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MICROBIOLOGY

Phytotoxic and antifungal compounds for agriculture are interestingly produced by the endophytic fungus *Fusarium* sp. (*Ascomycota*) isolated from *Piper* sp. (Piperaceae)

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Abstract: Our study focused on the phytotoxic and antifungal activities of metabolites isolated from *Fusarium* sp., an endophyte associated with the medicinal plant *Piper* sp. Chromatographic separations of the *Fusarium* sp. crude extract led to the isolation of three analogous compounds: anhydrofusarubin (**1**), 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (**2**), and javanicin (**3**). The structures of the isolated compounds were determined by high-resolution mass spectrometry (HRMS) analysis and direct comparison of ^{13}C NMR data with that reported in the literature. The isolated compounds were evaluated for phytotoxic activity against *Lactuca sativa* and *Agrostis stolonifera*. All compounds exhibited strong phytotoxic activity on both plant species, completely inhibiting seed germination at a concentration of 1 mg mL^{-1}. Additionally, the isolated compounds were evaluated against *Lemna paucicostata*, achieving a 50% growth inhibition (IC_{50}) at concentration of 64, 28, and 31 µM for compounds **1**, **2**, and **3**, respectively. The antifungal activity of these compounds was evaluated using a bioautography assay targeting the agricultural pathogen *Colletotrichum fragariae*. Among them, compound **2** demonstrated significant antifungal activity. Our results showed that tropical medicinal plants harbor an interesting endophyte that has a potential reservoir of bioactive compounds. Moreover, the structures of these compounds could serve as scaffold for the development of new pesticides.

Key words: Agriculture, endophytes, natural products, pesticides.

INTRODUCTION

Endophytic fungi are microorganisms that colonize plant tissues for part or all of their life cycle without causing any apparent adverse symptoms in the plants (Jia et al. 2016, dos Reis et al. 2022). In symbiosis with their host plants, these fungi can provide defense against pathogens and herbivores by producing pesticide metabolites, while acquiring nutrients and protection. These microorganisms can synthesize a wide variety of chemically distinct metabolites, exhibiting diverse activities relevant to medicinal, industrial, and agricultural applications (Asomadu et al. 2024). Once isolated and identified, these metabolites can be investigated for their potential as biopesticidal agents (Macías-Rubalcava & Garrido-Santos 2022).

The genus *Fusarium* Link (1809), classified within the fungal family *Nectriaceae* (*Ascomycota*), is a cosmopolitan group of filamentous fungi that harbors over 70 species (Singh et al. 2021, Villavicencio et al. 2021, Yadav

& Meena 2021). Recognized as one of the most dominant endophytic fungal genera in the world, *Fusarium* is ubiquitous in nature which are widely found in soil, plants, and organic substrates (Nucci & Anaissie 2007). Additionally, *Fusarium* taxa are responsible for producing toxic secondary metabolites known as mycotoxins (e.g., fumonisins), which can impair the immune system of infected plants, impede cell proliferation, disrupt plasma membrane function, cause apoptosis, and inhibit protein synthesis (Chukwudi et al. 2021, Abbas et al. 1993, Qin et al. 2017).

Agriculture is one of the main pillars of the global economy, and its ability to sustain a growing global population has been a constant concern and remains a top priority in global policies (Pilling et al. 2020). It is expected that the agricultural sector will face numerous challenges in ensuring food production and supply in the coming years, as the human population is projected to reach 9.7 billion by 2050 (Roser & Rodés-Guirao 2019). In the 1940s, with the onset of the chemical era in agriculture, the development and use of synthetic pesticides became widely adopted (Zimdahl 2015). They encompass the categories of herbicides, nematicides, fungicides, and insecticides, which are used to eliminate organisms considered undesirable in agriculture (Chopra et al. 2011). In the current global scenario, pesticides are indispensable for maintaining crop productivity and profits, as well as food security for the growing human population. Nonetheless, the intensive use of pesticides over the past seven decades has raised concerns regarding environmental contamination, as well as adverse effects on non-target organisms, including humans (Carvalho 2017). Similarly, with the increasing occurrence of weed and fungal resistance to herbicides and fungicides currently available in the market (Heap 2024, Corkley et al. 2022), this study aimed to investigate the potential phytotoxic and antifungal compounds produced by an endophytic fungus *Fusarium* sp.

MATERIALS AND METHODS

Fungus origin and preparation of extracts for biological assays

The fungus *Fusarium* sp. was obtained from the Collection of Microorganisms and Cells of the Universidade Federal de Minas Gerais (UFMG), with the reference number UFMGCB 15449. It was originally isolated as an endophyte from the medicinal tropical plant species *Piper* sp. collected in the Tropical Rain Forest Rio Doce State Park, Minas Gerais, Brazil, in July 2017 (Florindo 2019). The fungus was cultured on a large scale following the method established by Rosa et al. (2013). The strain was inoculated onto 660 Petri dishes containing potato dextrose agar (PDA, Sigma, USA) medium, with each dish measuring 90 x15 mm and containing 20 mL PDA. Two 5-mm fungal plugs were placed at the center of PDA plates and incubated for 15 days in a biological oxygen demand incubator at 25 °C. Subsequently, the fungal mycelia were fragmented and transferred to 125 mL Erlenmeyer flasks, frozen at -20 °C for 24 h, and subsequently subjected to the lyophilization process (Liofilizador Liobras – L108) for 5 days. Next, 120 mL of dichloromethane (Neon, Brazil) was added to each conical flask and allowed to settle for 7 days at ambient temperature. Later, the organic phase was filtered using filter paper (50 x 50 - 80 g) and transferred to pre-weighed scintillation vials. After drying the extract in a fume hood, it was preserved at -4 °C until the bioassay was conducted.

