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Investigation of Antimicrobial Compounds Produced by Endolichenic Fungi in Different Culture Media

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Continuous use of synthetic fungicides has led to explosive emergence of fungicide-resistant microbes. Therefore, there are urgent needs for environmentally friendly antimicrobial agents with novel modes of action. This study investigated endolichenic fungi (ELF) as a source of antimicrobial compounds against various plant pathogens. We utilized an One Strain MAny Compounds (OSMAC) approach to enhance the chemical diversity of fourteen ELF. This involved cultivation of ELF in four growth media and subsequently assessing antimicrobial activities of culture extracts. Nearly half of the culture extracts exhibited antimicrobial activity against a Gram-positive bacterium, but showed minimal activity against Gram-negative bacteria tested. Notably, culture extracts from two ELF, *Chaetomium globosum* and *Nodulisporium* sp., demonstrated significant inhibitory effects against plant pathogenic fungi. LC-MS/MS-based metabolome profiling confirmed the presence of known bioactive compounds like cyclic dipeptides and chaetoglobosins. These findings highlight the effectiveness of combining OSMAC and metabolomics for identifying antimicrobial agents for agricultural use.

Keywords **:** antimicrobial activity, biopesticide, endolichenic fungi, metabolomics, OSMAC

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Recent years have seen a rise in plant disease outbreaks, causing significant crop loss and quality reduction, which poses a threat to global food security and public health (Brooks et al., 2022; Ristaino et al., 2021). Furthermore, the increasing frequency of emerging plant diseases, coupled with climate change and global commerce is expected to exacerbate the problem in the coming years (Bebber et al., 2014; Brasier, 2008). As a consequence, there is a need for an integrated research agenda to prevent and combat emerging plant diseases and improve disease management strategies (Brasier, 2008). The search for new biologically active compounds to tackle the escalating issues in agriculture is ongoing (Brooks et al., 2022; Gorlenko et al., 2020; Lee et al., 2022). While plants have traditionally been the primary source of antimicrobial agents, excessive harvesting from their natural habitats to support drug production has led to their endangerment (Oksman-Caldentey and Inzé, 2004; Venkatasubramanian et al., 2018). Con-

sequently, alternative sources of antimicrobials have been investigated, including the identification of novel bioactive metabolites in endolichenic fungi (ELF) found in various lichen species.

Lichens are formed through a symbiotic relationship between fungi (mycobionts) and photosynthetic partners, namely green algae and/or cyanobacteria (photobionts) (Boustie and Grube, 2005). Lichen mycobionts are known to produce several biologically active metabolites that are significant for pharmaceutical and agricultural applications (Boustie and Grube, 2005; Paguirigan et al., 2022). Although production of lichen-derived bioactive compounds in heterologous hosts has been successful in some instances (Kealey et al., 2021; Kim et al., 2021), practical uses of many lichen substances have been limited due to their slow growth in axenic culture (Boustie and Grube, 2005). As an alternative, ELF—filamentous fungi that reside asymptomatically within lichen thalli (Kellogg and Raja, 2017)—have been investigated for their potential to produce bioactive secondary metabolites. These ELF, primarily within the Ascomycota (Pezizomycotina), have been found in various lichen species (Arnold et al., 2009; Kannangara et al., 2009; Kellogg and Raja, 2017; Suryanarayanan et al., 2017; U'Ren, 2011). Many ELF are valuable bioresources due to their ability to produce metabolites with diverse structures and wide-ranging biological activities (Li et al., 2015; Padhi et al., 2019; Yang et al., 2021). The bioactive metabolites produced by ELF originate from various biosynthetic pathways and encompass a range of chemical structural classes, including alkaloids, quinones, furanones, pyrones, benzopyranoids, xanthones, terpenes, steroids, peptides, and acyclic compounds (Cheon et al., 2013; Dou et al., 2014; Li et al., 2015; Lin et al., 2021; Yang et al., 2019b; Yuan et al., 2013, 2017). These compounds exhibit diverse bioactivities, such as anticancer, antiviral, antibacterial, antifungal, and anti-Alzheimer's disease properties, as well as serving as skin UV protectants (He et al., 2012; Kellogg and Raja, 2017; Li et al., 2015; Padhi et al., 2020; Santiago et al., 2021; Xie et al., 2020; Zhou et al., 2016).

