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Cercosporamide, a polyketide-derived fungal metabolite, serves as an antifungal agent against phytopathogenic fungi

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ABSTRACT

An endophytic fungus, *Phoma* sp. NG-25, produces a set of structurally related polyketides including cercosporamide, phomodione, and usnic acid, among which, cercosporamide has been reported to have strong antifungal and anticancer activities. In this study, *Phoma* sp. NG-25 was grown in seven growth media to determine the optimal culture condition conducive for cercosporamide production. Cercosporamide production peaked on the eighteenth day of incubation in beef peptone dextrose (BPD) broth media. The cercosporamide titer reached to an average of 77.5 μg/mL in BPD. Paper disk diffusion assay revealed that culture filtrate containing cercosporamide as a major constituent inhibited the growth of taxonomically diverse plant pathogens, including ascomycetous, basidiomycetous, and oomycete fungi. Cercosporamide exhibited strong antifungal activities against two pepper anthracnose pathogens, *Colletotrichum gloeosporioides* and *C. scovillei* with EC_{50} values of 3.8 and 7.0 μg/mL, respectively. This study suggests the potential application of cercosporamide as an effective antifungal agent in controlling anthracnose in pepper.

Keywords: antifungal activity, cercosporamide, *Didymella*, pepper anthracnose, secondary metabolite

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1. Introduction

Cercosporamide exhibits a variety of biological activities, including antifungal and anticancer activities. One of the molecular targets of cercosporamide is the protein kinase C (PKC) in *Candida albicans* causing a strong antifungal activity, that acts synergistically in combination with echinocandin-class compounds that inhibit the biosynthesis of fungal cell wall (Sussman et al., 2004). In most fungi, PKC is a single copy gene, and is implicated in the regulation of cell wall biosynthesis through a conserved MAP kinase signaling pathway (Heinisch & Rodicio, 2018). This suggests that the interaction between cercosporamide and PKC gene product leads to the inhibition of PKC activity, which is detrimental to fungal cell viability (Katayama et al., 2012; Penn et al., 2015). In addition, cercosporamide was shown to be a promising natural product for cancer therapy, suppressing proliferation and metastasis through inhibition of mitogen-activated protein kinase-interacting kinases (MNK1/2) in glioblastoma (Grzmil et al., 2016) and lung cancer cells (Konicek et al., 2011). Cercosporamide was also reported to block phosphorylation of MNK-eukaryotic initiation factor 4E (EIF4E), resulting in potent inhibitory effects on primitive leukemic progenitors (Altman et al., 2013) and human hepatocellular carcinoma (Liu et al., 2016). Recently, cercosporamide was shown to inhibit a bone morphogenetic protein receptor (BMPR) type I kinase activity in zebrafish embryos (Hoeksma et al., 2020).

Cercosporamide has been found in diverse fungi occupying different ecological niches. Cercosporamide was first isolated from culture extracts of a plant pathogenic fungus Cercosporidium henningsii and was characterized as a host-selective phytotoxin (Sugawara et al., 1991). Since then, cercosporamide was isolated from culture extracts of endophytic fungi, such as Cadophora orchidicola isolated from a Chinese medicinal plant Kalimeris indica (Wang et al., 2019), and two Phoma species isolated from Saurauia scaberrinae (Hoffman et al., 2008) and Arisaema erubescens (Wang et al., 2012). Also, some saprobic fungi and wood-decaying fungi are known to produce cercosporamide. These species belong to the genera Lachnum, Pseudaegerita, and Verruculina that grows on the rotten twig of a broadleaf tree or fern leaves in a cloud forest (Bunyapaiboonsri et al., 2020; Hosoya et al., 2011).

Alongside cercosporamide, usnic acid and phomodione represent two additional dibenzofuran derivatives produced by an endophytic fungus, *Phoma* sp. NG-25 (Hoffman et al., 2008). Usnic acid, the earliest identified dibenzofuran derivative, was first discovered by Knop in 1844. Usnic acid exhibits antiviral, antimicrobial, and anti-insect properties (Cetin et al., 2008; Luo et al., 2011; Luzina & Salakhutdinov, 2018). It can be synthesized by over six distinct li-

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chen genera, such as *Cladonia*, *Evernia*, *Usnea*, and *Xanthoparmelia* (Pizarro et al., 2020). Phomodione possesses a strong antibacterial property, as evidenced by its minimum inhibitory concentration of 1.6 μg/mL against *Staphylococcus aureus* (Hoffman et al., 2008). It also exhibited activity against a variety of microorganisms, including oomycetes, ascomycetes, and basidiomycetes, at concentrations of a few ppm (Hoffman et al., 2008).

Pepper (*Capsicum annuum* L.) is a valuable vegetable crop cultivated in nearly all countries worldwide (Jaiswal et al., 2021). However, anthracnose in pepper caused by *Colletotrichum* species leads to major agricultural production losses throughout Asia (Fu et al., 2021; Ren et al., 2020). Pepper anthracnose diseases exhibit the symptoms of sunken spots or lesions on infected fruits and shoots (Förster & Adaskaveg, 1999; Saxena et al., 2016). *Colletotrichum gloeosporioides* is one of the most common pepper pathogens that have devastated agricultural industry in Indonesia, Sri Lanka, Korea, and Thailand (De Silva, 2019). *Colletotrichum scovillei* is another problematic pepper pathogen prevalent in Brazil, China, Indonesia, Japan, Korea, Malaysia, and Thailand (Caires et al., 2014; Kanto et al., 2014; Oo et al., 2017; Zhao et al., 2016). Pathogenicity tests on chili pepper fruits showed that *C. scovillei* was more aggressive than *C. gloeosporioides,* especially when inoculated on non-wounded fruits (De Silva et al., 2021). *C. scovillei* has also been reported to cause significant yield losses on economically important fruit trees, such as mango and banana (Qin et al., 2019; Zhou et al., 2017).