Fungal identification

We employed molecular biology techniques for the *Fusarium* sp. UFMGCB 15449 identification.

DNA extraction was conducted following the protocol outlined by Rosa et al. (2009). Amplification of the transcribed internal spacer (ITS-5.8S) region for filamentous fungi was carried out as described by Rosa et al. (2009), utilizing the primers ITS1 and ITS4 (White et al. 1990). Additionally, amplification of the β-tubulin gene (Glass & Donaldson 1995), commonly employed for delineating fungal taxa with low intraspecific variation, was performed using the primers Bt2a/Bt2b, following the method outlined by Gonçalves et al. (2015). Representative consensus sequences of the fungal taxa were deposited in the NCBI GenBank database. To achieve species-level identification based on ITS and β-tubulin sequence data, NCBI BLAST was used to align the endophyte's consensus sequence with the most closely related species (Altschul et al. 1997). Fungal classification adhered to the guidelines of Kirk et al. (2008) and utilized the databases MycoBank (http://www.mycobank.org) and Index Fungorum (http://www.indexfungorum.org).

Assay for phytotoxic activity against *Lactuca sativa* and *Agrostis stolonifera*

The crude fungal extract, fractions obtained via silica gel column chromatography, and isolated pure compounds were evaluated for their phytotoxic effects on *Lactuca sativa* L. (lettuce, dicot) and *Agrostis stolonifera* L. (bentgrass, monocot) seeds, following the procedure outlined by Dayan et al. (2000). Seeds of *L. sativa* (Iceberg A Crisphead from Burpee Seeds) and *A. stolonifera* (Penncross variety, belonging to the creeping bentgrass species, sourced from Turf-Seed, Inc. in Hubbard, Oregon) underwent initial surface sterilization by immersion in a 2.5% sodium hypochlorite solution for 10 min. Subsequently, the seeds were thoroughly rinsed with sterile deionized water and air-dried in a sterile condition. Each well in a 24-well plate (Corning Inc., Corning, NY, USA) was filled with either *A. solonifera* seeds (10 mg), or *L. sativa* (5 seeds) placed on Whatman no. 1 filter paper. The test compounds and fractions were dissolved in a 10% acetone solution in sterile, deionized water. Then, 200 µL of the test solution was added to each well containing seeds, while the control wells received 200 µL of 10% acetone in water, and the negative control well received only 200 µL of water. A 1 mM acifluorfen solution (ChemServices, West Chester, PA, USA) served as the positive control. The plate was covered and sealed with Parafilm and placed in a Percival Scientific CU-36L5 incubator, under continuous light conditions at 24 °C with an average photosynthetically active radiation (PAR) of 120 µmol s^{-1} m^{-2}. Phytotoxic activity was qualitatively assessed visually by comparing seed germination in each well after 7 days for *L. sativa* and *A. stolonifera*, using a rating scale ranging from 0 to 5. A rating of 0 indicated no effect (all seeds germinated), while a rating of 5 indicated no seed germination. Each experiment was conducted in triplicate.

Phytotoxicity-guided fractionation and isolation of phytotoxic compounds

Initially, 4.09 g of dichloromethane (DCM) extractables from the fungi was dissolved in 160 mL MeOH/H_2O (90:10, v/v) and subjected to liquid/liquid partitioning with 60 mL of hexane (3 times), yielding 2.06 g of hexane extractables. The remaining partition (MeOH/H_2O, 90:10, v/v) was adjusted to 70:30 (v/v) MeOH/H_2O by adding 22.8 mL of H_2O and partitioned with 30 mL of chloroform (three times), resulting in 964.7 mg of chloroform ($CHCl_3$) extractables. The remaining MeOH was removed using rotary evaporation, followed by the addition of 40 mL of ethyl acetate (three times), resulting in 405.8 mg of ethyl acetate extractables. The water extract was rotary evaporated to remove any residual organics, and the water was lyophilized,