The ELF that reside inside the healthy lichen thalli produce metabolites that can protect the host under various stress conditions (Nguyen et al., 2013). Since the discovery of secondary metabolites from ELF in 2007 (Paranagama et al., 2007), several other potent bioactive molecules have been identified worldwide (Agrawal et al., 2020; Padhi et al., 2020; Santiago et al., 2021; Xie et al., 2020; Yuan et al., 2013). Within the past two decades, numerous bioactive metabolites have been identified for ELF, including phomol, 16-α-D-mannopyranosyloxyisopimar-7-en-19-oic acid, and 8-methoxy-1-naphthyl-β-glucopyranoside isolated from *Hypoxylon fuscum*, which exhibits a weak antibacterial activity against *Staphylococcus aureus* (Basnet et al., 2019). In addition, carbonarone A and fonsecinone A exhibited antibacterial activity against the plant pathogenic bacteria, *Dickeya solani* and *Pseudomonas syringae* pv. *maculicola*, respectively (Padhi et al., 2020). Consequently, there has been a significant increase in interest in producing bioactive natural products from ELF (Kellogg and Raja, 2017). With an estimated 20,000 species of lichens globally (Feuerer and Hawksworth, 2007), there exists a substantial pool of potential ELF to explore, offering a vast library of compounds for further investigation.

In this study, we took advantage of the ELF collection deposited in the Korean Lichen Research Institute (KoLRI), which were isolated from three lichen thalli of *Parmotrema tinctorum* on pine trees (*Pinus thunbergii*) from Songho beach located in Haenam-gun, South Korea (Yang et al., 2022). The ELF collection included 1,029 isolates classified into 58 genera (Yang et al., 2022). Among the isolated ELF, we selected 14 ELF that have not yet been investigated for their chemical diversity or the antimicrobial activities of their metabolites through literature surveys (Table 1). ELF isolates were identified by sequencing of a region of the internal transcribed spacer (ITS), using an ITS4 and ITS5 primer pair (Supplementary Table 1). We adapted an OSMAC (One Strain MAny Compounds) approach to increase the diversity of secondary metabolites produced by 14 ELF by culturing them in four different growth media: (1) beef peptone-dextrose broth (BPDB—dextrose 10 g, beef peptone 8 g [Difco, Detroit, MI, USA] in 1 liter of distilled water, pH adjusted to 5.3), (2) malt-yeast extract broth (MEB—malt extract 3 g [Difco], yeast extract 3 g [Difco], peptone 5 g [Difco], dextrose 10 g in 1 liter of distilled water, pH adjusted to 5.3), (3) potato dextrose broth (PDB; Difco, pH adjusted to 5.3), and (4) oatmeal (50 g oatmeal flakes [Quaker, Chicago, IL, USA], moistened with 10 ml of distilled water). For the three broth culture (BPDB, MEB, and PDB), ELF were cultured in 200 ml of liquid media in 500 ml flasks. The growth media were inoculated with agar blocks containing hyphae of ELF that had been grown on potato dextrose agar (PDA; Difco). The cultures were incubated for 21 days at 23ºC on orbital shaker (150 rpm) in 12 h light and dark cycle. For oatmeal media, cultures were grown under a static condition.

