Antimicrobial Activity of Endophytic and Rhizospheric Fungi Associated with Soft Fern (*Christella* sp.) and Cinderella Weed (*Synedrella nodiflora*) Inhabiting a Hot Spring in Los Baños, Laguna, Philippines

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ABSTRACT

Background. The growing resistance of microorganisms to antimicrobial agents is a pressing public health issue. Bioprospecting efforts have mainly focused on well-known environments such as soil and animal gut in search for microorganisms with antibiotic production or antimicrobial activity, or terrestrial ecosystems for endemic plants with bioactive compounds. However, microbial communities thriving in stressed environments such as hot springs, are potential sources of metabolites that can be screened for antimicrobial activity. There is a need for research on bioprospecting of fungi as potential sources of antimicrobials.

Objectives. The study aimed to test the antimicrobial activity of endophytic and rhizospheric fungi associated with soft fern (*Christella* spp.) and Cinderella weed *(Synederella nodiflora)* inhabiting a hot spring in Los Baños, Laguna, Philippines.

Methods. A total of 23 endophytic and rhizospheric fungi isolated from soft fern and Cinderella weed were purified and phenotypically identified. These isolates were subjected to agar well diffusion and agar plug diffusion methods as preliminary assays for antimicrobial activity against *Bacillus subtilis* var. *spizizenii* (ATCC® 6633™), *Staphylococcus aureus* (ATCC® 25923™), four multi-antibiotic resistant *Escherichia coli* (OT11, OT16, OT18, OT22), and *Cladosporium cladosporioides*. Based on the results of the preliminary screening, ethyl-acetate extracts of selected fungal isolates were subjected to broth microdilution assay to determine the minimum inhibitory concentrations (MICs) for antibacterial activity, as well as poisoned food technique to determine the percent mycelial inhibition for antifungal activity. The nearest phylogenetic affiliations of fungal isolates with higher antimicrobial activities were determined.

Results. Ten rhizospheric fungal isolates from Cinderella weed and seven rhizospheric and six endophytic fungal isolates from soft fern were phenotypically identified as *Aspergillus*, *Coniothyrium*, *Fusarium*, *Penicillium*, *Talaromyces*, and *Trichoderma* species. Ethyl acetate extracts from endophytic fungal isolates UL1 (*Trichoderma* sp.) and UL2 (*Trichoderma* sp.) and rhizospheric fungal isolates UR1 (*Trichoderma* sp.) and UR3 (*Trichoderma* sp.) showed activity against the test bacteria at 128-256 µg/mL concentrations. Isolates UL1, UL2, and UR3, which exhibited higher antibacterial activities, were sequenced and confirmed to be most phylogenetically related to *Trichoderma virens*. Eleven fungal isolates belonging to *Aspergillus* spp., *Coniothyrium* spp., *Fusarium* spp., *Penicillium* spp., and *Talaromyces* spp. demonstrated antagonism against *C. cladosporioides*. The rhizospheric fungal isolate FCRU4 (*Talaromyces* sp.), from where ethyl acetate extracts were recovered for testing mycelial inhibition, was confirmed to be most phylogenetically related to *Talaromyces islandicus*.

Conclusion. Endophytic and rhizospheric fungi associated with Cinderella weed (*Synedrella nodiflora*) and soft fern (*Christella* sp.) from a hot spring in Los Baños, Laguna, Philippines have antimicrobial activity.

Keywords: endophytic fungi, rhizospheric fungi, CHRISTELLA sp., S. NODIFLORA, antimicrobial activity, hot spring

Paper presented during the 1st International Symposium and Training Workshop on Microbial Utilization in conjunction with 20th MSP Anniversary and 6th Annual AnMicro Conference and iCollect Workshop on April 27, 2019 in SEARCA, University of the Philippines Los Baños, Laguna, Philippines.

