisms was recorded using a microplate reader (Labsystems Multiskan MCC/340) at 620 nm every 2 h during the 12 hour culture incubation (28 ± 2 °C). Assays were performed in triplicate, and statistical significance was assessed using the nonparametric Mann-Whitney test ($P \leq 0.05$).

Evaluation of the inhibition effect of virulence factors on *Chromobacterium violaceum* 553 (biosensor strain)

The effects of the fungal extracts on two virulence factors in *C. violaceum* 553 (biofilm and violacein production inhibition) were measured. Prior to evaluation, the sub-inhibitory concentrations of the extracts (62.5, 125, 250, and 500 µg/mL) were determined to rule out bactericidal or bacteriostatic effects. The sub-inhibitory concentrations were calculated based on the effect of each extract. First, 20-hour cultures of *C. violaceum* 553 were obtained in liquid medium (LB) in 50 mL Falcon tubes, following the methodology described by Pérez-López *et al*. (2018). The evaluation was carried out in triplicate in 96-well round-bottom plates. For the tests, five µL of the fungal extract was added to reach final concentrations of 62.5, 125, 250, and 500 µg/mL, along with 195 µL of the bacterial culture. The plates were incubated at 150 rpm for 48 h at 28 ± 2 °C. To quantify violacein production, the contents of each well (200 µL) were transferred to 0.5 mL Eppendorf tubes with the addition of ethyl acetate (200 µL). The samples were then centrifuged to separate the two phases. The absorbance of the controls was considered 100% violacein production, as described by Pérez-López *et al*. (2018). Anacardic acid (obtained from hexane extracts of dried cuachalalate (*Amphipterygium adstringens*) bark, fractionated on silica gel columns and purified by preparative thin layer chromatography) at 125 µg/mL was used as a positive control (Castillo-Juarez *et al*., 2013), and 2.5% DMSO served as a negative control (Castillo-Juárez *et al*., 2013). For the biofilm formation inhibition assay, the methodology described by Pérez-López *et al*. (2018) was followed using 96-well round-bottomed plates. Five µL of the fungal extract was added to obtain final concentrations, along with 195 µL of culture. The plates were incubated at 150 rpm and 28 ± 2 °C for 24 hours. The biofilm was stained with 1% crystal violet and 80% ethanol and the absorbance was read at 540 nm using a microplate reader. Triplicates of the assays were performed, and statistical significance was determined using nonparametric Mann-Whitney tests ($P \leq 0.05$).

RESULTS AND DISCUSSION

Morphological and molecular-genetic characterization of the fungal strain

Specimens of the genus *Xylaria* are among the most abundant within the group of ascomycetes, particularly those that inhabit tropical forests. They exhibit various morphological and molecular-genetic characteristics depending on the species in question (San Martín and Rogers, 1989; 1995; 2005). The growth of the isolated strain in the Petri dish was radial, with the mycelium center changing from white to dark as it aged. After 60 days of incubation, black, erect, scaly textured, cylindrical teleomorphic ectostomes measuring 15-110 x 1-2 mm were observed (Figure 1a). At the end of the incubation period, the growth of ectostomes was observed and counted in liquid medium supplemented with rice and PDA medium. In total, 20 ectostomes were counted for both media, and in the liquid medium supplemented with rice, the mature ectostomes were divided in half, measured with a micrometer ruler from apex to base, and observed under a Carl Zeiss Primo Star microscope. In the same incubation period but in a rice-supplemented medium, the ectostome reached a length of 15-145 x 1-3 mm (Figure 1b), showing similar characteristics to those described by Rakshith *et al*. (2020). The cylindrical stipe displayed white shades. When transverse sections were made, reproductive structures were identified under a microscope. Additionally, some areas of blackened, dotted perithecia containing ascospores were observed. The perithecia were prominently oval in shape, dark in color, and 0.8 to 1.3 mm in size. The ascospores had ellipsoidal-inequilateral forms measuring 14-16 (17) x 5-6 mm, with a brown germ line running over the entire length of the spore. Based on these characteristics, it is suggested that the species we are dealing with could be *Xylaria multiplex*.