We initially evaluated chemical diversity of culture extracts from 14 ELF, using high-performance liquid chromatography (HPLC) analysis. Fungal mycelia were macerated using a homogenizer. For extraction, 200 ml of ethyl acetate was added to the homogenized cultures (approximately 200 ml) and the mixture was sonicated three

Table 1. Endolichenic fungi isolated from thalli of *Parmotrema tinctorum*

Institutional code	ITS accessions	BLASTn best hits	Species	% Coverage	% Identity
CNC11_W06	N/A[a]	MH793573	*Nemania diffusa* AgF2-4-4	99	99.31
CNC11_W13	MZ855422	KP689127	Sordariomycetidae sp. N133	100	99.67
CNC12_B16	MZ855437	PP757928	*Pestalotiopsis clavata* FJ1-1	99	100
CNC12_P14	MZ855441	LT796902	*Pseudochaetosphaeronema* sp.	100	98.93
CNC12_P24	MZ855447	MZ423054	Sordariomycetes sp. NTOU 4496	99	97.67
CNC13_H08	MZ855423	MT107902	*Microcera larvarum* ICMP 5444	99	98.26
CNC14_H02	MZ855406	OW986721	*Exophiala phaeomuriformis*	98	93.24
CNC23_B05	N/A[a]	MH858130	*Chaetomium globosum*	100	98.83
CNC24_P08	MZ855435	OR781497	*Pestalotiopsis rhodomyrti* JGS20c	100	99.84
CNC24_W03	MZ855391	LC798884	*Coprinellus radians*	100	99.71
CNC32_P05	MZ855451	KX611052	*Thielavia* sp. A84	99	99.83
CNC32_P20	N/A[a]	GQ906948	*Nodulisporium* sp. JP60-3	99	98.74
CNC35_W02	MZ855366	EF488415	*Annulohypoxylon atroroseum* B38	97	99.89
CNC35_P04	N/A[a]	OR761515	*Xylaria longipes* isolate 80310	93	100

[a]Internal transcribed spacer sequences can be found in Supplementary Table 1.

times for 10 min in a sonicator bath. The solvent layers were separated by filtration using two layers of Whatman papers and evaporated using a rotary evaporator. The resulting crude culture extracts were transferred to vials and air-dried in a fume hood. Then, culture extracts were reconstituted with methanol to yield a final concentration of 1 mg/ml. HPLC analysis was performed using a YMC-Pack ODS-A column (150 × 4.6 mm, 5 μm particle size, 12 nm pore diameter) at 40°C on an HPLC system (Prominence Modular High Performance Liquid Chromatography LC-20A, Shimadzu, Kyoto, Japan). UV-active metabolites were monitored from 200 to 800 nm using a diode array UV detector. The mobile phase consisted of distilled water with 0.1% trifluoroacetic acid for pump A and methanol with 0.1% trifluoroacetic acid for pump B. A gradient program for the mobile phase was set as follows: 0-30 min, 20-100% B; 30-40 min, 100% B; 40-52 min, 20% B, with a flow rate of 1.0 ml/min. The HPLC analyses of different culture extracts from 14 ELF exhibited peaks of UV-active metabolites, many of which were exclusively found in a specific growth medium (Supplementary Fig. 1).

With a total of 56 culture extracts (14 ELF × 4 growth media), we evaluated antibacterial and antifungal activities against plant pathogenic bacteria and fungi. Examined were one Gram-positive bacterium, seven Gram-negative bacteria, 10 ascomycetes fungi, two basidiomycetes fungi, and two oomycetes fungi, all of which can cause devastating diseases on fruits and vegetables of agricultural importance in South Korea (Supplementary Table 2). Bacterial and fungal strains were obtained from the Korean Agricultural Culture Collection (KACC), National Academy of Agricultural Sciences (NASS), otherwise mentioned. The bacterial strains were cultured on tryptic soy broth (BD Bioscience, Beford, MA, USA) at a room temperature, and the fungal strains were maintained on PDA at a room temperature. To test antibacterial activities of ELF extracts, bacterial suspensions were prepared with a final concentration of 1×10^5 colony-forming units (cfu)/ml. One hundred milliliters of tryptic soy agar (TSA; BD Bioscience) was cooled down, mixed with 1 ml of bacterial suspension, and then poured into a 90 mm Petri dish to solidify. Forty microliters of culture extracts at a concentration of 1 mg/ml were applied to 8 mm paper discs (Advantec Toyo Kaisha, Tokyo, Japan). Negative control was 100% methanol, while positive controls were prepared using 200 ppm streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA). After drying, the discs were placed onto TSA culture plates containing bacterial suspensions. These plates were incubated at 30°C for 1-3 days until bacterial growth was visible. Each experiment was performed twice independently, with three replicate plates each time. The half of the ELF cultures grown in BPDB, MEB, PDB, and oatmeal media (28 out of 56) showed substantial inhibitory activities against a Gram-positive bacterium, *C. michiganensis* subsp. *michiganensis* (Fig. 1A). However, most of the ELF cultures were not effective in suppressing