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INTRODUCTION

Discovery and development of new antimicrobials are urgently needed to address the growing concern on antimicrobial resistance (AMR) worldwide.[1] In 2017, the World Health Organization (WHO) provided a priority list of antibiotic-resistant microorganisms.[1] The list includes 12 families of bacteria, grouped into critical, high, and medium urgency (Table 1).

Table 1. Priority list of antibiotic-resistant microorganisms

Urgency	Examples
Critical	Carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant and ESBL-producing *Enterobacteriaceae*
High	Vancomycin-resistant *Enterococcus faecium*
Medium	Penicillin-non-susceptible *Streptococcus pneumoniae*

Source: WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017.[1]

WHO urges researchers to prioritize discovery and development of new antibiotics against drug-resistant Gram-negative bacteria.[1] As part of the global effort to combat AMR, WHO also launched the Global Antimicrobial Resistance Surveillance System (GLASS), a standardized approach for the collection, interpretation, and dissemination of data to improve the understanding of the impacts of AMR on human health.[2] In response, the Philippines in 2015 launched the "National Action Plan to Combat Antimicrobial Resistance: One Health Approach" for AMR surveillance, antimicrobial use, and antimicrobial stewardship programs for human and animal health.[2]

Endophytic and rhizospheric fungi are rich sources of bioactive metabolites that have potential applications in medicine.[3,4] At least 30% of the bioactive compounds that have been discovered from microorganisms so far, are produced by fungi.[5] Many bioactive compounds from fungi, such as alkaloids, terpenoids, polyketides, steroids, quinones, phenols, coumarins, and peptides were reported to have antimicrobial activity.[6] This underscores the importance of fungi in the pipeline for drug discovery.[7]

Endophytic fungi are microorganisms that thrive in plant tissues without causing any disease to the plant.[8] They inhabit a wide range of hosts such as ferns, grasses, herbaceous and woody plants, and even algae.[9] Endophytic fungi may assist their host plant in biological processes such as nitrogen-fixation.[10] Rhizospheric fungi, on the other hand, inhabit the plant root area where they metabolize chemical secretions.[11,12] One of the known functions of rhizospheric fungi is phosphate solubilization, which increases the availability of soil phosphates for plant utilization.[13]

Several studies confirmed the antimicrobial activity of some endophytic and rhizospheric fungi. Antibacterial activity of the extracts of endophytic fungi *Acremonium*, *Colletotrichum*, and *Pestalotiopsis* associated with *Canarium ovatum*, a plant endemic to the Philippines, was reported against *Staphylococcus aureus, Escherichia coli, Serratia marcescens, Micrococcus luteus, Bacillus subtilis,* and *Bacillus megaterium*.[14] Extracts of endophytic fungi isolated from medicinal plants *(Gliricidia sepium*, *Canna indica* and *Gardenia jasminoides*) in the province of Bulacan yielded flavonoids, terpenoids, and phenols that have antioxidant property.[15] The antagonistic activity of rhizospheric fungi *Gibellulopsis*, *Plectosphaerella*, *Trichoderma*, *Fusarium*, and *Chaetomium* against the fungal agent of root-rot disease was also confirmed.[16] The correlation between the antimicrobial activity of some medicinal plants and the proportion of their antagonistic endophytes was reported, suggesting that the phytochemical components of these plants may be directly or indirectly related to their interactions with symbiotic microorganisms.[17]