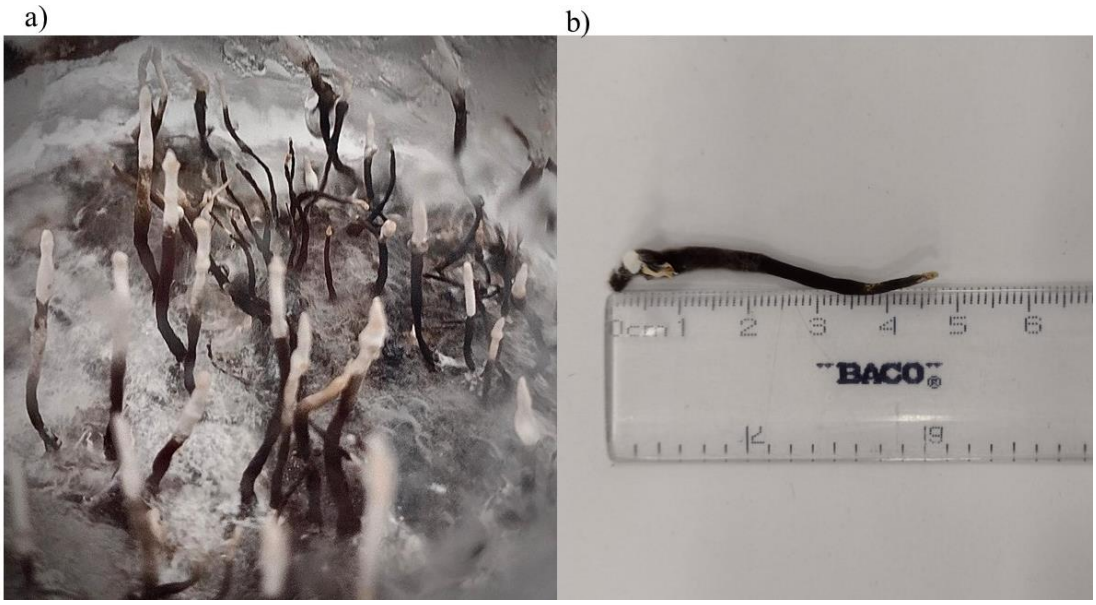

Figure 1. a) Development of ectostomes in PDA medium after 60 days of incubation, where thin and short bodies without maturation were observed; b) Strain of *Xylaria* sp. grown in liquid medium supplemented with rice substrate under stress conditions (60 days of incubation), where stromata were observed with an approximate length of 4.7 cm, narrow tip, slightly brown color, and wide and dark base.

When the *Xylaria* strain was grown in a liquid medium supplemented with rice substrate under stress conditions (60 days of incubation), stromata were produced with an approximate length of 4.7 cm, featuring a narrow tip and slightly brown color, as well as a wide and dark base. It is worth noting that *Xylaria* species are considered the most abundant species in the State of Veracruz (Medel *et al*., 2008), where up to 45 species have been recorded. Only 32 have been identified as lignicolous dicotyledonous wood, while the rest are endophytic microorganisms.

Since the morphological characterization results were not entirely conclusive for establishing the species-level taxonomic classification of the strain, the analysis was complemented with a concatenated nucleotide sequence (556 bp) formed from the transcripts of the ITS1 and ITS2 genes and the 5.8 S rRNA gene. Additionally, a short complementary EF-1α sequence was included. Thus, the consensus DNA sequence was molecularly similar to a fragment of the Xylariales genome. A BLASTn analysis was performed to identify potential matching organisms. Consequently, NCBI accessions corresponding to species with a higher percentage identity were filtered out. The accessions were: 1) MH003462: *Xylaria* sp. (90.29%); 2) MF435124: *Xylaria multiplex* (90.25%); 3) KR534704: *Xylaria adscendens* (90.11%); and 4) JX427059: *Xylaria hypoxylon* (90.60%), which mostly aligned some of their conserved regions in ITS and elongation factor genes with the concatenated sequence (obtained by conventional PCR using the respective molecular marker). The results were confirmed through comparative analysis using logarithmic expectation (Madeira *et al*., 2022). Detailed bioinformatic characteristics of the previously described sequence can be found in the NCBI nucleotide database under accession; GenBank: OQ732919.1

Molecular taxonomic evolution (phylogenetic reconstructions)

Figures 2 and 3 display the results of phylogenetic reconstructions (optimal molecular evolutionary analysis by neighbor-joining (NJ) and maximum likelihood (MLE) statistical methods, respectively) of the concatenated nucleotide sequence formed from ITS1 and ITS2 gene transcripts, the 5.8 S rRNA gene, and a fragment of a short complementary EF-1α sequence, as described above. Figure 2 shows a radial phylogenetic reconstruction supported by the NJ statistical method (Saitou and Nei, 1987). The optimal tree had a sum of the lengths of each branch of the 0.00925465 substitutions. The percentage of replicate trees in the associated taxa grouped in the bootstrap test was represented by 500 replicates (Felsenstein, 1985), and the evolutionary distances were calculated using the composite maximum likelihood method (Tamura *et al*., 2004), obtaining a total of 1,924,318 positions in the final dataset. Of the total number of taxa that made up the reconstruction, at least 44 were evolutionarily closest to the native strain, with the most representative (without regrouping by clades) being *X. necromorpha*, which forms a monophyletic group together with the native strain. Species belonging to the genera *Zopfia*,

Nothophoma, *Alternaria*, *Penicillium*, and *Aspergillus* have also been found, among many others. Taxa mostly close to the native strain are highlighted with a gray background, whereas the remaining taxa farther away are distinguished by a blue background. It should be noted that in the cladogram, there are several monophyletic groups with poly-and dichotomous branches, as well as internal nodes, and inferred common ancestors.