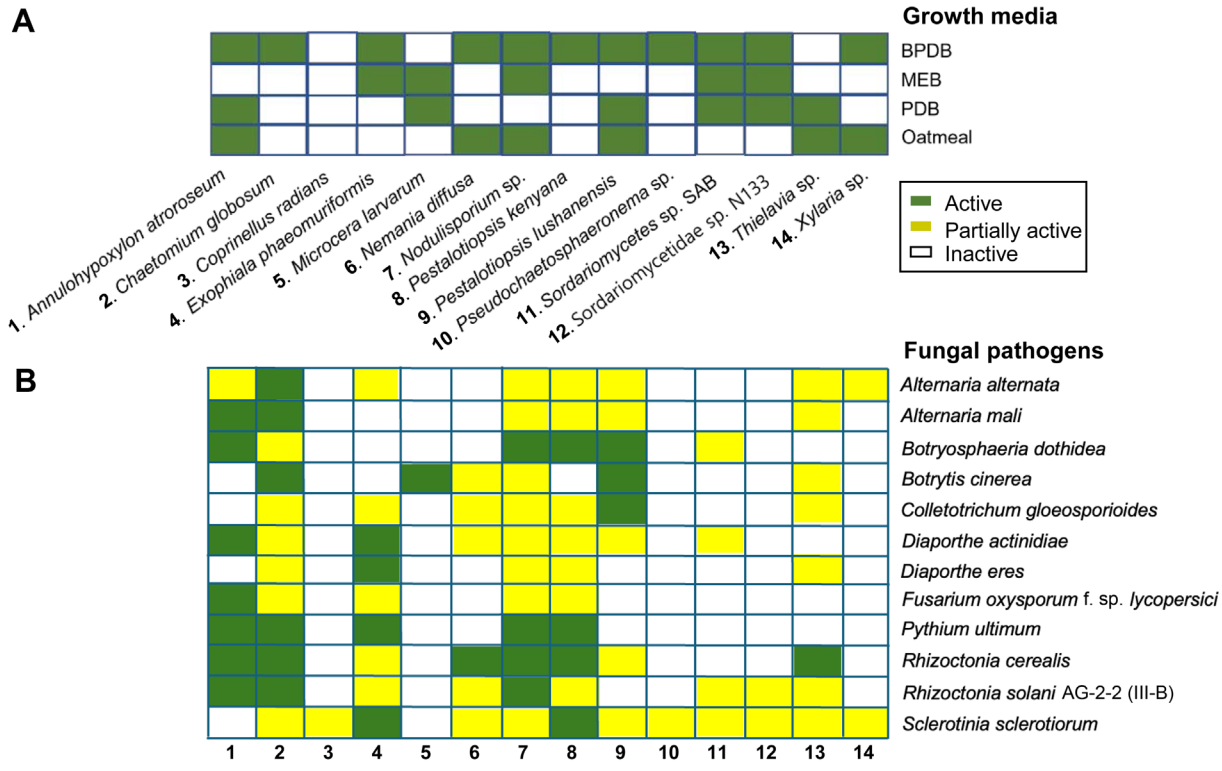

Fig. 1. Degrees of antimicrobial activities of 14 endolichenic fungi (ELF). (A) Antibacterial activity against *Clavibacter michiganensis* subsp. *michiganensis*. Crude extracts (40 μg) of 14 ELF culture grown in four different growth media were tested. (B) Antifungal activity against twelve plant pathogenic fungi and oomycetes. Crude extracts (40 μg) of 14 ELF cultures grown in four different growth media (beef peptone-dextrose broth [BPDB], malt-yeast extract broth [MEB], potato dextrose broth [PDB], and oatmeal) were tested. Degrees of antifungal activity are shown as a heatmap with green and yellow colors when at least one of ELF cultures grown in four different media exhibited antifungal activities. The numbers 1 to 14 are ELF as shown in panel A.