Rhizospheric and endophytic microorganisms sustain symbiotic relationships by maintaining the health of their host plants.[9,18-20] Being sessile, plants are exposed to a wide range of biotic and abiotic stresses that limit their growth and development.[18] These microorganisms manufacture, or induce the host plant to produce, biologically active compounds that help them adapt to biological stresses.[19] The likelihood of finding endophytic or rhizospheric microorganisms with unique biochemical properties is greatest in untapped environments (e.g., deserts, hot springs, alpine areas, and the like).[21,22] The plants of interest in this study, soft fern (*Christella* sp.) and Cinderella weed (*Synedrella nodiflora* L.), were collected from a hot spring.[23,24] Most species of *Christella* grows in wet, dense or semi-open forests at relatively low elevations, mainly in tropical regions.[24-27] *Christella* spp. were reported to demonstrate antibacterial activity against *Staphylococcus* spp. and *Pseudomonas aeruginosa*, as well as antifungal effects against causative agents of fungal diseases in groundnuts.[26,28] There is limited information about the traditional medicinal uses of these ferns, with one species, *Christella dentata*, reportedly used in Bangladesh as an antihyperglycemic and analgesic.[29-31] On the other hand, *S. nodiflora* L. is a native of the tropical parts of the American continent, but now grows in many parts of the world especially in Africa and Southeast Asia. The plant has been traditionally used to treat cardiac problems, wounds, headaches, earaches, and stomachaches, and as liniment for rheumatism.[32-34] It is also known to contain flavonoids that have antimicrobial, antioxidant, and anti-inflammatory activities.[28,29] Phenolic compounds isolated from the aerial parts of the plant were reported to have antibacterial and antifungal activities.[35]

This study aimed to screen endophytic and rhizospheric fungi isolated from Cinderella weed (*S. nodiflora*) and soft fern (*Christella* sp.) for antibacterial and antifungal activity. Specifically, the study aimed to: (1) identify the isolates based on phenotypic traits; (2) test the antimicrobial activity of these isolates against a selection of bacteria and fungi; and (3) confirm the molecular identification (based on nearest phylogenetic affiliations) of the isolates with higher antimicrobial activity.

MATERIALS AND METHODS

Isolation of endophytic and rhizospheric fungi

The study used previously isolated but unidentifed endophytic (n=6) and rhizospheric (n=17) fungi from host plants Cinderella weed (*S. nodiflora)* and soft fern (*Christella* sp.) thriving in Libis ng Nayon, Los Baños, Laguna. The plant samples were collected from two sites: (1) "stressed sites" with water temperature of at least 41˚C and pH of 6 (i.e., with the plant's root system submerged in water); and (2) "unstressed sites" with soil temperature of at least 29.5˚C and pH of 6. The differentiation between "stressed sites" and "unstressed sites" was based on temperature. The optimum temperature range for plant growth is 28–30˚C, and 41˚C can be considered as a stressful condition considering that heat stress denotes temperatures that exceed the optimum values by around 10–15˚C.[36] In a temperate climate, heat stress is within the temperature range of 35–40˚C.[37]

Endophytic fungi were isolated from the leaves while rhizospheric fungi were isolated from the soil surrounding the roots of the plants. For isolation, samples from unstressed sites were incubated at room temperature for 5–7 days while those from stressed sites were incubated at 42˚C for 5–7 days.

Phenotypic identification of fungal isolates

Pure fungal isolates were inoculated onto potato dextrose agar (PDA) plates and were incubated at room temperature for 5–7 days. Whenever growth was observed, the fungi were sub-cultured using the Riddell method. After 7 days, cover slips with hyphal growth were recovered and subjected to microscopic observation. The morphology (i.e., form, size, elevation, margin, surface, opacity, color) of the colonies was noted. In addition, the hyphae of the isolates were observed as to form, type of reproduction, and container and structure of spores. Based on these observations, the isolates were identified up to at least genus level using classification guides for fungi.[38-40]

Isolates with putative phenotypic identifications were screened for antimicrobial activity by performing preliminary screening (agar well diffusion method and agar plug diffusion method), broth microdilution assay, and poisoned food technique. All experiments were carried out in three replications with negative and positive controls.