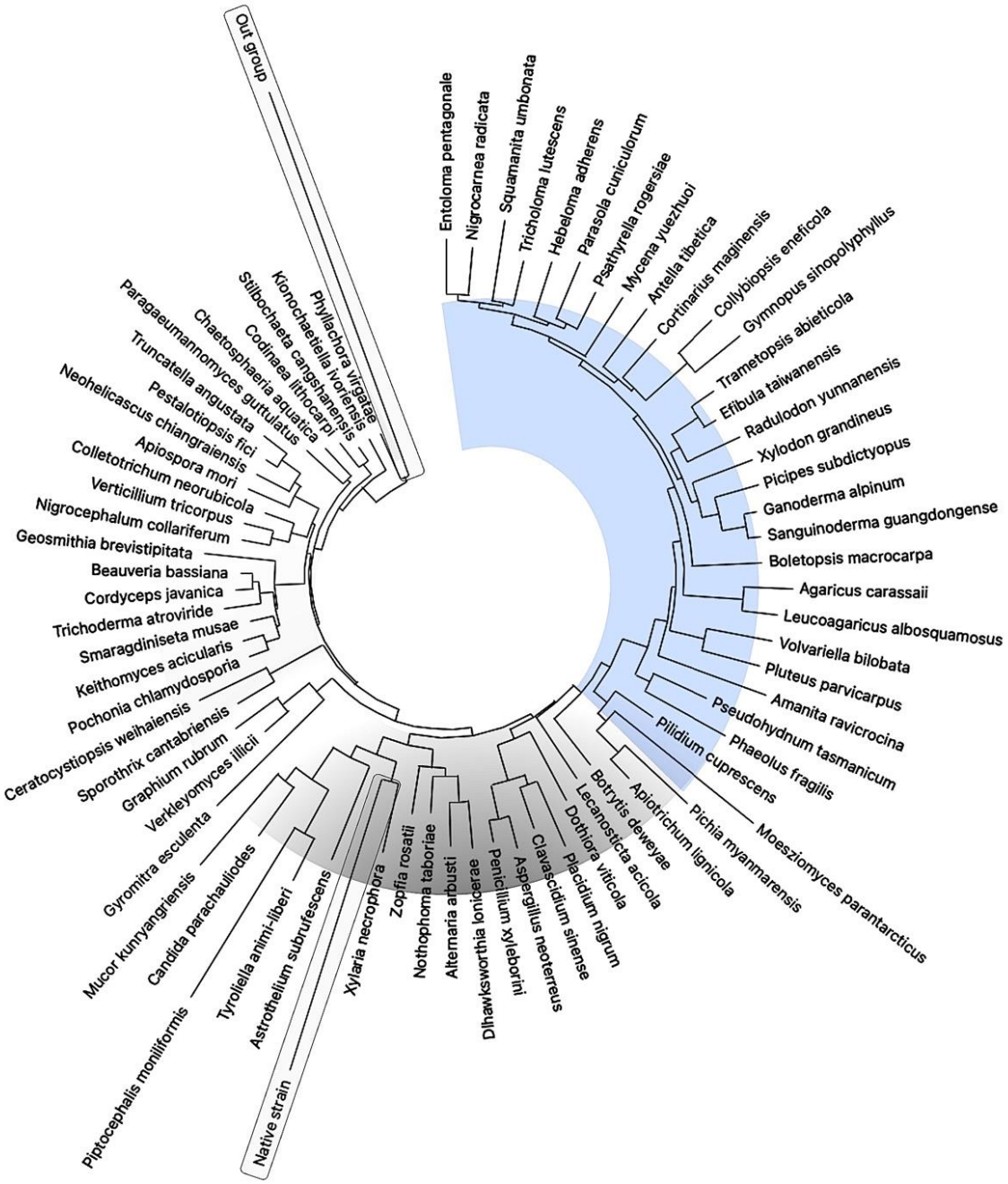

Figure 2. Radial phylogenetic reconstruction was supported by the neighbor-joining (NJ) statistical method (Saitou and Nei, 1987). The optimal tree with the branch length sum was 0.00925465. The percentage of replicate trees in the associated taxa grouped in the bootstrap test was represented by 500 replicates (data not shown) (Felsenstein, 1985). Evolutionary distances were calculated using the composite maximum likelihood method (Tamura *et al*., 2004) (shown in units of number of base substitutions per site). In total, 1,924,318 positions were observed in the final dataset. Evolutionary analyses were performed using MEGA7 (version 7.0; Kumar *et al*., 2016). The positions of the native strain and outgroup are boxed. Taxa closer to the native strain are highlighted with a grey background, and those farther away with a blue background are highlighted.

Figure 3 shows a linear phylogenetic reconstruction supported by the MLE statistical method using the Tamura-Nei model (Tamura and Nei, 1993). The cladogram yielded the highest possible log likelihood (-26,345,147.21 substitutions). The reconstruction was generated by a heuristic search obtained automatically by applying the NJ algorithm to a matrix of pairwise estimated distances, complemented by the maximum likelihood compositional model (MCL), and selecting the topology with the highest closeness value. To scale, the reconstruction shows the measured branch lengths in the number of substitutions per site. In total, 1,824,218 positions were observed in the final dataset. The native strain is highlighted in a box and regrouped with taxa belonging to one of the major clades (clade A). Clade B comprised of several of the remaining taxa. Both major clades come from an ancestor-descendant lineage, and interestingly, each is, in turn, descended from its adjacent secondary clade consisting of at least four paraphyletic taxa. Similar to radial reconstruction, it highlights the presence of poly-and dichotomous branches as well as several internal nodes and inferred common ancestors (Helaly *et al*., 2018; Ma *et al*., 2022; Osorio-Navarro *et al*., 2022; Raymundo *et al*., 2014, 2017).

Considering the above results, it is pertinent to mention that traditional approaches to fungal classification are often ambiguous and sometimes impossible (Koch *et al*., 2017; 2018). Consequently, accurate identification frequently requires highly technical laboratory work and may be complemented by extensive bioinformatics resources. For example, the most commonly used tools today are based on the study of different molecular markers (*i.e*., any gene whose expression and conserved regions allow a quantifiable or observable effect (phenotypic characteristics) and, above all, are easily detectable) (Shamim *et al*., 2017).

On the other hand, because current approaches to fungal taxonomy tend to adhere to heterogeneous heuristic models, each lineage is considered to be directly proportional and dependent on its evolutionary processes. For this reason, it is very challenging to unequivocally identify fungi, as various taxonomic approaches often struggle to resolve varying degrees of heterogeneity. Consequently, to date, there are no universal tools for reliable identification of these microorganisms (Oliveira and Azevedo, 2022). Given the results of the morphological and molecular characterization of the fungal strain under study, it was concluded that although the fungus belongs to the genus *Xylaria*, the species should be considered indeterminate. This leaves open the possibility of future complementary characterizations that will allow the taxonomic level of the species to be reached. Even so, *Xylaria multiplex* would be considered the "possible" species to which this fungus belongs.