the growth of Gram-negative bacteria; only cultures of an ELF (CNC35_W02: *Annulohypoxylon atroroseum*) grown in oatmeal media exhibited antimicrobial activities against two Gram-negative bacteria, *Xanthomonas euvesicatoria* and *Ralstonia solanacearum*. The culture extracts of *A. atroroseum* grown in oatmeal showed several peaks that have not been detected in the other three broth media during HPLC analysis (Supplementary Fig. 1), suggesting that these unidentified UV-active compounds may account for the observed antimicrobial activities.

ELF extracts were screened for their antifungal activities against a panel of plant pathogenic fungi (Supplementary Table 2). To perform the antifungal assays, a small piece of agar containing mycelia of phytopathogenic fungi was placed in the center of a plate containing quarter-strength PDA. After 3 days, when the fungi had grown to cover about half of the plate, 40 μl of culture extracts (1 mg/ml) were applied to 8 mm paper discs and placed around the edge of the plate. Eight micrograms of chlorothalonil (Sigma-Aldrich) were applied to discs to be served as the positive control, while 100% methanol was used as a negative control. The plates were then incubated for an additional 3 days at 23°C until the fungi had fully covered the plates, after which the inhibition zones of fungal growth were measured. This experiment was conducted independently three times, with each trial including three replicate plates. The antifungal activities were considered 'Active' when fungal mycelia did not reach to paper discs containing culture extracts, or considered 'Partially active' when fungal mycelia did touch, but cannot cover paper discs containing culture extracts.

Many ELF culture extracts exhibited antifungal activities against plant pathogenic fungi and oomycetes (Fig. 1B). The antifungal potential varied based on the culture media used, with MEB yielding a higher number of cultures with inhibitory effects (Supplementary Fig. 2). Six ELF culture extracts showed strong antifungal activities against *Rhizoctonia cerealis*, while only one extract exhibited a strong activity against *Alternaria alternata, Colletotrichum gloeosporioides*, *Diaporthe eres*, and *Fusarium oxysporum*