Fungicide application is the main practice to control anthracnose of pepper. However, fungicide resistance of *Colletotrichum* spp. to commercially available chemical fungicides is being increasingly reported (Hu et al., 2015; Materatski et al., 2019; Ramdial et al., 2016). Moreover, synthetic fungicides cause environmental pollution and ecological degradation (Bhusal & Mmbaga, 2020). Therefore, organic or biochemical fungicides against anthracnose pathogens need to be developed to protect global chili pepper industries and sustainably increase pepper production. In this study, we obtained a fast-growing filamentous fungus known as *Phoma* sp. NG-25, which produces a series of structurally similar dibenzofurans, namely cercosporamide, phomodione, and usnic acid. These three dibenzofurans exhibited antifungal properties, albeit with varying degrees of effectiveness. The objectives of this study were (i) to select the best culture condition of *Phoma* sp. NG-25 for production of cercosporamide (ii) and to evaluate a spectrum and degree of antifungal activity of the three dibenzofurans produced by *Phoma* sp. NG-25 against agriculturally important phytopathogenic fungi, with primary emphasis on the causal agents of pepper anthracnose, *C. gloeosporioides* and *C. scovillei*.

2. Materials and methods

2.1. Chemical profile of Phoma sp. NG-25

Phoma sp. NG-25 strain was generously provided by Angela M. Hoffman from the University of Portland, Portland, Oregon, USA (Hoffman et al., 2008). Reanalysis of its rDNA ITS sequence (GenBank EU180709) indicates that of the strain is closely-related to species in the genus *Didymella*. To establish culture conditions improving production of cercosporamide, fungal mycelia of *Phoma* sp. NG-25 as a form of agar block (2–3 mm) were inoculated into 30 mL of growth media in a 100 mL baffled flask. For an OSMAC approach (Wei et al., 2010), a selection of liquid media were compared including: 1) beef peptone dextrose medium [BPD, 3 g/L beef extract and 5 g/L peptone (Nutrient broth, Difco), and 10 g/L glucose], 2) potato dextrose broth medium [PDB (Difco, USA)], 3) soya peptone maltose dextrose medium [SMG, 10 g/L soya peptone (MBcell, Korea), 5 g/L maltose, and 10 g/L dextrose], 4) malt extract yeast extract medium [MEYE, 3 g/L malt extract and 3 g/L yeast extract (Difco, USA), 5 g/L peptone (Difco, USA), and 10 g/L dextrose], 5) Lilly & Barnett medium (Muggia et al., 2017) plus 10 g/L ribitol (LBR), 6) Lilly & Barnett medium plus 10 g/L erythritol (LBE), and 7) Lilly & Barnett medium plus 10 g/L sorbitol (LBS). Fungal cultures were agitated at 150 rpm and 12 h light and dark intervals at a room temperature of 20 °C. After two wk of incubation, fungal mycelia were harvested by filtration using four layers of sterilized Miracloth (Merck Millipore, MA, USA) and extracted with 30 mL of acetone. The crude extracts were reconstituted in 300 μL acetone and filtered through a 0.4 mm syringe membrane. Separation of culture extracts was carried out using a YMC-Pack ODS-A column (150 × 4.6 mm, particle size 5 μm, pore diameter 12 nm, at 40 °C; YMC Co., Ltd., Kyoto, Japan) on an HPLC system (Prominence Modular High Performance Liquid Chromatography LC-20A; Shimadzu, Kyoto, Japan). The UV-active metabolites were monitored from 200 to 800 nm by a diode array UV detector. The mobile phase consisted of distilled water/trifluoroacetic acid (99.9:0.1, v/v) for pump A and methanol/trifluoroacetic acid (99.9:0.1, v/v) for pump B. A gradient program was employed for the mobile phase as follows: 0–30 min, 20–100%; 30–40 min, 100%; 40–52 min, 20% of pump B, with a flow rate maintained at 1.0 mL per min. For mass detection, the crude extract of BPD culture was analyzed by liquid chromatography mass spectrometry (LC-MS) as previously described (Kim et al., 2021).

2.2. Time-course experiment of cercosporamide production

Phoma sp. NG-25 mycelia (agar blocks) were inoculated into 500-mL flasks containing 200 mL of BPD. Twenty-two flasks were prepared for the time-course experiment. Mycelia were collected from two separate culture flasks in replicates every 3 d for the detection of cercosporamide. For chemical extraction, mycelia were homogenized, after which, 200 mL of ethyl acetate (EtOAc) was added for extraction. The organic layer was separated, filtered, dried using a rotary evaporator, reconstituted with acetone, and monitored by HPLC employing the solvent system as described above. For quantification, peak areas corresponding to cercosporamide were calculated at 257 nm.

2.3. Purification of cercosporamide and phomodione

Cercosporamide and phomodione were detectable in both the mycelia and the broth. Here, cercosporamide was extracted from culture filtrate of *Phoma* sp. NG-25 cultivated in BPD medium for 18 d. In contrast, phomodione was extracted at 5 wk after growth because it displayed a higher yield during that period. For chemical extraction, 200 mL of EtOAc was added to 200 mL of BPD cultures. Culture extracts were subjected to preparative TLC with dichloromethane-methanol (10:1, v/v) as eluent. The fraction (R_f, retention factor = 0.56) containing UV-active compound was collected separately by scraping silica on preparative TLC plates and reconstituted with acetone. The fraction was then subjected to preparative HPLC, and separation was achieved at a flow rate of 2.2 mL/min, using the semi-preparation Kromasil C18-column (250 × 10 mm, 5 μm, 10 nm; at 40 °C). Mobile phases for cercosporamide and phomodione were 65% and 80% acetonitrile buffered with 0.1% trifluoroacetic acid, respectively, yielding 14.7 mg of cercosporamide (t_R, retention time = 11.32 min) and 7.7 mg of phomodione (t_R = 11.35 min). Purity of cercosporamide was below 95%, and thus a second round of separation was conducted using 55% methanol buffered

with 0.1% trifluoroacetic acid, as a mobile phase, at a flow rate of 1 mL/min on the YMC-Pack ODS-A column (150 × 4.6 mm, 5 μm, 12 nm; at 40 °C), yielding 8.4 mg of cercosporamide (t_R = 8.02 min).