Evaluation of *Xylaria* growth capacity in various culture media supplemented with natural substrates

The growth capacity of the *Xylaria* strain was assessed in three culture media that were supplemented with natural substrates. The highest radial proliferation rate occurred in REA medium (0.227 mm/h), followed by OM (0.213 mm/h), PDA (0.197 mm/h), and CM (0.183 mm/h) respectively. Figure 4 displays the obverse view of the cultures in Petri dishes using different substrates, illustrating variations in colonial morphology. Similar studies have reported the growth of X. *hypoxylon* (L: Fr.) Grev on malt extract agar, corn flour, potato dextrose agar, and oat flour, with moderate mycelial development and an optimal PDA medium (Ahmed *et al*., 2018). Natural substrates have also been employed in other fungal species, such as *Alternaria solani* (Cooke) Wint, where growth has been observed on OMA and PDA (Koley, 2015). The highest radial growth rate was recorded on the REA medium, which was attributed to its rich content of carbohydrates, proteins, and significant amounts of vitamins B_3 and B_{12}. In our study, the growth of the native strain was adequate at pH 7.0, whereas studies conducted with *Xylaria papulis* Lloyd reported optimal cultures at pH 6.0, when induced with substrates such as rice and cornmeal (López *et al*., 2022).

Determination and evaluation of Minimum Inhibitory Concentration (MIC) of *Xylaria* extracts against phytopathogenic bacteria

The extracts derived from *Xylaria* were examined for their activity against four distinct phytopathogenic bacteria, which showed promising inhibitory effects on both biomass growth and broth culture. The highest inhibitory capacity in biomass extracts was observed within a MIC range of 1.95 to 7.81 µg/mL, exhibiting the greatest percentage of inhibition in *P. syringae* A20 (88%), followed by *P. syringae* pv. *tabaci tobacco* (87%), *Chryseobacterium* sp. B1-5 (85.3%), and *P. putida* M11 (79.25%) (Figure 5). Conversely, the extracts obtained from broth displayed the highest percentage of inhibition with MICs ranging from 7.81 µg/mL to 3.9 µg/mL. The descending order of inhibitory effects was *P. syringae* A20 (84.37%) and *P. syringae* pv. *tabaci* (84%), *Chryseobacterium* sp. B1-5 (73.85%), and *P. putida* M11 (56.74%) (Figure 6). According to the ranges established by Popiolek *et al*. (2015), crude extracts are considered inactive if the MIC is >1,000 µg/mL. Activity was mild at MIC values between 501-1,000 µg/mL, moderate between 126-500 µg/mL, good between 26-125 µg/mL, strong between 10-25 µg/mL, and very strong at values < 10 µg/mL. Consequently, the broth and biomass extracts of *Xylaria* sp. used in this study exhibit very strong MICs and should be considered for their antibacterial activity. Significant statistical differences were observed in all the cases.