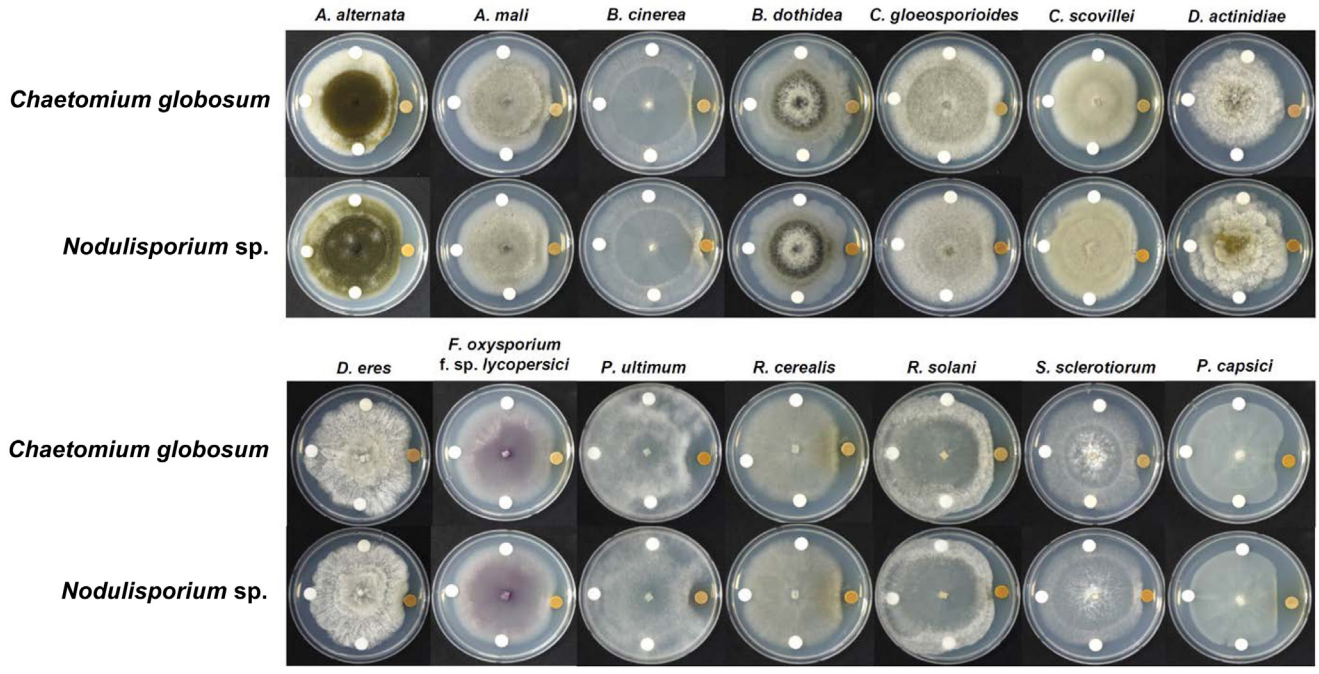

Fig. 2. Antifungal activities of culture extracts of *Chaetomium globosum* and *Nodulisporium* species. Paper disks containing culture extracts (40 μg) of the two endolichenic fungi grown in malt-yeast extract broth were placed on the right side of the plates. Left disks containing a positive control (8 μg of chlorothalonil), top disks containing a medium control, Bottom disks containing a solvent control (M, 5% methanol). Plant pathogenic fungi and oomycetes were inoculated on the center of the plates containing quarter-strength potato dextrose agar. Tested plant pathogenic fungi are as follows: *Alternaria alternata*, *A. mali*, *Botrytis cinerea*, *Botryosphaeria dothidea*, *Colletotrichum gloeosporioides*, *C. scovillei*, *Diaporthe actinidiae*, *D. eres*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pythium ultimum*, *Rhizoctonia cerealis*, *Rhizoctonia solani* AG-2-2 (III-B), *Sclerotinia sclerotiorum*, and *Phythophthora capsici.*

f. sp. *lycopersici* (Supplementary Fig. 3). This indicated a high degree of selectivity of ELF metabolites on growth inhibition of fungal species. Among the ELF cultures, the extracts of *Chaetomium globosum* and *Nodulisporium* species exhibited a broad spectrum of antifungal activities (Fig. 1B). Therefore, we repeated the antifungal assays, including two additional pepper pathogens, *Colletotrichum scovillei* and *Phytophthora capsici*, driven by our interest in developing a novel, environmentally safe fungicide to control pepper diseases (Liu et al., 2024). *Colletotrichum scovillei* strain KC05 was isolated from an infected pepper fruit in Gangwon province, South Korea (Fu et al., 2021). The MEB culture of both *C. globosum* and *Nodulisporium* sp. demonstrated strong antifungal activities against *C. scovillei* and *P. capsici*, comparable to their effects on other plant pathogenic fungi (Fig. 2). It was noteworthy that the growth of *A. alternata* was strongly suppressed by the MEB culture of *C. globosum*, which was not significantly affected by culture extracts of the other ELF examined in this study (Fig. 1B). This suggested that as-yet-unknown antifungal compounds in the ELF culture can be used to control the pepper anthracnose and blight, as well as various plant diseases caused by *A. alternata*, which is one of the problematic pathogens due to development of fungicide resistance in fields (Chitolina et al., 2021; Wang et al., 2022; Yang et al., 2019a).