Preliminary screening for antimicrobial activity

Agar well diffusion method was performed to determine if the antibacterial activity of the fungal isolates had extracellular origin (i.e., antibacterial compound as filtrate or released in the medium).[41] Test bacteria were standardized to 0.5 McFarland (approximately 1.5 x 10^8 cells/mL) using 0.9% saline solution, and then lawned onto Mueller Hinton agar (MHA) plates. Three wells (6 mm in diameter) were bored in each of the MHA plates. Meanwhile, fungal isolates grown for 7 days in potato dextrose broth (PDB) were vortexed. One mL of this was transferred to a microcentrifuge tube and spun at 13500 rpm for 4 minutes. Forty µL of the supernatant, as well as negative control (PDB) and positive control (ampicillin), were inoculated in their respective wells. The MHA plates were then incubated at 37^{0}C. The zones of inhibition were measured using a caliper.[42] The test bacteria included *B. subtilis* var. *spizizenii* ATCC 6633, *S. aureus* ATCC 25923, and four strains of multi-antibiotic resistant *E. coli* (isolates OT11, OT16, OT18, OT22).

Agar plug diffusion method was performed to determine if the antibacterial activity of the fungal isolates had intracellular origin (i.e., antibacterial compounds produced by the mycelia).[43] Test bacteria were standardized to 0.5 McFarland using 0.9% saline solution, and then lawned onto Mueller Hinton Agar (MHA) plates. Agar plugs (6 mm in diameter) containing cultures of the isolates were obtained from PDA plates incubated at 25–27˚C for 7 days. The MHA plates with agar plugs and antibiotic discs as controls were then incubated at 37˚C for 24 hours.[39] Antibiotics used in control set-ups included erythromycin (i.e., positive control for *E. coli*), chloramphenicol, and ertapenem. The zone of inhibition formed by agar plugs and discs on MHA were measured using a caliper.

For both agar well and agar plug diffusion methods, antibacterial activity was evaluated as inactive (<10 mm), partially inactive (10–13 mm), active (14–19 mm), and very active (>19 mm) based on measured zones of inhibition.[21]

The antagonistic activity of the fungal isolates was tested against *Cladosporium cladosporioides*. Dual culture bioassay was performed wherein each fungal isolate and *C. cladosporioides* were inoculated on PDA plate and observed for growth for at least 7 days until a margin of inhibition was seen.[42] Observed growths were primarily interpreted as no inhibition (-) or with inhibition (+), and different types of interactions between the two fungi were assessed following Wheeler and Hocking (1993).[44]

Table 2. Types of interaction between two fungi

Type	Description
Type A	Mutual intermingling growth, where both fungi grew into each other without any macroscopic signs of interaction
Type B	Mutual inhibition or space between colonies at a distance <2 mm
Type C	Inhibition of one species on contact, the inhibited species continued to grow at a significantly reduced rate, while the inhibitor species grew at a slightly, reduced rate or unchanged
Type D	Mutual inhibition at a distance >2 mm
Type E	Inhibition of one species on contact, the inhibitor species continuing to grow at a reduced rate through the inhibited colony
Type F	Inhibition of one species on contact or at a distance, the inhibitor species then continuing to grow at an unchanged rate through or over the inhibited colony

Source: Wheeler KA, Hocking AD. Interactions among xerophilic fungi associated with dried salted fish. Journal of Applied Microbiology. 1993. 74(2):164-169.[44]

Minimum Inhibitory Concentration (MIC) Assay

Based on results of the agar plug diffusion and agar well diffusion methods, five fungal isolates (UL4, UL5, UR1, UR2, UR4) were subjected to MIC Assay using the same set of test microorganisms.

Agar plugs (6 mm diameter) obtained from 5-day PDA cultures of fungal isolates were transferred into flasks containing 300 mL of PDB.[45,46] The flasks were incubated at 25-27˚C for 30 days under static and shaking conditions (at a speed of 180 RPM).[3,42] After incubation, extracts were obtained from the isolates. Briefly, 250 mL ethyl acetate (EtOAc) was added to the broth culture and left overnight.[46] After 24 hours, the filtrate with the EtOAc was removed from the mixture using a separatory funnel for aqueous layer partitioning.[46] The mycelia were soaked in 150 mL mixture of water and EtOAc overnight. After 24 hours, the mycelia underwent mechanical grinding, filtration, and partitioning. Crude extracts from both the filtrate and mycelia were recovered through rotary evaporation and then dissolved to a concentration of 10 mg/mL in dimethyl sulfoxide (DMSO).[46]