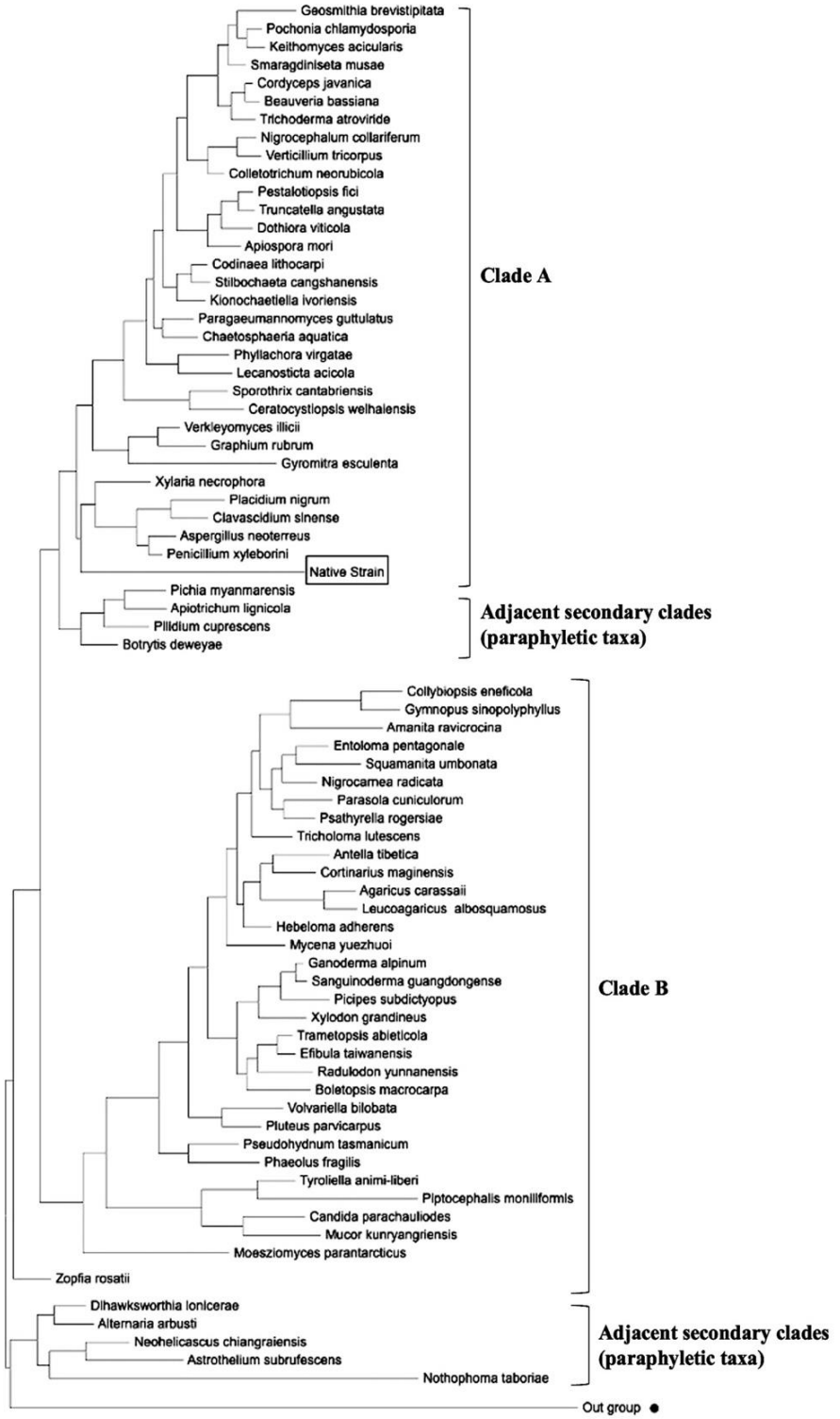

Figure 3. Linear phylogenetic reconstruction was supported by the maximum likelihood statistical method (MLE) using the Tamura-Nei model (Tamura and Nei, 1993). The cladogram shows the highest log-likelihood (-26,345,147.21). The reconstruction was generated through a heuristic search obtained automatically by applying the union-neighborhood and BioNJ algorithms to a matrix of estimated pairwise distances using the maximum likelihood of composition (MCL) method by selecting the topology with a higher log-likelihood value (1,000 bootstrap replicates) (data not shown). The cladogram was scaled with branch lengths measured in the number of substitutions per site. In total, 1,824,218 positions were observed in the final dataset. Evolutionary analyses were performed using MEGA7 (version 7.0) (Kumar *et al*., 2016). The native strain is highlighted in the box. All taxa were regrouped into two distinct main clades (A and B) in addition to the presence of two adjacent secondary clades (paraphyletic taxa).

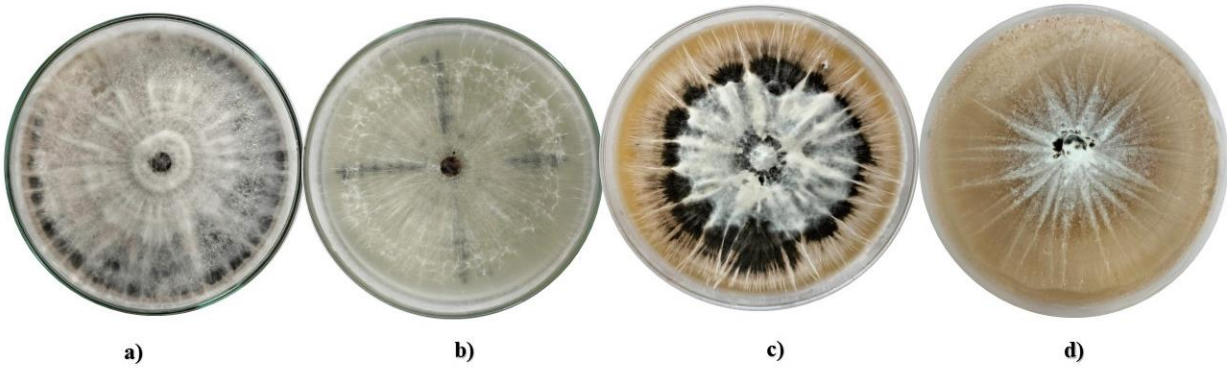

Figure 4. Growth of *Xylaria* sp. OG-03 in Petri dishes using various culture media: a) Potato dextrose agar, b) Oat-agar, c) Rice agar, and d) Corn flour agar.