Chemical structures of cercosporamide and phomodione were confirmed by NMR and MS data. ^{1}H NMR experiments were recorded with a JEOL JNM ECZ600R NMR spectrometer. Cercosporamide (**1**): yellow power, ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 13.56 (1H, s), 10.61 (1H, br s), 8.25 (1H, s), 7.53 (1H, s), 6.21 (1H, s), 6.14 (1H, s), 2.56 (3H, s), 1.72 (3H, s) (Supplementary Fig. S1), observed *m/z* value: 330.0607 [M-H]$^-$. The ^{1}H NMR data of purified cercosporamide was comparable to previously published data (Sugawara et al., 1991). Phomodione (**2**): light tan power, ^{1}H NMR (600 MHz, CD_3OD) δ_H 3.92 (3H, s), 3.44 (3H, s), 2.61 (3H, s), 2.07 (3H, s), 1.85 (3H, s), 1.72 (3H, s), 1.29 (2H, m) (Supplementary Fig. S2), ^{13}C NMR (150 MHz, CD_3OD) δ_C 203.7, 193.6, 179.2, 164.5, 163.9, 159.2, 145.4, 138.7, 118.8, 113.8, 112.1, 105.2, 62.3, 57.8, 50.9, 31.7, 30.3, 23.0, 15.6, 9.2 (Supplementary Fig. S3), observed *m/z* value: 389.1233 [M-H]$^-$. The ^{1}H NMR and ^{13}C NMR data of purified phomodione were comparable to published data (Hoffman et al., 2008).

Usnic acid (**3**): yellow power, t_R = 11.25 min. The authenticity of usnic acid was confirmed by our in-house lichen substance database (Huneck & Yoshimura, 1996). Commercially purchased usnic acid (Sigma-Aldrich, St. Louis, MO, USA) was also used as a reference (Supplementary Fig. S4). HPLC analysis employed a Shimadzu liquid chromatography system. The chromatographic conditions were as follows: Column, YMC-Pack ODS-A; mobile phase, methanol/distilled water/ phosphoric acid (80:20:1, v/v/v) for pump B, flow rate, 1.0 mL/min; column temperature, 40 ˚C; a photodiode array detector, SPD-M20A, range 200–800 nm.

2.4. Calculation of the cercosporamide titer in BPD

Purified cercosporamide was prepared in a series of dilutions, ranging from 20 μg/mL to 250 μg/mL. These dilutions were then analyzed using an HPLC system, as previously outlined. Peak areas for each concentration were quantified, enabling the construction of a standard curve for estimating the cercosporamide concentration. For each batch of experiments, *Phoma* sp. NG-25 was cultivated in thirty flasks, each containing 200 mL of BPD medium for a duration of 18 d. These fermentations were conducted in three independent batches, with each batch yielding 5 L of culture filtrate. To ascertain the initial titer of cercosporamide in the BPD medium, a small volume (200 μL) of the culture filtrate was aliquoted into a microcentrifuge tube and analyzed using HPLC. The concentration of cercosporamide in each batch was subsequently calculated by referencing the established standard curve.

For the liquid-liquid extraction experiment, approximately 1 L of BPD culture filtrate was collected. Initially, the concentration of cercosporamide in the culture filtrate was determined by referencing the standard curve. To assess the effectiveness of liquid-liquid extraction, an equivalent volume (125 mL) of EtOAc was employed to extract the cercosporamide from the BPD culture filtrate. A small volume (200 μL) of the EtOAc phase was aliquoted into a microcentrifuge tube and analyzed using HPLC. The concentration of cercosporamide in the EtOAc phase was calculated based on the standard curve.

To evaluate extraction efficiency of different organic solvents in solid-liquid (mycelia-solvent) extraction, mycelia were harvested from BPD culture and aliquoted into four tubes. Approximately 1 g of dried mycelia was ultrasonically extracted with 20 mL of four different solvents; acetone, EtOAc, hexane, and methanol. Organic phases were collected and vacuum-dried to obtain crude extracts, which were reconstituted with 5 mL of acetone and were subjected to HPLC analysis.

2.5. Sources of phytopathogenic fungi

The plant pathogenic fungi used in the study included *Alternaria alternata* (KACC 40019), *Colletotrichum gloeosporioides* (KACC 40896), *Fusarium oxysporum* f. sp. *lycopersici* (KACC 40038), *Diaporthe actinidiae* (KACC 48275), *Phytophthora capsici* (KACC 40157), *Pythium ultimum* (KACC 40705), *Rhizoctonia cerealis* (KACC 40154), *Rhizoctonia solani* AG-2-2 (KACC 40151), and *Sclerotinia sclerotiorum* (KACC 40457). *Colletotrichum scovillei* strain KC05, isolated from an infected pepper fruit in Gangwon province, South Korea, was generously provided by Dr. Kyoung Su Kim (Kangwon National University, Chuncheon, South Korea) (Fu et al., 2021). while the *Diaporthe eres* strain D-5, isolated from an infected kiwifruit in Boseong county of South Korea, was provided by Dr. Kwang-Yeol Yang (Chonnam National University, Gwangju, South Korea) (Gi et al., 2022). Among these taxonomically diverse phytopathogenic fungi and oomycetes, *C. gloeosporioides*, *C. scovillei*, *Phytophthora capsici*, and *Pythium ultimum* are known to be pathogenic to pepper, causing serious problems in South Korea and many other pepper-growing countries. All fungal strains were maintained on potato dextrose agar media (PDA; Difco, USA) at 23 °C.

2.6. Paper disk diffusion assay of Phoma sp. NG-25

The evaluation of *Phoma* sp. NG-25 culture filtrate's antifungal activity followed this process: Agar blocks (0.45 cm) containing mycelia of phytopathogenic fungi were placed at the center of quarter-strength PDA (Q-PDA) plates and allowed to grow for 2–6 d until the fungi covered half of the plates. Four paper disks were positioned along the edge of the plates, each containing 40 μL of one of the following: approximately two times concentrated BPD culture filtrate with 200 μg/mL of cercosporamide, 200 μg/mL of chlorothalonil as a positive control (Sigma-Aldrich), methanol as a solvent control, or approximately two times concentrated BPD medium as a negative control. The plates were incubated for an additional 1–3 d at 23°C until the fungi completely covered the negative control.