Fifty µL of the ethyl acetate extracts prepared from shaking and static conditions was dispensed in assigned wells and were serially diluted two-fold to reach the final concentrations of 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, and 16 µg/mL after the addition of 50 µL bacterial suspensions (i.e., test bacteria, standardized to 0.5 McFarland).[46-48] Mueller Hinton Broth (MHB), which was used for serial dilution, served as blank, the standardized bacterial suspension as negative control, and ampicillin and chloramphenicol as positive controls. The cultures were incubated at 37˚C for 15 hours. After incubation, 10 µL of 0.18% resazurin dye was added to each well. To determine the MIC, plates were checked for color change 3–5 hours after the addition of the resazurin dye.

Poisoned Food Technique

Based on the demonstrated antagonistic activity of the fungi, the filtrate ethyl acetate extracts of isolate FCRU4 prepared from shaking and static conditions were tested for activity against *C. cladosporioides.*[49,50] From a 10 mg/mL stock solution of the extract, 750, 1500, and 3000 uL were added to 30 mL of molten PDA media, and then poured into Petri plates to obtain 250, 500 and 1000 ug/mL extract concentrations, respectively.[49,50] Each solution was mixed thoroughly and then poured into sterilized plates to solidify. A 5 mm disc of *C. cladosporioides*, obtained from a 5- to 10-day-old culture grown on PDA, was inoculated at the center of the plate.[49] Plates were incubated at 28˚C and the diameter (in mm) of fungal colony was measured using a caliper every 24 hours until growth was observed in the control set-ups (sterile water and nystatin).[48] Percent growth inhibitions of the three extract concentrations were calculated using the equation:[51]

$$\% \text{ Growth inhibition} = \frac{\text{radial diameter of fungus growing on the control plate} - \text{radial diameter of fungus on the experimental plate}}{\text{radial diameter on the control plate}}$$

Molecular identification of fungal isolates

Four isolates (UL1, UL2, UR3, FCRU4), which exhibited higher antimicrobial activities, were selected for confirmation of molecular identification. To extract fungal DNA from these isolates, a lump of mycelia was added to 500 µL of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). The tube was left for 10 minutes at room temperature before adding 150 µL of potassium acetate solution. The solution was briefly vortexed, centrifuged at >10000 x g for 1 minute, and the supernatant was transferred to a microcentrifuge tube where an equal volume of isopropanol was added. The tube was spun at >10000 x g for 2 minutes before the supernatant was discarded. The DNA pellet was washed in 300 µL of 70% ethanol. The solution was spun at 10000 rpm for 1 minute before discarding the supernatant. The DNA pellet was air-dried and dissolved in 50 µL of 1x Tris-EDTA.[52]

Primer pair ITS 1 (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGAT-ATGC-3') was used for the amplification of the ITS-5.8S rDNA.[53] The conditions for polymerase chain reaction (PCR) were: 5 minutes initial denaturation at 95˚C; 35-37 cycles of (1) denaturation at 95˚C for 30 seconds, (2) annealing at 55˚C for 1 minute, (3) extension at 72˚C for 1 minute; and final extension phase at 72˚C for 6 minutes. Electrophoresis of PCR products in TAE (Tris-acetate-EDTA) buffer was performed on agarose gel (1%) at 8 V/cm with DNA ladder (KAPA Universal ladder). Target DNA bands were 560 bp.[54] PCR products were processed for purification, DNA quantification, and sequencing. Phylogenetic analyses were done using MEGA X (Molecular Evolutionary Genetics Analysis ver. 10).