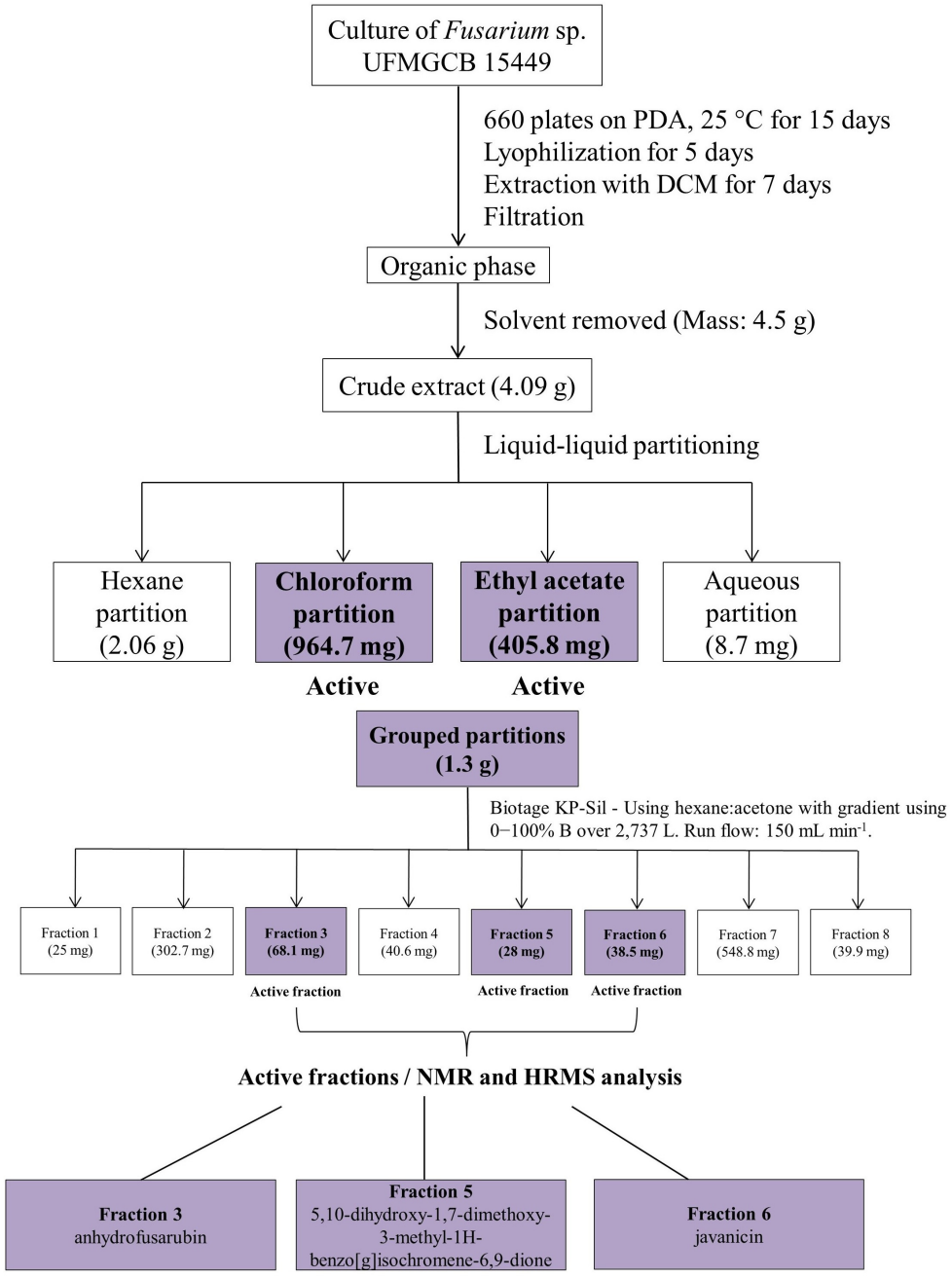

Figure 1. Flowchart of bioassay-guided chemical fractionation of the methylene chloride extract from the fungus *Fusarium* sp. UFMGCB 15449.

obtaining 8.7 mg of water extractables (Figure 1). Guided by the lettuce and creeping bentgrass bioassays, the chloroform and ethyl acetate partitions showed activity, and had similar TLC profiles. So, these partitions were combined and subjected to column chromatography using an Isolera One system (Biotage), equipped with a dual wavelength UV detector (220 and 340 nm) and an automatic fraction collector. Separation was performed using normal-phase column chromatography on a *Biotage Sfar* 100g column (60 µm). The separation was performed using a linear gradient of hexane (solvent A) and acetone (solvent B), from 0–100% B over 2,737 L. The flow rate was 150 mL min^{-1}. Portions of 20 mL each were collected in 16 × 150 mm test

tubes. According to TLC and UV chromatogram profiles, the fractions were recombined into nine fractions, named F_1-F_9. These fractions were evaluated for their phytotoxicity activities, with fractions F_3 (**1**), F_5 (**2**), and F_6 (**3**) had the highest phytotoxicity.

Compound **1** (anhydrofusarubin) as purple powder. High-resolution DART positive *m*/*z* 289.0710 $[M + H]^+$, calculated for $C_{15}H_{13}O_6$ 289.0712. ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.90, 177.82, 161.51, 159.93, 157.59, 157.53, 132.91, 122.66, 110.84, 109.88, 107.90, 94.61, 62.90, 56.66, 20.12 (Kurobane et al. 1980). Compound **2** (5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione) as purple solid. High-resolution DART positive *m*/*z* 319.0823 $[M + H]^+$, calculated for $C_{16}H_{15}O_7$ 319.0817. ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.66, 175.45, 161.42, 161.27, 159.83, 158.42, 132.52, 121.21, 111.37, 110.07, 107.70, 94.66, 94.54, 56.79, 55.89, 20.73 (Parisot et al. 1989). Compound **3** (javanicin) as purple powder. High-resolution DART positive *m*/*z* 291.0868 $[M + H]^+$, calculated for $C_{15}H_{15}O_6$ 291.0868. ^{13}C NMR (101 MHz, $CDCl_3$) δ 203.92, 184.44, 177.78, 161.43, 160.67, 160.38, 142.58, 134.28, 109.73, 109.68, 108.45, 56.86, 41.28, 30.11, 12.93 (Maharjan et al. 2020).