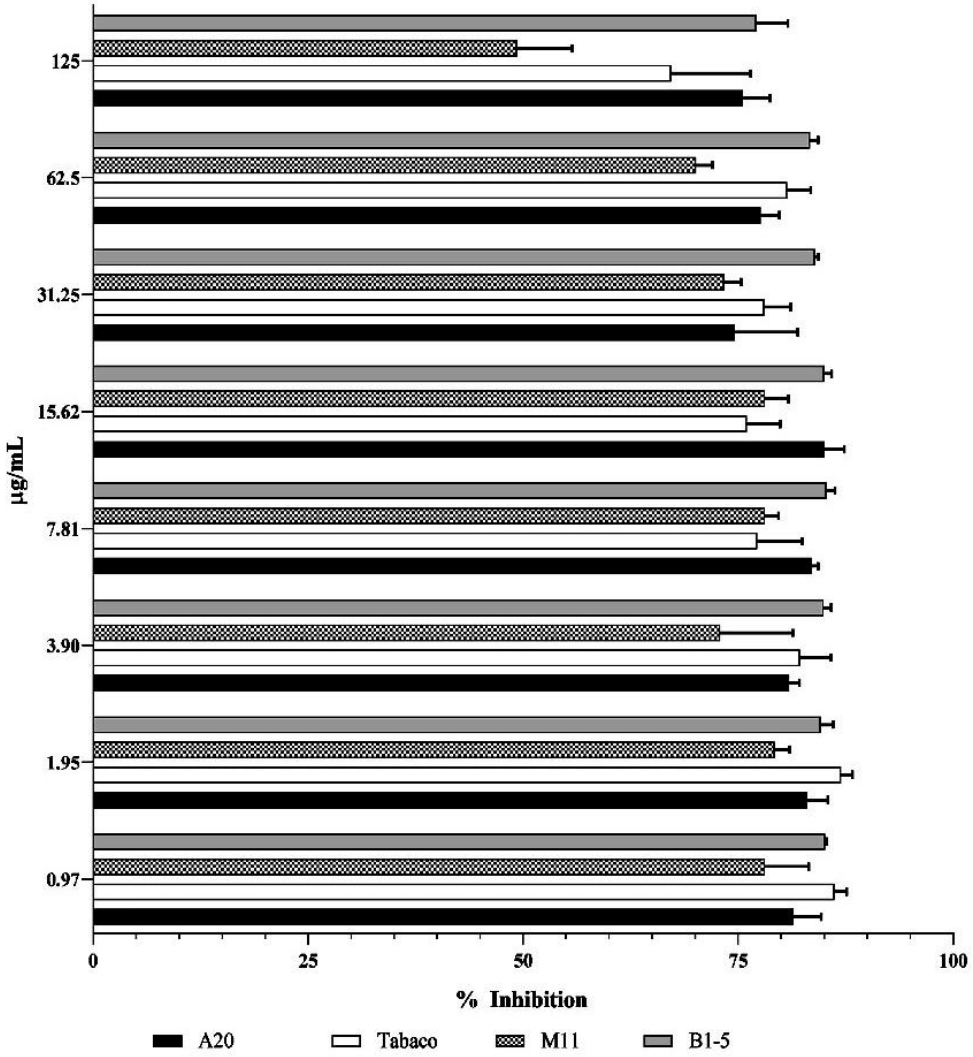

Figure 5. Inhibitory activity of *Xylaria* sp. biomass extracts in chloroform-methanol (1:1) against the following bacteria: *Pseudomonas syringae* pv. *syringae* (A20) and *P. syringae* pv. *tabaci* (tobacco), *Pseudomonas putida* (M11) and *Chryseobacterium* sp. (B1-5) in LB liquid medium. Standard deviations are shown for all cases (n=3).

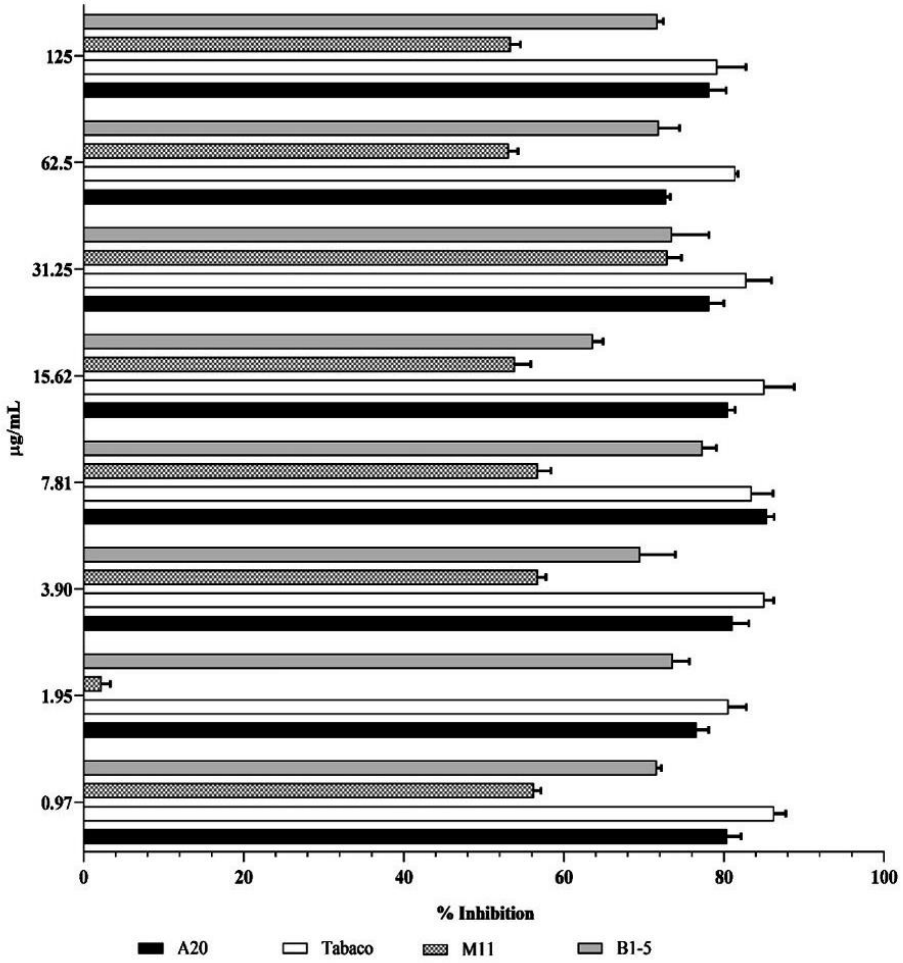

Figure 6. Inhibitory activity of *Xylaria* sp. broth extracts in chloroform-methanol (1:1) against the bacteria *Pseudomonas syringae* pv. *syringae* (A20) and *P. syringae* pv. *tabaci* (tobacco), *Pseudomonas putida* (M11) and *Chryseobacterium* sp. (B1-5) in LB liquid medium. Standard deviations are shown for all cases (n=3).