In addition, the evaluation of the antifungal activity of *Phoma* sp. NG-25 oatmeal culture extracts involved the subsequent procedure: oatmeal culture extracts were collected from ground mycelia of *Phoma* sp. NG-25 inoculated in 200 g of autoclaved oatmeal flakes (The Quaker Oats Company, Chicago, IL, USA), and grown for 3 wk. Oatmeal flakes colonized with *Phoma* sp. NG-25 were extracted with 200 mL of EtOAc, dried using a rotary evaporator, reconstituted with 5 mL of methanol and subjected to HPLC analysis. The HPLC sample preparation and chromatographic conditions were as follows: Oatmeal crude extracts were filtered through a 0.4 mm syringe membrane. Culture extracts were separated using a YMC-Pack ODS-A column on an HPLC system. A 52 min gradient program for the mobile phase was used. The flow rate was 1.0 mL per min. Paper disk diffusion assay was performed with paper disks containing 40 μL of oatmeal culture extract with oatmeal media extract as the negative control. This activity was repeated three times.

2.7. Determination of the median effective concentration (EC_{50})

We obtained pure compounds of cercosporamide and phomodione, using a preparative TLC and HPLC methods. In addition,

commercially available usnic acid was purchased for this study. To determine the median effective concentration of cercosporamide, phomodione, and usnic acid, agar blocks (0.45 cm) containing mycelia of *C. gloeosporioides* or *C. scovillei* were placed at the center of Q-PDA plates amended with different concentrations of each of the dibenzofuran compounds, which were made from two-fold serial dilution (3.125, 6.25, 12.5, 25, 50, or 100 μg/mL) (Paguirigan et al., 2022). Control Q-PDA plates contained 5% methanol. Colony diameter of *C. gloeosporioides* or *C. scovillei* growing on media containing the different compounds was measured in triplicate when fungal colony reached to the edge of the control plates. This measurement facilitated the evaluation of colony diameter values for each phytopathogen, obtained from triplicate tests using various concentrations of cercosporamide. Inhibition percentages were calculated using the formula: $I(\%) = ([(C-d)-(T-d)])/((C-d)) \times 100$, where d was diameter of the initial agar plug containing mycelia, I was the inhibition (%), and C and T were the average colony diameters in the control plates and in the plates containing compounds, respectively. The EC_{50} values were estimated using a probit regression model in the SPSS program (Lish et al., 2019).

3. Results

3.1. An OSMAC approach for cercosporamide production

The One-Strain-Many-Compounds (OSMAC) approach varies culture conditions to modulate and stimulate secondary metabolite production in fungi. Hence, we evaluated seven different growth media to examine the differential production of metabolites in *Phoma* sp. NG-25. The three previously reported compounds, cercosporamide, phomodione, and usnic acid, were detected in BPD, PDB, and LBR, however, only traces of these metabolites were detected in the other four growth media, indicating that BPD, PDB, and LBR were suitable for production of dibenzofurans in *Phoma* sp. NG-25. Notably, the cultivation of *Phoma* sp. NG-25 in PDB and LBR media resulted in cercosporamide production levels comparable to those achieved in BPD media. While, both PDB and LBR culture extacts of *Phoma* sp. NG-25 contained an unknown peak as a major constituent (Fig. 1A).

For chemical profiling, the BPD culture extract was evaluated by LC-MS analysis. The observed mass-to-charge ratio (m/z) of the purified compounds corresponded to the previous reports for cercosporamide, phomodione, and usnic acid (Hoffman et al., 2008). Chemical structures of cercosporamide and phomodione were confirmed by NMR and MS data (Fig. 1B; Supplementary Figs. S1–S3). The authenticity of usnic acid was confirmed by comparing its relative retention time and the UV spectrum in HPLC analysis to those in our in-house lichen substance database (Huneck and Yoshimura, 1996), as well as that of the commercially purchased standard (Supplementary Fig. S4). Given that cercosporamide was the major secondary metabolite produced in BPD, a time-course experiment was conducted to monitor the progress of cercosporamide production in BPD. The production of cercosporamide peaked at 18 d after incubation, and thereafter the titer of cercosporamide declined, presumably due to biotransformation and degradation of the compound (Fig. 1C).

3.2. Titer and extraction efficiency of cercosporamide

Cercosporamide was purified from BPD culture extracts, with a purity of greater than 95% (see Materials and Methods). To assess the titer of cercosporamide produced in BPD, we established a standard curve based on UV absorbance and cercosporamide concentrations ($R^2 = 0.998$; Fig. 2A). In addition, three independent batch