Phytotoxicity assay with *Lemna paucicostata* (duckweed)

Using the slightly modified method of Michel et al. (2004), *Lemna paucicostata* Hegelm. cultures, originating from a single colony that included a mother and two daughter fronds, were cultivated in a beaker with Hoagland's No. 2 Basal Salt Mixture (Sigma H2395, San Luis Obispo, CA, USA) at a concentration of 1.6 g L^{-1}. Iron was supplemented by adding 1 mL of 1000× FeEDTA solution per liter of Hoagland media. The pH of the medium was adjusted to 5.5 using 1N NaOH and subsequently filter-sterilized through a 0.2 µm filter. The *L. paucicostata* cultures were grown in approximately 100 mL sterile jars with vented lids in a Percival Scientific CU-36L5 incubator, maintaining continuous light conditions at 24 °C and an average of 120 µmol s^{-1} m^{-2} PAR. The doubling time for the plants was approximately 24 to 36 h. For assays, nonpyrogenic polystyrene sterile six-well plates (Costar 3506, Corning Incorporated, Wilmington, NC, USA) were used. Each well contained 4950 µL of Hoagland's media and 50 µL of water, solvent, or the compound dissolved in the appropriate solvent, resulting in a final solvent concentration of 1% by volume. Atrazine was used as the positive control. Two three-frond plants of the same age (4 to 5 days old) and approximate size were inoculated into each well. All six-well plates were placed in the Percival incubator at 24° C and an average PAR of 120 µmol s^{-1} m^{-2}. The LabScanalyzer (LemnaTec GmbH, Aachen, Germany), an image analyzer, was used to measure the frond surface area. Measurements were recorded on day 0 and day 7. The dose-response analysis and calculation of the half-maximal inhibitory concentration (IC_{50}) were performed with R software version 4.2.1, supported by the drc package.

Antifungal bioautography assay

The antifungal activity of pure compounds was evaluated against *Colletotrichum fragariae* (isolate CF63), a fungal pathogen affecting various vegetables and fruits, using a TLC bioautography method as described by Stappen et al. (2015). Briefly, the fungus was grown for 7-10 days at 27 ± 1 °C in PDA medium. Fresh condia were collected by flooding the culture plate with 10 mL sterile water and gently scraping them off using a sterile, L-shaped spatula and filtered through sterile double Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) to remove mycelia. Spore concentration was determined using a Countess 3 cell counter (Invitrogen), and the resulting suspension was centrifuged at 1968 rcf for 10 min to collect spores, with the

supernatant discarded. The inoculum for the bioautography assay was prepared by adding PDB-TLC media (12.5 g PDB, 0.5 g agar, 0.5 mL Tween 80 in 500 mL of water) to achieve a spore concentration of 3×10^5 spores mL^{-1}. Each test compound dissolved in acetone (10 mg mL^{-1}) was pipetted onto a silica gel plate (Analtech, Inc. Silica Gel GHLF, 250 µm) in two volumes, 1 µL (10 µg of solute) per spot, and 10 µL (100 µg of solute), in triplicates. After solvent evaporation, TLC plates were sprayed with *C. fragariae* inoculum until uniformly dampened. Inoculated TLC plates were then placed in a moisture chamber box under conditions of 100% relative humidity and incubated in a growth chamber for 3 days at 27 ± 1 °C with a 12-ho photoperiod and photon flux of 60 ± 5 µmol $s \cdot m^{-2}$ s^{-1} to optimize fungal growth. Antifungal activity was determined by the presence of inhibition zones (clear white zones with no fungal growth). For positive control plates, we used technical grade fungicides fludioxonil (>99.5%, Chem Service, Inc., West Chester, PA, USA) and captan (>98%, Chem Service, Inc., West Chester, PA), applying 1 µL from a 2 mg mL^{-1} stock solution, which is equivalent to 2 µg of solute. For natural product-based fungicides, thymol (analytical grade 99-101%, Sigma-Aldrich, Inc.) and carvacrol (natural, 99%, FG, Sigma-Aldrich, Inc.), we used a 10 mg mL^{-1} stock solution and applied 2 µL, equivalent to 20 µg of solute. Ethanol was used as the solvent for all solutions.