RESULTS

Phenotypic identification of endophytic and rhizospheric fungi

A total of 23 fungal isolates were phenotypically identified. Ten rhizospheric fungal isolates (SRF1, SFR2, SFR3, SFR4, SFR5, SFR6, SFR7, SFR8, SFR9 & SFR10) from Cinderella weed (*S. nodiflora*) were phenotypically identified as *Aspergillus* with four morphospecies, while seven rhizospheric fungal isolates from soft fern (*Christella* sp.) were phenotypically identified as *Penicillium* spp. (FCRU1), *Coniothyrium* sp. (FCRU2), *Aspergillus* sp. (FCRU3), *Talaromyces* sp. (FCRU4) and *Trichoderma* sp. (UR1, UR2, and UR3). Also, six endophytic fungal isolates from soft fern (*Christella* sp.) were phenotypically identified as *Aspergillus* sp. (FCLBU1), *Penicillium* spp. (FCLBU3), *Fusarium* sp. (FCLBU2), and *Trichoderma* spp. (FCLDU1, UL1, UL2) (Table 3). No endophytic fungi were isolated from Cinderella weed (*S. nodiflora*).

Table 3. Endophytic and rhizospheric fungal isolates

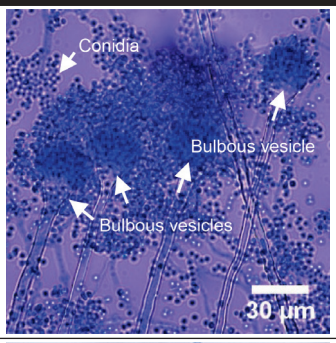
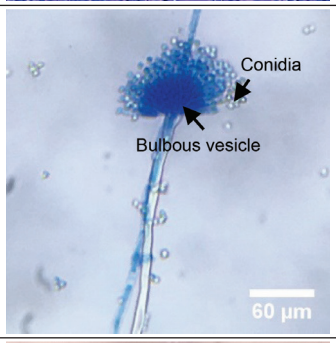
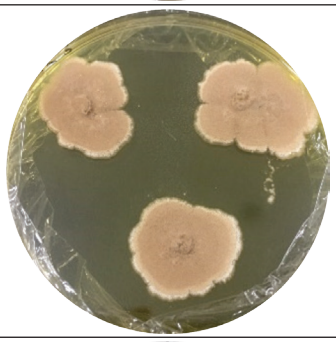
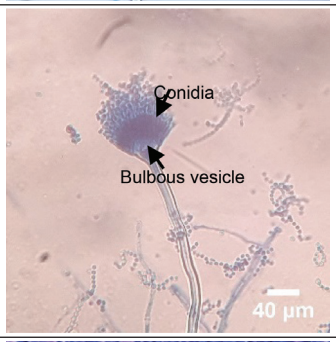
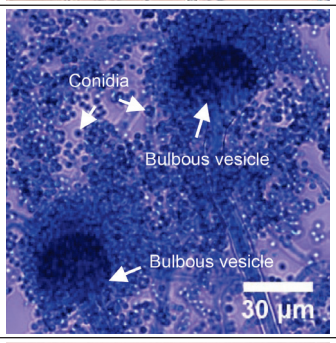
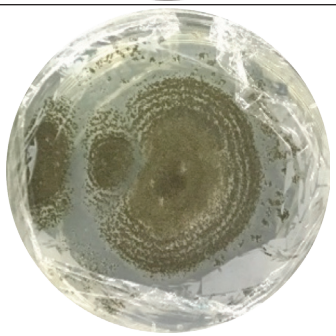
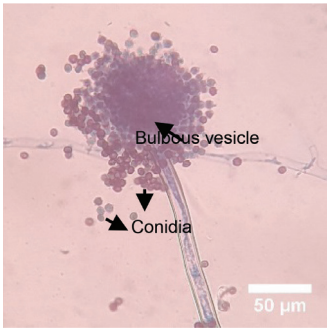
Isolate code	Source	Culture	Structures	Phenotypic Identification
SFR1	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR2	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR3	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR4	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR5	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.