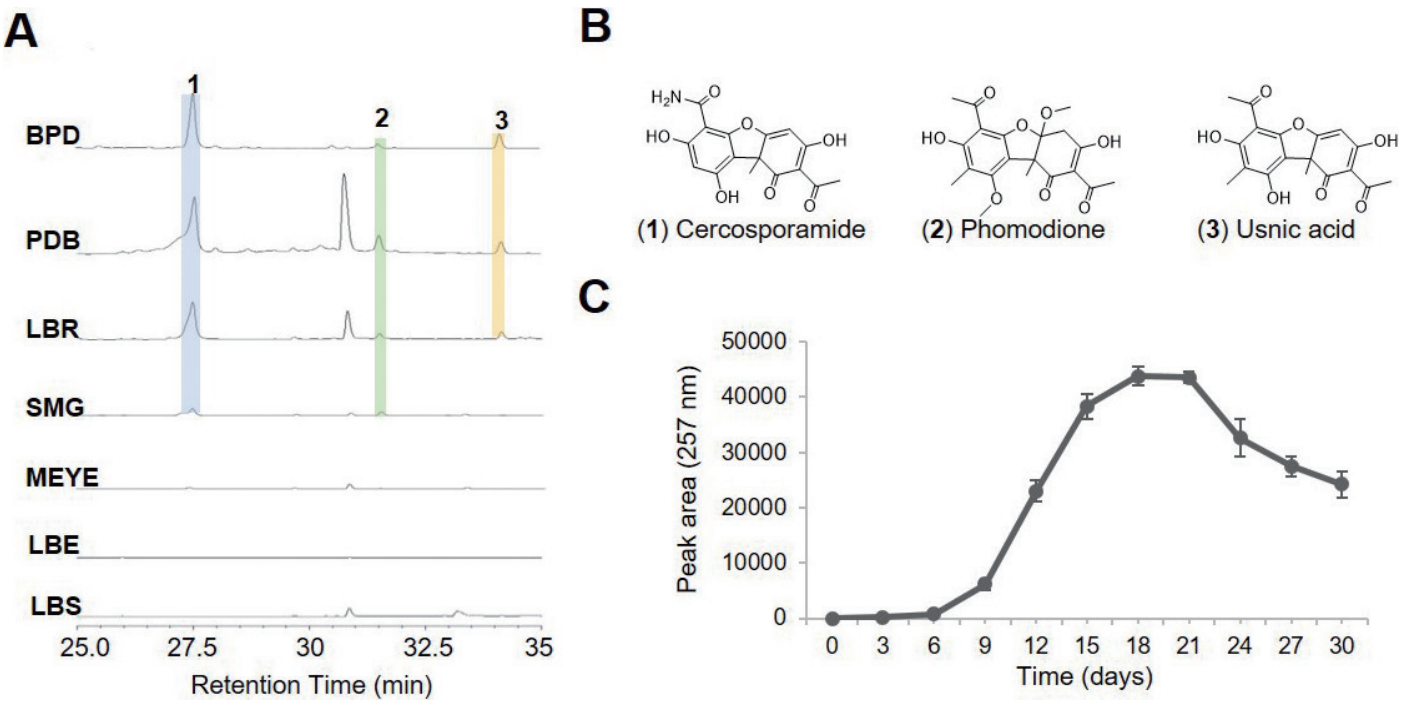


Fig. 1 – Cercosporamide production in various growth media. A: HPLC profiles of culture extracts of *Phoma* sp. NG-25 cultivated in different growth media: Beef peptone dextrose medium (BPD), potato dextrose broth (PDB), soya peptone maltose glucose medium (SMG), malt extract and yeast extract medium (MEYE), Lilly & Barnett (LB) medium containing ribitol as a carbon source (LBR), LB medium containing erythritol (LBE), and LB medium containing sorbitol (LBS). LC-MS analysis of culture extracts confirmed the production of cercosporamide (**1**, observed m/z value: 330.0607 $[M\text{-}H]^-$), phomodione (**2**, observed m/z value: 389.1233 $[M\text{-}H]^-$), and usnic acid (**3**, observed m/z value: 343.0814 $[M\text{-}H]^-$). B: Chemical structures of the three dibenzofurans produced by *Phoma* sp. NG-25. C: Time-course production of cercosporamide in beef peptone dextrose medium. Each data point is the averaged peak area of cercosporamide at 257 nm wavelength in two replicate samples, and bars indicate standard deviation.

cultures of *Phoma* sp. NG-25 were grown for 18 d and analyzed for cercosporamide production. The titers of cercosporamide in each of the five-liter batch cultures were 67.9, 71.8, and 92.8 μg/mL (Fig. 2B).

Next, we evaluated the efficacy of solvent extraction for cercosporamide using a BPD culture filtrate, which initially contained 86.1 μg/mL of cercosporamide. This culture filtrate held a total of 10.76 mg of cercosporamide in 125 mL. We added an equal volume of EtOAc to the culture filtrate for a liquid-liquid extraction, resulting in the recovery of 5.65 mg of cercosporamide. The EtOAc extraction efficiency for cercosporamide was calculated as 53% (Table 1).

In addition, to confirm the presence of cercosporamide in mycelia and assess the efficiency of solvent extraction for cercosporamide, we systematically evaluated the performance of various organic solvents for extracting cercosporamide from fungal mycelia, employing a comparative approach. Our findings revealed that methanol was the best extractant for solid-liquid extraction from mycelia (Fig. 2C). Thus, while EtOAc was suitable for liquid-liquid extraction; methanol proved to be the optimal choice for solid-liquid (mycelia-solvent) extraction.

3.3. Broad spectrum antifungal activity of substances in Phoma sp. NG-25 cultures

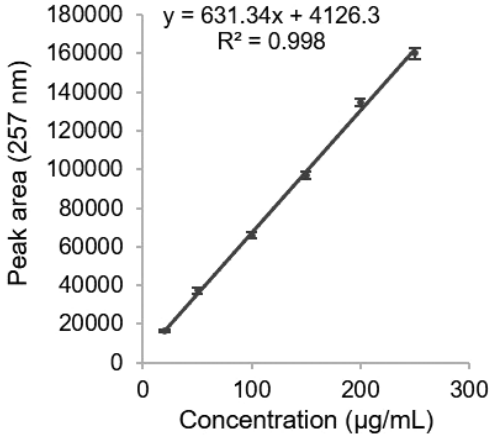
Antifungal activities of culture filtrate of *Phoma* sp. NG-25 were tested against a panel of taxonomically diverse phytopathogenic fungi causing significant yield losses of crops and fruit trees in China and South Korea: Ascomycota (*Alternaria alternata, C. gloeosporioides*, *Diaporthe actinidiae*, *Diaporthe eres*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*), Basidiomycota (*Rhizoctonia cerealis* and *Rhizoctonia solani* AG-2-2), and Oomycota (*Phytophthora capsici* and *Pythium ultimum*). With the paper disk diffusion assay, the BPD culture filtrate, containing approximately 200 μg/mL of cercosporamide, exhibited a strong antifungal activity against the tested fungal species, suggesting the presence of a potent antifungal substance in the culture (Fig. 3).