RESULTS AND DISCUSSION

Fungal identification

Fusarium sp. UFMGCB 15449 underwent molecular biology techniques for its taxonomic elucidation. The ITS sequence BLAST analysis showed that the closest sequence (98% query cover and 97.75% identity) belonged to the fungus *Fusarium* sp. (AF178399). Additionally, a comparison of the fungal ITS sequence with the closest species deposited in the GenBank (Figure 2a) indicated a phylogenetic proximity to *Fusarium protoensiforme* (AF178399)/ *Neocosmospora protoensiformis* (telomorph). The β-tubulin sequence BLAST analysis showed 100% query cover and 96.41% identity with the species *Fusarium witzenhausenense* (MG237871). Further comparison of the fungal β-tubulin sequence with the closest species deposited in the GenBank (Figure 2b) formed a separate cluster. The phylogenetic analysis using both sequences was not sufficient to conclusively identify the endophyte *Fusarium* sp. UFMGCB 15449 to the species level, and further detailed taxonomic studies, including classical morpho-physiological techniques, will be necessary.

Anamorphic fungal taxonomy is traditionally based on the comparative macro- and micromorphology of asexual reproductive structures. The bioactive taxon *Fusarium* sp. UFMGCB 15449 was cultivated in different culture media and exhibited distinct asexual structures exclusive to the genus *Fusarium*. Molecular biology techniques confirmed the genus but did not allow identification at the species level. However, this is not unusual, as *Fusarium* has a confusing and variable taxonomic record, resulting from the unchecked and inconsistent application of species epithets to toxigenic, pathogenic, and endophytic isolates (Campos et al. 2011). Species belonging to the genus *Fusarium* (*Hypocreaceae*, *Ascomycota*) are usually described as phytopathogens of various plants. However, some species can inhabit plant tissues (Souza et al. 2004, Campos et al. 2011) or grow as saprobes on plant debris and in soil (Glenn et al. 2007).

Phytotoxicity-guided fractionation and isolation of phytotoxic compounds

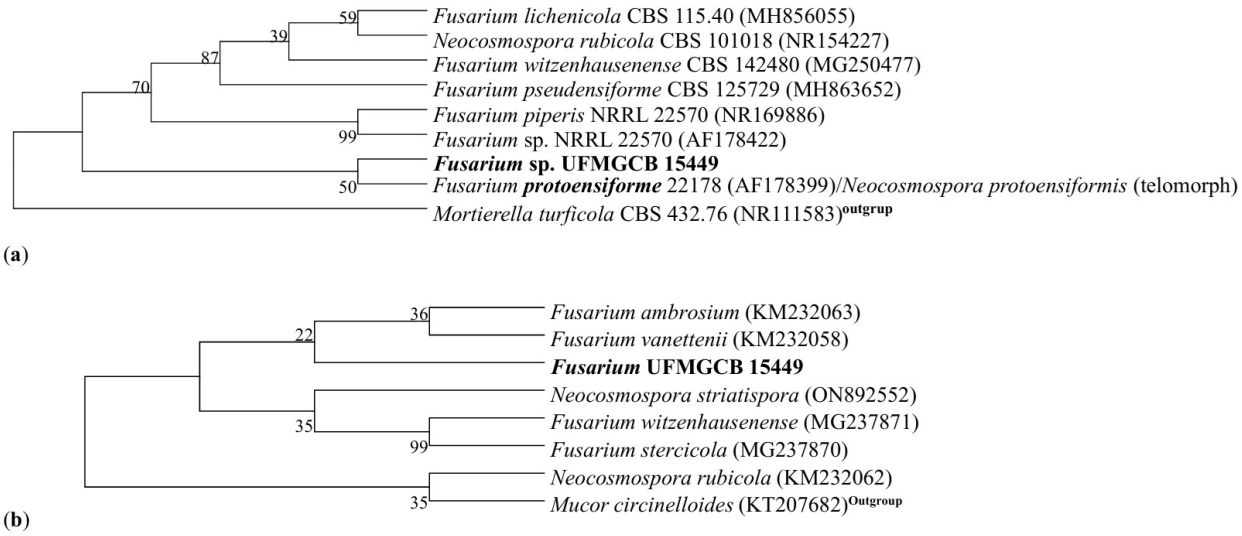

Figure 2. Phylogenetic analysis of the sequences of endophytic fungus *Fusarium* UFMGCB 15449 (in bold) compared with type sequences of the closest species following BLAST analysis, deposited in the GenBank database. The trees were constructed based on the (a) ITS and (b) β-tubulin region sequences using the maximum composite likelihood method. The sequences of *Mortierella turficola* (NR111583) and *Mucor circinelloides* (KT207682) were used as out-group.

Despite the different ecological roles of *Fusarium*, several studies report on its biological activities as endophytes, mainly for medical purposes. *Fusarium* endophytes recovered from coffee plants (Sette et al. 2006) and the medicinal *Dioscorea zingiberensis* (Xu et al. 2007) displayed antimicrobial activities against Gram-positive and Gram-negative bacteria. In addition, *Fusarium* endophytes have demonstrated antifungal activities against clinical strains of *Paracoccidioides brasiliensis* and *Cladosporium sphaerospermum* (Campos et al. 2011).