The phytopathogenic bacteria investigated in this study are known to affect various crops, causing economic losses and posing food safety concerns (Oliveira *et al*., 2011; Ratnaweera *et al*., 2014). *Pseudomonas syringae*, for example, attacks onion, almond, peach, and marigold crops, causing spot diseases (Maldonado-Bonilla *et al*., 2018). This species is also the causative agent of the "wildfire" disease in tobacco plants, resulting in necrotic white spots (Ichinose *et al*., 2023). *Pseudomonas putida* can survive and thrive under adverse conditions, and has been reported to act as a platform for antibiotic resistance gene exchange with *P. aeruginosa* (Peter *et al*., 2017). Additionally, *Chryseobacterium* sp. has been identified as the causative agent of necrosis in chayote (*Chayota edulis* Jacq.) (San Martin-Romero *et al*., 2014).

Phytopathogenic bacteria belonging to the genus *Pseudomonas* commonly attack plants by utilizing virulence factors, such as toxins, exopolysaccharides, and cell wall-degrading enzymes. They also produce effector proteins that enter plant cells via the type III secretion system and can mimic plant hormone-like compounds such as auxins (Xin *et al*., 2018). One example is the conversion of 3-indolacetonitrile (IAN) to indole-3-acetic acid (IAA), an essential plant growth factor. IAA biosynthesis from tryptophan (Trp) proceeds via indole-3-pyruvic acid (IPyA) and indole-3-acetaldehyde (IAAld) in *Ustilago esculenta*. Because IAN is an intermediate for IAA production, the synthesis of this biomolecule is not exclusive to higher plants, and several indole compound intermediary pathways operate in the metabolism of thousands of bacteria colonizing the plant rhizosphere. These pathways include IPyA, indole-3-acetamide (IAM), tryptamine (TAM), and IAN (Duca and Glick, 2020).

Xylaria species produce secondary metabolites with antimicrobial, antitumor, and acetylcholinesterase inhibitory properties (Oliveira *et al*., 2011; Ratnaweera *et al*., 2014). Recent studies on *Xylaria* sp. endophytes in the Chinese medicinal plant *Sophora tonkinensis*

identified xylaftalide as an antibacterial compound effective against *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Shigella dysentariae*, with MIC values ranging from 12.5 to 25 µg/mL (Zheng *et al*., 2018a). Another study on *Xylaria* sp. isolated from the same plant species demonstrated the ability of the fungus to produce a 2-pyranone-derived compound exhibiting antibacterial activity against *E. coli* and *S. aureus*, with MIC values of 50 µg/mL (Zheng *et al*., 2018b). Additionally, type A xylapeptides with antibacterial properties against *B. subtilis* and *B. cereus*, displaying an MIC value of 12.5 µg/mL, have been reported (Xu *et al*., 2017). Similar to plants colonized by *Xylaria* sp. strains (GDGS-77B), the compound xylarcalasin B exhibited antibacterial activity against *B. subtilis* and *E. coli* with MICs of 25 and 12.5 µg/mL, respectively (Zhao-Long *et al*., 2022).

Suryelita *et al*. (2021) conducted a study using extracts of *Xylaria* sp. isolated from *Andrographis paniculata* (Sambiloto), an ancestral oriental medicinal plant. The biological material demonstrated antibacterial activity against *Micrococcus luteus* (MIC = 12.5 µg/mL), *Streptococcus pyogenes* (MIC = 25 µg/mL), and *E. coli* (MIC = 25 µg/mL). Similarly, Liu *et al*. (2008) observed the production of 7-amino-4-methylcoumarin by *Xylaria* sp. isolated from *Ginkgo biloba* L., which displayed *in vitro* activity against various bacterial strains, with MICs ranging from 4 to 40 µg/mL. Indarmawan *et al*. (2016) showed that *Xylaria psidii* KT30, a marine-derived strain, transcribes an extracellular protein capable of inhibiting the growth of *B. subtilis* and *S. aureus*. Furthermore, helvolic acid production has been reported in an endophytic *Xylaria* species isolated from the orchid *Anoectochilus setaceus* endemic to Sri Lanka, which exhibits antibacterial activity against *B. subtilis* (Ratnaweera *et al*., 2014).

Xylaria sp. KYJ-15, isolated from *Illigera celebica*, has been reported to produce two distinct steroid-type biomolecules: xylarosteroids and xylarglucosides (A and B for both). Xylarosteroids exhibited MIC values of 2 mg/mL against *B. subtilis*, whereas xylarglucosides showed MICs of 4 and 2 mg/mL against *S. aureus* (Dong *et al*., 2023). Moreover, extracts from the fruiting bodies of *Xylaria curta* have been found to possess antibacterial activity against drug-resistant strains of *S. aureus* and *P. aeruginosa* at an MICs of 200 mg/mL (Veluchamy *et al*., 2012). Notably, the fungal strain used in our study was also isolated from a medicinal plant that was not previously reported to exhibit antibiotic activity against gram-negative phytopathogenic bacteria. The obtained MIC values were < 9 µg/mL, demonstrating consistency with prior findings and highlighting the ability of *Xylaria* to produce bioactive compounds when colonizing various plant types.

Evaluation of inhibitory activity of virulence factors in *Chromobacterium violaceum* 553 (biosensor strain)

The biomass extract of *Xylaria* sp. was evaluated at sub-inhibitory concentrations (62.5, 125, 250, and 500 µg/mL), where from 62.5 µg/mL a 47.28% decrease in biofilm formation was observed, whereas at 125, 250, and 500 µg/mL, the inhibition was 62, 62.5, and 60%, respectively, surpassing the anacardic acid control (Figure 7a). Figure 7b shows an inhibition of violacein at 62.5 µg/mL (56.32%), this activity coincides with the effect of anacardic acids, which were used as positive control at 125 µg/mL for each assay (biofilm and violacein).