In addition, we examined antifungal activities of *Phoma* sp. NG-25 cultured in oatmeal flakes and observed that the antifungal activity of the oatmeal flakes culture was as equally good as the BPD culture filtrate, with respect of growth inhibition of the fungal plant pathogens (Fig. 4B). It was observed that cercosporamide production was the main chemical constituent of *Phoma* sp. NG-25 grown in oatmeal flakes (Fig. 4A).

3.4. Cercosporamide as a potent antifungal agent controlling anthracnose of pepper

To precisely measure the antifungal activities of the three dibenzofurans, namely cercosporamide, phomodione, and usnic acid, we initially succeeded in acquiring the individual compounds (see Materials and methods). Our choice of two pepper pathogens was motivated by the increasing reports of fungicide resistance in *Colletotrichum* spp. against commercially available chemical fungicides. Hence, the antifungal activity of the three compounds were tested against *C. gloeosporioides* and *C. scovillei*, given their relevance to pepper anthracnose. The dose-response curve based on the probit regression model was generated for estimation of EC_{50} values of

A



B

Experiments	Concentration (μg/mL)
First batch	92.8
Second batch	67.9
Third batch	71.8
Mean ± standard deviation	77.5 ± 13.4

C

Solvent	Weight recovered (mg)
Methanol	0.72
Acetone	0.48
EtOAc[a]	0.13
Hexane	Not detected

[a] EtOAc, ethyl acetate

Fig. 2 – Production and extraction of cercosporamide. A: A standard curve was established based on the peak areas corresponding to cercosporamide (UV monitoring at 257 nm). Each data point represents the average peak area of three replicates, and bars indicate standard deviation. B: The titer of cercosporamide in the culture filtrates of *Phoma* sp. NG-25 was measured, which was cultivated in a beef peptone dextrose medium and harvested 18 d after incubation. C: Solvent extraction efficiency of cercosporamide from one gram of *Phoma* sp. NG-25 mycelia.

Table 1 Solvent extraction efficiency for cercosporamide from culture filtrate of *Phoma* sp. NG-25.

	Concentrations (μg/mL)	Volume (mL)	Amounts (mg)	Extract Efficiency (%)
Culture filtrate	86.1	125, water	10.76	—
EtOAc[a] extract	45.2	125, EtOAc	5.65	52.5

[a] EtOAc, ethyl acetate

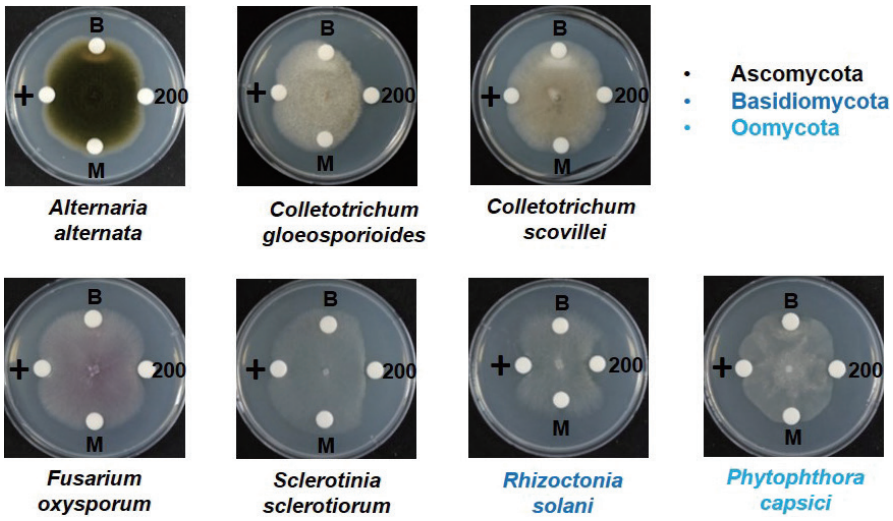


Fig. 3 – Antifungal activities of culture filtrate of *Phoma* sp. NG-25. Paper disks containing culture filtrate of *Phoma* sp. NG-25 were placed on the right side of the plates (labelled as 200, beef peptone dextrose media containing 200 μg/mL of cercosporamide). Left disks containing a positive control (+, 200 μg/mL of chlorothalonil), top disks containing a medium control (B, approximately two times concentrated beef peptone dextrose medium), Bottom disks containing a solvent control (M, 100% of methanol). Plant pathogenic fungi and oomycetes were inoculated on the center of the plates, quarter strength potato dextrose agar.