In our study, after phytotoxicity-guided fractionation, three compounds were isolated from the active fractions of *Fusarium* sp. UFMGCB 15449, anhydrofusarubin (**1**), 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (**2**), and javanicin (**3**) (Figure 3). The isolated compounds were evaluated for phytotoxicity against lettuce (dicotyledonous) and bentgrass (monocotyledonous) at a concentration of 1 mg mL^{-1} and 1 mM, respectively. At a concentration of 1 mg mL^{-1} (ca. 4 mM), all compounds demonstrated phytotoxic activity against both tested model plants, achieving a ranking of 5, indicating 100% inhibition of seed germination. This activity was comparable to that of synthetic herbicide acifluorfen at the same concentration. However, at a concentration of 1 mM (ca. 0.26 mg mL^{-1}), the compounds did not exhibit any phytotoxic activity.

The compounds were further assessed with a dose-response bioassay using duckweed. Compound **1** exhibited a 50% growth inhibition (IC_{50}) at a concentration of 64 µM, while compounds **2** and **3** demonstrated IC_{50} values of 28 and 31 µM, respectively (Figure 4). The genus *Lemna* is commonly used by the pesticide industry, environmental toxicologists, and others as a model for investigating the effects of phytotoxins and herbicides (Einhellig et al. 1985, Michel et al. 2004, Grossmann et al. 2012). Due to extensive prior testing of various herbicides using this method, it has become feasible to compare the isolated compounds with these established products (Amagasa et

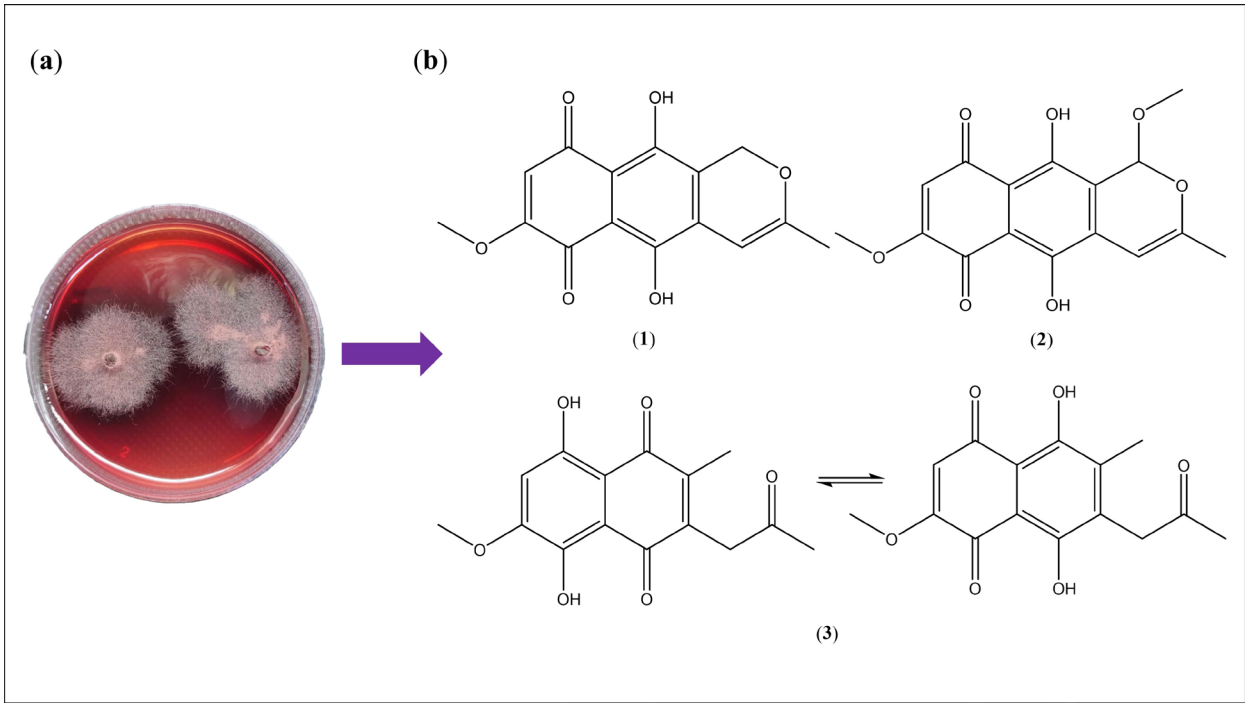

Figure 3. (a) Culture of *Fusarium* sp. UFMGCB 15449 on a PDA plate. (b) The chemical structures of quinone compounds purified from the active fraction: anhydrofusarubin (1), 5,10-dihydroxy-1,7- dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (2), and javanicin (3).

al. 1994, Michel et al. 2004). Under comparable experimental conditions, the phytotoxicity demonstrated by the isolated compounds surpassed that of certain synthetic herbicides like glyphosate and clomazone, which exhibited IC_{50} values of 388 and 126 µM, respectively, in the same bioassay (Michel et al. 2004). Lower IC_{50} values indicate that these compounds are potent enough to achieve the desired inhibitory effect which can lead to reduced cost, environmental impact, and potentially fewer non-target effects compared to compounds with higher IC_{50}. All three compounds appeared to cause hormesis (greater growth than the control) at one or more concentrations below those that inhibit growth. Hormesis is common with both natural and synthetic phytotoxins (Belz et al. 2007, Belz & Duke 2017).