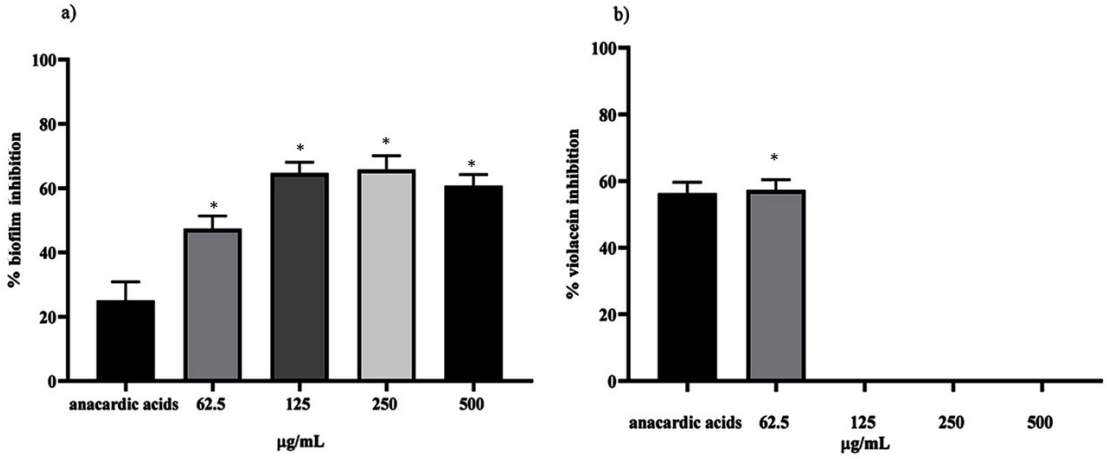

Figure 7. Effect of *Xylaria* sp. biomass extracts in chloroform-methanol (1:1) (540 nm) on *C. violaceum* 553: a) inhibition of biofilm formation; b) Violacein was not inhibited at concentrations of ≥125 µg/mL. Anacardic acid was used as a positive control. Dimethyl sulfoxide (DMSO) was used as a negative control, and the inhibition results were completely null (data not shown). (*) represents a significant difference (P=0.01) respect to the positive control. Standard deviations are shown for all cases (n=3).

Various medicinal mushrooms have been reported to produce biomolecules with antiviral potential. For example, *Tremella fuciformis* extracts at a concentration of 5 µg/mL decreased violacein production by *C. violaceum* CV02 (Zhu and Sun, 2008). Pigments from the edible fungus *Auricularia auricular* at concentrations of 0.3, 0.6, 0.9, and 1.2 mg/mL have been reported to inhibit this metabolite in the same strain (Zhu *et al*., 2011), similar to *Phellinus igniariu*s, a lignin-degrading saprotrophic fungus. The wild fungi *Amanita rubescens* and *Lactarius* sp., collected from the Black Sea in Turkey, inhibited violacein production at 312.5 and 625 µg/mL in *C. violaceum* ATCC 12472 (Tabbouche *et al*., 2017). Furthermore, ascomycetes such as *Aspergillus ochraceopetaliformis* SSP13, isolated from Indian *Carica papaya*, have been shown to affect pigment production in *C. violaceum* MTCC 2656 at concentrations of 250, 500, and 750 µg/mL (7.59, 84.01, and 94% inhibition, respectively) (Pattnaik *et al*., 2018). In this study, we showed that the fungal extract of *Xylaria* sp. isolated from oregano can inhibit virulence factors associated with quorum sensing in the violacein-overproducing strain *C. violaceum* 553. Research on the biological activities of *Xylaria* sp. has only covered its ability to inhibit bacterial growth at minimal inhibitory concentrations. To our knowledge, no study has described its effect on the inhibition of virulence factors at subinhibitory concentrations.

CONCLUSIONS

Xylaria sp. extracts obtained from oregano possess antibacterial potential against phytopathogenic bacteria of agricultural and food interest. This suggests that the strain under study is an indeterminate taxon belonging to the genus *Xylaria*, demonstrating its biological capacity. This fungal microorganism showed antibiotic activity against phytopathogenic bacteria and it was also able to inhibit virulence factors associated with quorum sensing in *C. violaceum* 553. Because secondary metabolites at low concentrations are involved in the regulation of virulence in bacterial communities, it can be concluded that there are still biomolecules at sub-inhibitory concentrations that need to be discovered and thoroughly characterized. To extend the results of this study, it is necessary to carry out further chemical analyses of the biological extracts and identify the majority of their compounds, as the molecular structures of each atomic aggregate, as well as the different fungal genomic natures, are determinants of the biological activities in question.

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Conflict of interest. The authors declare no conflict of interest during or after the execution of this study.

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Compliance with ethical standards. Does not apply.

Data availability. The data are available upon request to the corresponding author.

Author Contribution Statement (CRediT). D. Luis-Yong – Conceptualization, methodology, Writing – original draft. **O. Guzmán-López -** Investigation, Writing –Review & editing, **A. Salinas-Castro -** Investigation, Writing –Review & editing, **E. San Martín-Romero -** Investigation, Writing –Review & editing, **J. Zamora-Palma –**Methodology, Writing – Review & editing, **J. Ricaño-Rodríguez -** Project administration, writing – original draft, Writing – review & editing, formal analysis.

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