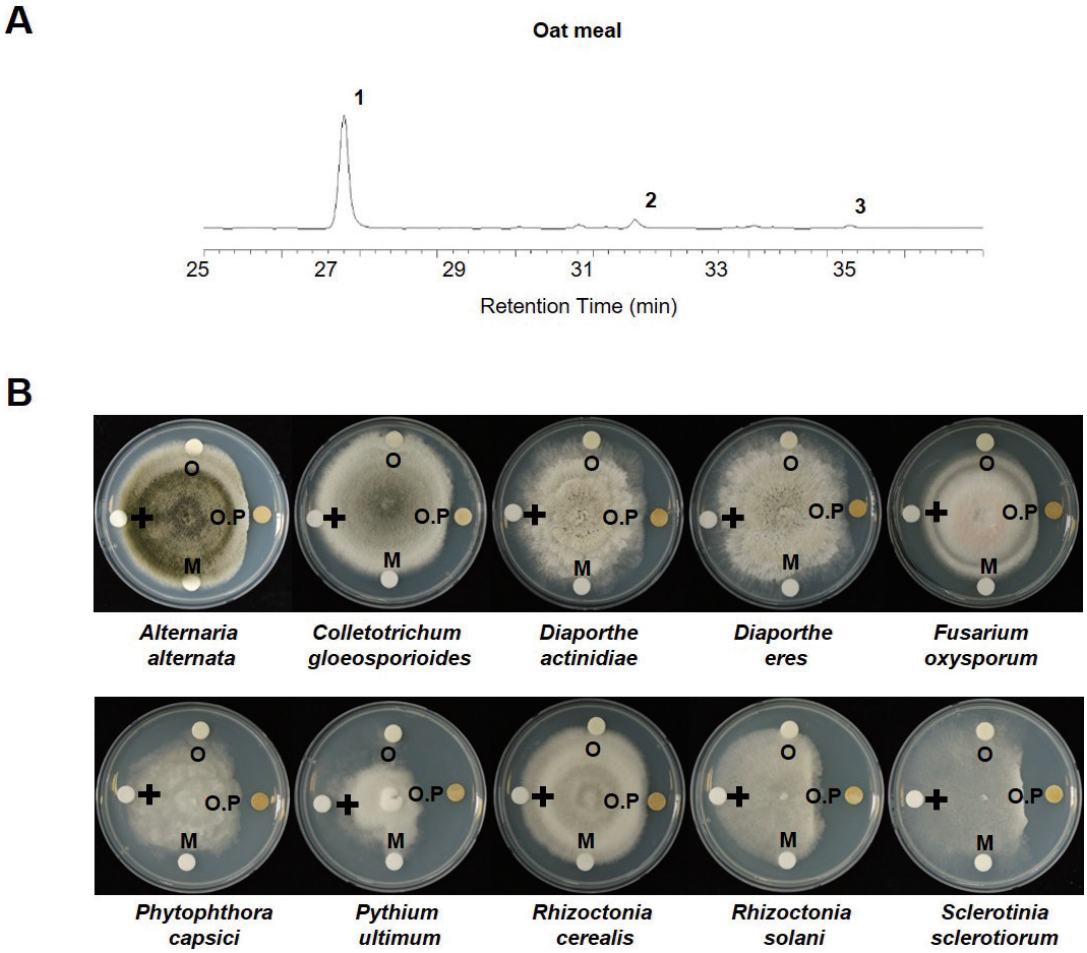


Fig. 4 – Antifungal activities of culture extracts of *Phoma* sp. NG-25 grown on oatmeal flakes. A: Chemical profile of the crude extract of *Phoma* sp. NG-25 grown on oatmeal flakes. Oatmeal culture extract were separated using a YMC-Pack ODS-A column on an HPLC system. Three dibenzofurans were cercosporamide (**1**), phomodione (**2**), and usnic acid (**3**). B: Paper disks containing culture extracts of *Phoma* sp. NG-25 cultivated in oatmeal flakes were positioned on the right side of the plates. Disks on the left contained positive control (200 μg/mL of chlorothalonil), top disks containing oatmeal flakes medium extract, and bottom disks containing a solvent control (100% of methanol). Plant pathogenic fungi and oomycetes were inoculated on the center of the PDA plates.

cercosporamide (Supplementary Fig. S5; Supplementary Table S1). Cercosporamide exhibited strong antifungal activity against both *C. gloeosporioides* and *C. scovillei*, with EC_{50} value of 3.8 μg/mL and 7.0 μg/mL, respectively (Fig. 5A, B). Phomodione displayed modest activity against *C. gloeosporioides*, but it was ineffective in inhibiting the growth of *C. scovillei*, with an EC_{50} exceeding 100 μg/mL (Fig. 5A, B). In the case of usnic acid, it was challenging to determine the EC_{50} value for *C. gloeosporioides* due to the fungus exhibited greater sensitivity to lower concentrations of usnic acid compared to higher concentrations (Fig. 5A). Nonetheless, usnic acid exhibited only weak effects on both pepper pathogens.

4. Discussion

In recent years, the global production and application of biological or biochemical fungicides in agriculture have increased owing to adverse effects of synthetic fungicides on human health, food safety, and environment (Carvalho, 2017; Kumar, 2012). This is further reinforced by the escalating emergence of fungicide-resistant phytopathogens due to excessive and repetitive application of synthetic fungicides (Peng et al., 2021; Ramdial et al., 2016). However, developmental costs and technological challenges in large-scale production are major hindrances to the development of commercial products based on biological or biochemical fungicides. In this study, a laboratory-scale culture of *Phoma* sp. NG-25 resulted to a maximum titer of cercosporamide of approximately 80 μg/mL. However, we observed aggregation and clumping of mycelia when *Phoma* sp. NG-25 were grown in liquid media, which cause problems in large-scale fed-batch culture systems. 1) This issue can potentially be addressed by elevating the speed of orbital shakers, enhancing the culture medium's viscosity, or introducing oat flour into the liquid medium. Oxygen is a limiting factor, and increased aeration can be beneficial until reaching saturation. 2) On an alternative note, to mitigate this issue, solid-state culture can serve as a cost-effective alternative for large-scale production. We found that oatmeal flakes as a solid medium supported extensive growth of *Phoma* sp. NG-25, as well as production of cercosporamide as a major constituent (Fig. 4A). This observation suggested