Anhydrofusarubin (**1**) and javanicin (**3**), two naphthoquinone pigments, are synthesized primarily by various species of the fungus *Fusarium* (Tatum & Baker 1983, Khan et al. 2018). These compounds can also be obtained by heating the compound fusarubin (Ammar et al. 1979). Both compounds have demonstrated promising antibiotic properties and significant cytotoxic efficacy against certain types of cancer (Khan et al. 2016, Moni et al. 2022, Pankin et al. 2023). Although these compounds have shown phytotoxic effects on radish and pea seedlings, the literature on their phytotoxic activity is very limited (Baker et al. 1981, Medentsev & Akimenko 1992). Additionally, we found no previous publication on the phytotoxicity of 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (**2**), making this study the first to report phytotoxic activity of this compound.

Natural napthoquinones such as plumbagin have been explored as structural templates for synthesis of new herbicides (Durán et al. 2019), however, similar efforts have not been

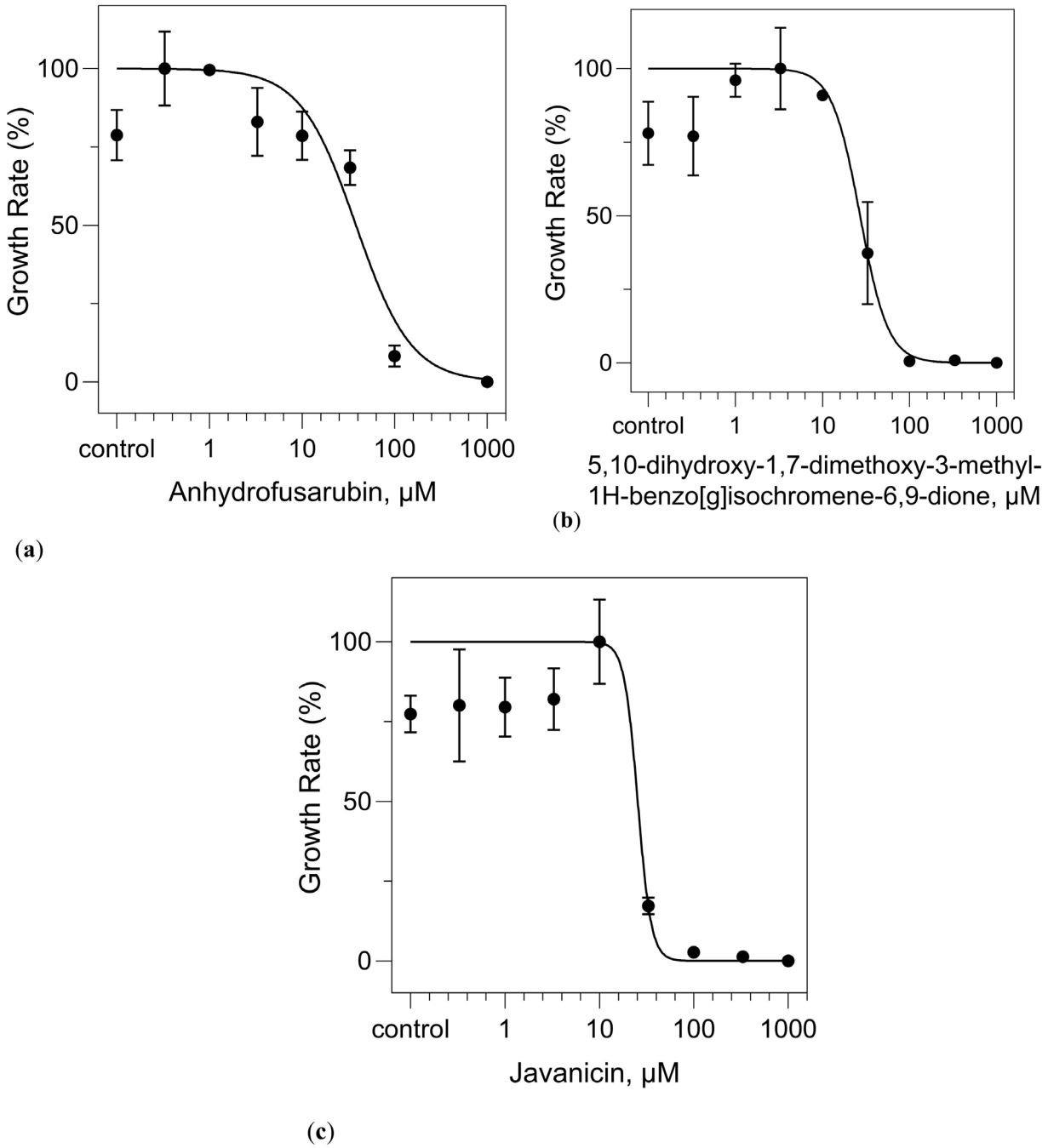

Figure 4. Phytotoxicity assay with *Lemna paucicostata* (duckweed). Effects of (a) anhydrofusarubin (1), (b) 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (2), and (c) javanicin (3) on the growth (% of initial frond area) of *L. pausicostata* at varying concentrations after 7 days of exposure. Each treatment was carried out in triplicate. Error bars are ±1 standard error of the mean.

published using the compounds that we report in this paper. The herbicidal effects of 2-hydroxy-3-alkyl-1,4-naphthoquinones is inhibition or photosystem II (PSII) of photosynthesis by binding to the quinone binding site of the D1 protein of PSII (Jewess et al. 2002). This is a likely target for the compounds reported in this paper, however, further study is required for

the confirmation. There are no quinone-based commercial herbicides, and there is a need for new PSII inhibitor herbicides because of the evolution of resistance, toxicity concerns, and other limitations of current commercial PSII inhibitor herbicides (Twitty & Dayan 2024).