LC-MS-based metabolomics coupled with computational data analysis methods have been used to identify known compounds in complex biological samples, quickly and efficiently (Nothias et al., 2020). To annotate known bioactive compounds that may be responsible for the observed antifungal activities in cultures of *C. globosum* and *Nodulisporium* sp., LC-MS analysis was performed on a Waters Acquity I-Class UPLC system coupled to a Waters VION IMS QTOF mass spectrometer (Waters Corp., Milford, MA, USA), which was equipped with an electrospray ionization interface. The mobile phase was comprised of 0.1% formic acid in water (pump A) and acetonitrile (pump B). A stepwise gradient method at a constant flow rate of 0.3 ml/min was used to elute the column with the following conditions: 10-100% of pump B (0-12 min), followed by 3 min of washing and 3 min of reconditioning. Tandem MS analyses were performed in data-independent acquisition (MS^E), negative and positive ion mode with the *m/z* 50-

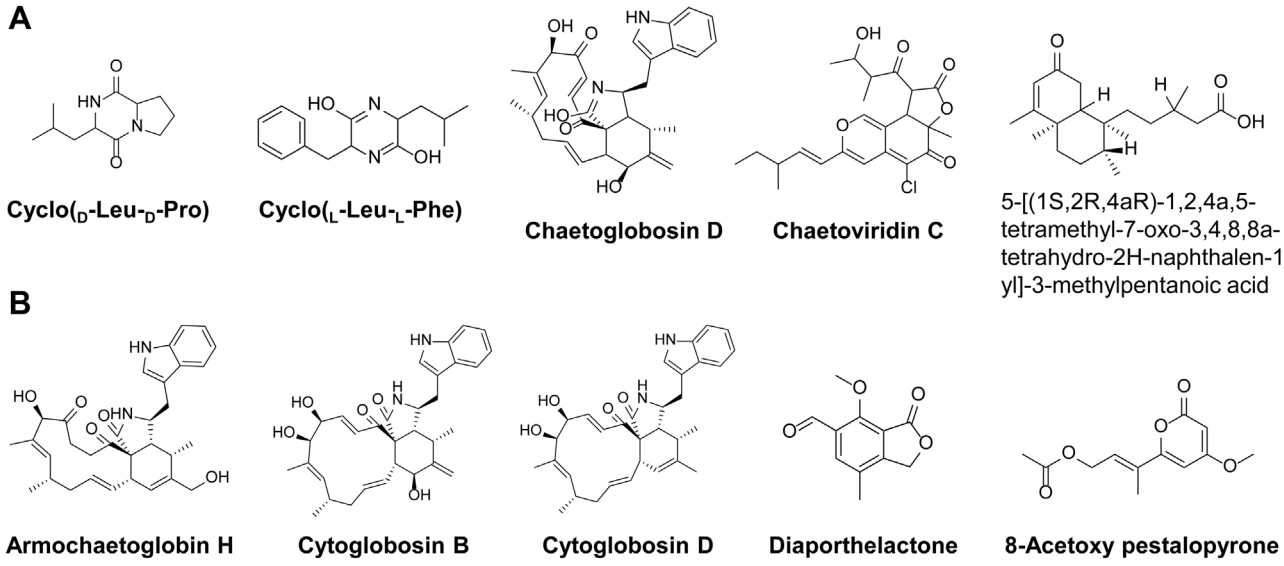

Fig. 3. Metabolic dereplication of culture extracts of *Chaetomium globosum* and *Nodulisporium* species. (A) Secondary metabolites identified by Global Natural Product Social Molecular Networking (GNPS) spectral library matches. (B) Secondary metabolites identified by *in silico* NPAtlas structural library matches.

1,500 Da range and acquisition times of 0.2 s. The low collision energy for the detection of the precursor ions was set to 6 eV, while the high collision energy for fragmentation was set to 20-40 eV. Detailed ionization and other operational conditions can be found in our previous work (Kim et al., 2021). MS/MS spectral data matching of major compounds detected in transformants was performed by the feature-based molecular networking workflow (Nothias et al., 2020) in the Global Natural Product Social Molecular Networking (GNPS) environment (Wang et al., 2016) after the preprocessing using MS-DIAL (Tsugawa et al., 2015).