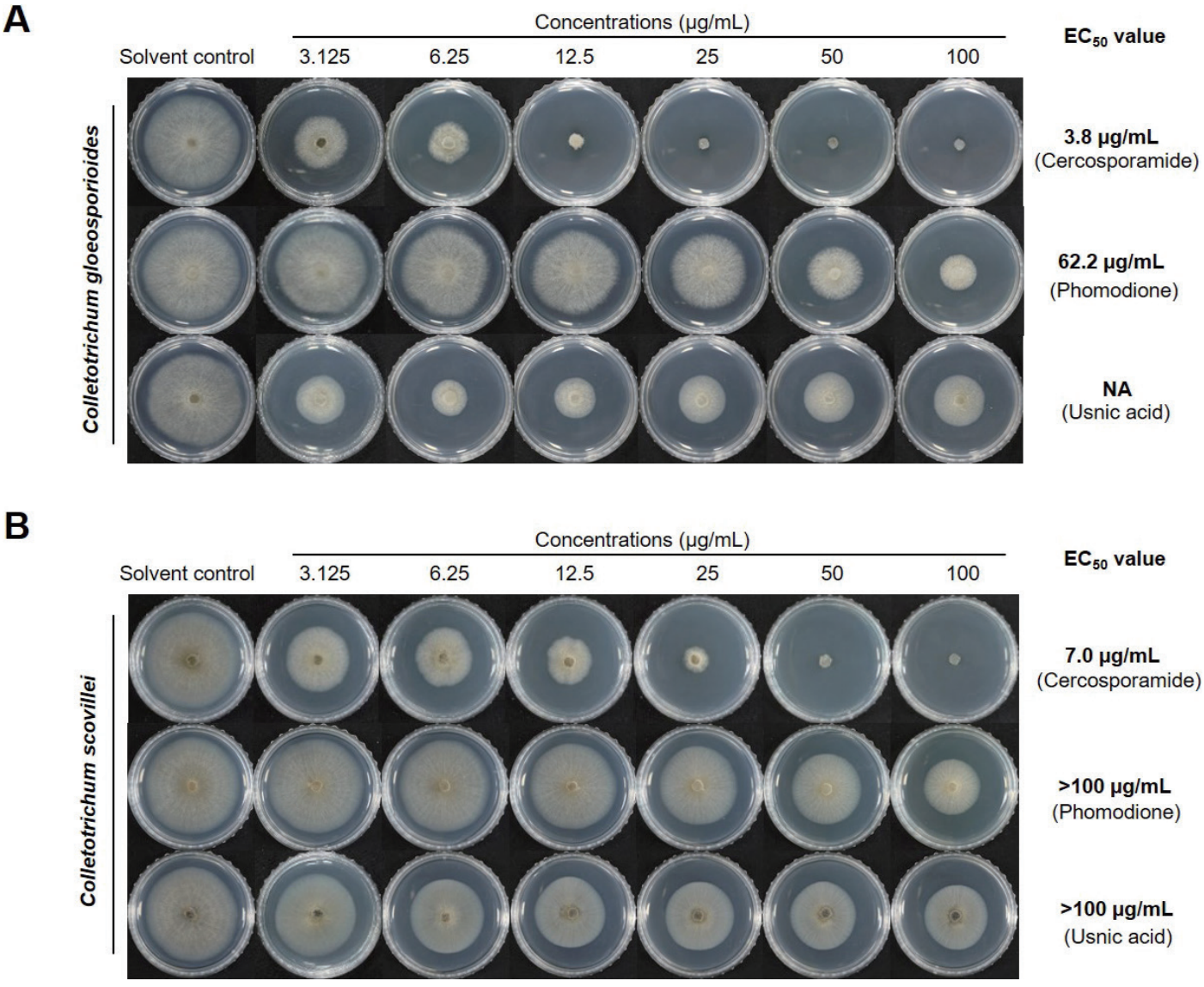


Fig. 5 – Antifungal activities of purified dibenzofurans in *Phoma* sp. NG-25. The effective concentration (EC_{50}) of cercosporamide, phomodione, and usnic acid that inhibits the growth of the causal agents of pepper anthracnose, *Colletotrichum gloeosporioides* (A) and *C. scovillei* (B). For solvent control, 5% methanol was amended to growth media. NA, not applicable.

that various agro-industrial cereals and wastes (straws and husks) as organic substrates can be used for solid-state culture for cercosporamide production in *Phoma* sp. NG-25. To further our research, we can explore options like brown rice, wheat seeds, millet seeds, and cracked corn for solid media. Additionally, we should assess various factors such as temperature, moisture levels, incubation duration, and combinations of organic substrates to enhance the production of cercosporamide in solid media. 3) Moreover, Singh et al. reported a remarkable 100-fold rise in the production yield of cercosporamide from the LV-2841 fungal culture by introducing 3% V/V of the adsorptive polymeric resin HP-20, resulting in consistent titers ranging from 200 to 600 μg/mL (Singh et al., 2010). This culture-based strategy presents a viable alternative for augmenting production yields in our endophytic strain.

The BPD culture filtrate and oatmeal media extracts obtained from *Phoma* sp. NG-25 exhibited a broad spectrum of antifungal activity against various plant pathogenic fungi. Notably, cercosporamide inhibited strong inhibitory effects on *C. gloeosporioides* and *C. scovillei*, the causative agents of anthracnose in pepper, with EC_{50} values of 3.8 and 7.0 μg/mL, respectively. Cercosporamide's mode of action is associated with its ability to selectively bind to a fungal PKC as previously reported (Sussman et al., 2004). We observed that cercosporamide exhibited potent antifungal properties against *C. gloeosporioides* and *C. scovillei*, and this broad-spectrum antifungal activity can be attributed to its mode of action targeting PKC. Future research endeavors may delve into whether cercosporamide employs a similar mechanism to hinder the growth of pepper pathogens. Its mode of action might also be linked to processes such as cell wall biosynthesis and PKC. In addition, cercosporamide exhibited markedly enhanced antifungal activity when applied synergistically with an echinocandin analog (a β-1,3-glucan synthase inhibitor) (Sussman et al., 2004). Combinational or rotational use of cercosporamide with commercial fungicides with different modes of action might also be highly effective in controlling anthracnose of pepper. Also, it is notable that cercosporamide exhibited strong anti-oomycete activity against *Phytophthora capsici* and *Pythium ultimum*, the causal agents of pepper blight and damping-off, respectively, which are agriculturally significant diseases in pepper (Arora et al., 2021; Kim et al., 1989).

In our initial experiment last summer, we assayed plants utilizing cercosporamide at a concentration of 80 μg/mL. Notably, this concentration did not result in the formation of lesions on pepper plants, indicating that it appears to be non-toxic to peppers. Nevertheless, the current study should be extended to *in vivo* studies to measure the efficiency of cercosporamide and validate its efficacy in managing anthracnose in pepper plants. Additionally, the formulation of cercosporamide for optimal delivery merits exploration in order to effectively translate its potential into tangible agricultural applications.

Conclusions

Overall, this study has successfully established improved culture and extraction methods that increased the yield of cercosporamide. Furthermore, it characterized the extent of antifungal efficacy demonstrated by cercosporamide, phomodione, and usnic acid against several plant pathogenic fungi. The strong antifungal activity exhibited by cercosporamide validates its efficacy as a potential biochemical fungicide for addressing anthracnose in pepper cultivation.

Disclosures

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they are performed.

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