Antifungal bioautography assay

The antifungal properties of the isolated compounds were evaluated at 10 and 100 µg/spot against the plant pathogenic fungus *C. fragariae* using a thin-layer chromatography bioassay (Figure 5). Among them, compound **1** exhibited no activity, compound **3** displayed weak antifungal activity, revealing an inhibition zone measuring 8 ± 0.5 mm at 100 µg and 4 ± 0.5 mm at 10 µg. Compound **2** demonstrated significantly greater antifungal activity, with an inhibition zone measuring 12 ± 1 mm at 100 µg and 5 ± 0.5 mm at 10 µg. The commercial fungicides captan and fludioxonil exhibited inhibition zones of 9 and 16 mm, respectively, at 2 µg $spot^{-1}$, while carvacrol and thymol showed inhibition zones of 8 and 9 mm, respectively, at 20 µg $spot^{-1}$.

We found no previous reports of the antifungal activity of 5,10-dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (**2**), suggesting this to be the first report of antifungal activity of this compound. Javanicin (**3**) has previously documented antifungal activity (Kharwar et al. 2009). Isolated from the fungus *Chloridium* sp., javanicin exhibited potent antifungal effects against agricultural pathogens such as *Cercospora arachidicola*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Verticillium dahliae*, with MIC values spanning from 5 to 20 µg mL^{-1} (Kharwar et al. 2009). Anhydrofusarubin (**1**) has been described as having no antifungal activity against *Aspergillus niger* (Moni et al. 2022), though Ammar et al. (1979) reported the mild antifungal activity of anhydrofusarubin against *A. niger*. The results obtained in this work are consistent with those published in the literature.

Napthoquinones have studied for possible fungicide use for decades (e.g., Fieldgate & Woodcock 1973) and are of great interest as possible medicinal fungicides (Futuro et al. 2018). Several natural napthoquinones (e.g., juglone and plumbatin) are reported to be fungicidal, as reviewed by Martínez & Benito (2005). Meazza et al. (2003) found several natural napthoquinones to be fungitoxic to more than one *Colletothrichum* species. Structural

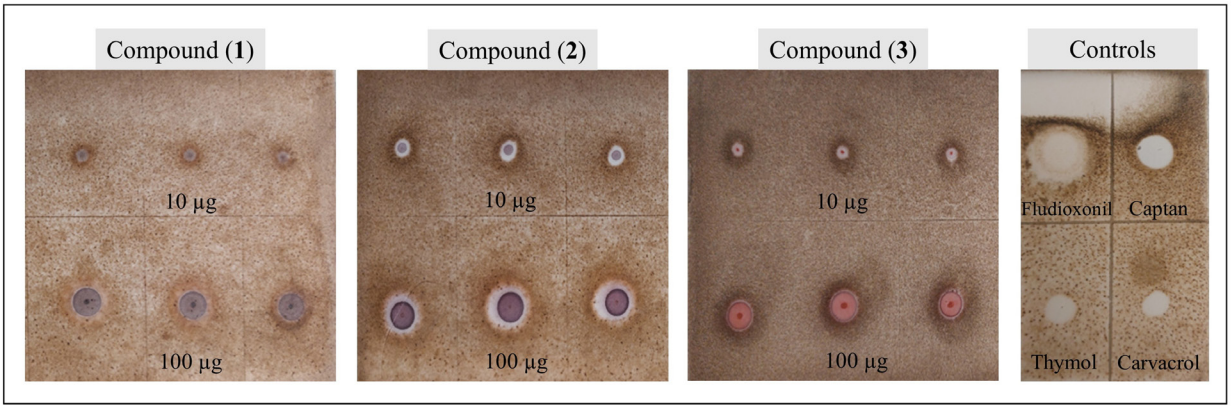

Figure 5. Thin layer chromatography bioautography of (a) anhydrofusarubin (1), (b) 5,10- dihydroxy-1,7-dimethoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione (2), (c) javanicin (3), and (d) controls against the phytopathogenic target *Colletotrichum fragariae*.

alteration of compound **2** of the present study could improve the fungicidal activity.

CONCLUSIONS

Our results indicated that endophytes isolated from a tropical medicinal plant represent an interesting reservoir of bioactive compounds. These endophytes can produce phytotoxic and fungicidal compounds, potentially valuable for the development of new pesticides. Our study marked the first report of compound **2** displaying biological activity. The structures of these compounds could prove valuable for the development of new pesticides; however, further studies, including determination of the molecular targets, are essential to fully understand their potential.

Acknowledgments

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