GNPS spectral library matches revealed the presence of cyclo($_D$-Leu-$_D$-Pro) (t_R 2.81 min), cyclo($_L$-Leu-$_L$-Phe) (t_R 4.12 min), chaetoglobosin D (t_R 6.13 min), and chaetoviridin C (t_R 8.11 min) in the MEB culture of *C. globosum* (Fig. 3A, Supplementary Figs. 4 and 5). The results of the GNPS molecular networking analysis can be found in the following links: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c2032c8e915f4c1da6e0e07008433e6f (negative mode) and https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ca5f7cc6164240a8a49ced23c3ed6a2a (positive mode). Although evidence for metabolite annotation is not strong as GNPS spectral library matches, we also used *in silico* Network Annotation Propagation (NAP) tool (da Silva et al., 2018) with the NPAtlas structural library (van Santen et al., 2022) to further annotate compounds whose reference spectra are absent in the GNPS library. The NAP tool annotated armochaetoglobin H (t_R 5.35 min), cytoglobosin B (t_R 5.76 min), and cytoglobosin D (t_R 7.56 min) in the culture extract of *C. globosum* (Fig. 3B, Supplementary Figs. 4 and 5). Intriguingly, we also annotated cyclo($_D$-Leu-$_D$-Pro) (t_R 2.80 min) and cyclo($_L$-Leu-$_L$-Phe) (t_R 4.08 min) in the MEB culture of *Nodulisporium* species (Supplementary Figs. 4 and 5). Additionally, we annotated 5-[(1S,2R,4aR)-1,2,4a,5-tetramethyl-7-oxo-3,4,8,8a-tetrahydro-2H-naphthalen-1-yl]-3-methylpentanoic acid (t_R 8.65 min) via GNPS spectral library matches (Fig. 3A, Supplementary Fig. 4). Through *in silico* NAP annotation, we annotated diaporthelactone (t_R 8.65 min) and 8-acetoxy pestalopyrone (t_R 8.65 min) (Fig. 3B, Supplementary Figs. 4 and 5) in the MEB culture of *Nodulisporium* species. The results of the *in silico* NAP analysis can be found in the following links; negative mode: https://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task=f6ba694179c2487cae10047ab643b71f and positive mode: http://proteomics2.ucsd.edu/ProteoSAFe/status.jsp?task=03839c6781064e2e8dc8dc55ab662651.

Natural products derived from ELF have become important sources of novel metabolites that hold potential pharmaceutical and agricultural applications (Agrawal et al., 2020; Zhang et al., 2024). We are currently investigating unknown metabolites with antifungal properties in the MEB culture extracts of *C. globosum* and *Nodulisporium* species through metabolic dereplication. While the specific metabolites responsible for the observed broad-spectrum antifungal activities in the two ELF are not yet identified,

the cyclic dipeptide cyclo($_D$-Leu-$_D$-Pro) has been previously reported to exhibit antifungal activities (Kumar et al., 2013; Salman et al., 2022; Yan et al., 2004). Importantly, a recent study demonstrated that chaetoglobosin D can be used as an effective preservative for maintaining tomato quality and flavor by mitigating postharvest decay caused by *A. alternata* (Du et al., 2024), consistent with our finding that the MEB culture extract of *C. globosum* can inhibit the growth of *A. alternata* (Fig. 2). In addition, chaetoviridin C was shown to be effective in controlling Cotton Verticillium wilt disease (Zhang et al., 2021).

There have been growing challenges of plant disease outbreaks, which threaten global food security and public health. Emerging new plant diseases are further compounded by climate change and ever-growing global commerce. These global issues necessitate an integrated research agenda to manage plant diseases effectively. ELF are highlighted as promising sources of novel bioactive compounds with potential applications in agriculture and pharmaceuticals, producing diverse secondary metabolites with various biological activities, including antibacterial and antifungal properties. Here we focused on identifying and analyzing the antimicrobial activities of ELF metabolites, particularly from *C. globosum* and *Nodulisporium* species, which enable us to annotate bioactive compounds in crude extracts of ELF cultures. This study underscores the potential of ELF as valuable bioresources for developing novel antimicrobial agents and utilizing previously known compounds to combat plant pathogens and enhance crop protection strategies.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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