Table 3. Endophytic and rhizospheric fungal isolates *(continued)*

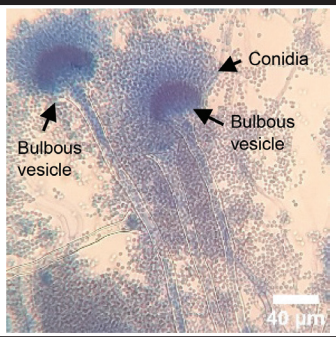
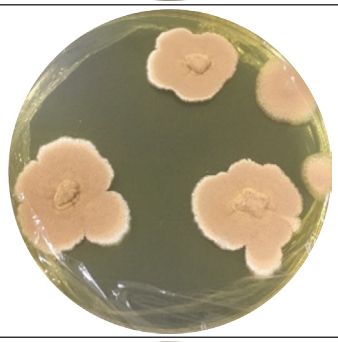
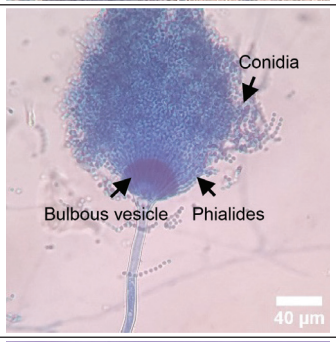
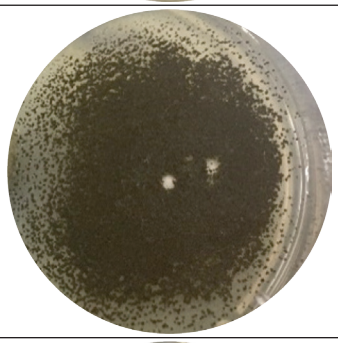
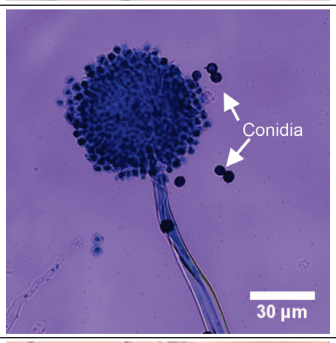
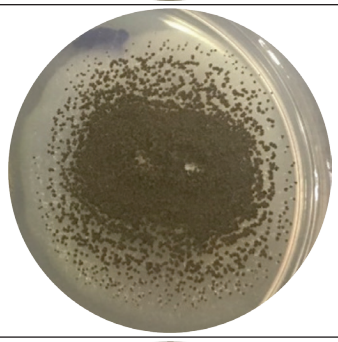
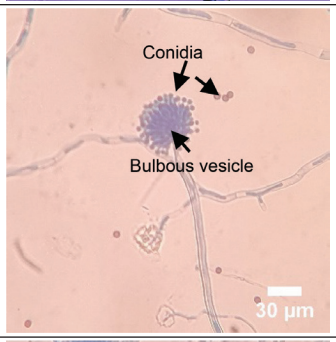
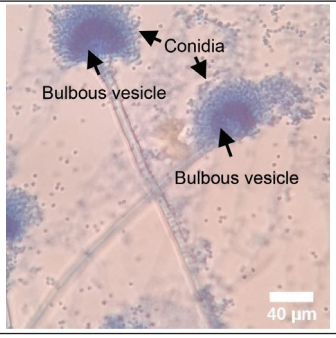
Isolate code	Source	Culture	Structures	Phenotypic Identification
SFR6	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR7	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR8	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR9	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.
SFR10	*S. nodiflora* (rhizospheric)			*Aspergillus* sp.

Table 3. Endophytic and rhizospheric fungal isolates *(continued)*

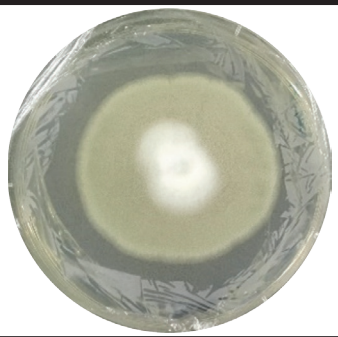
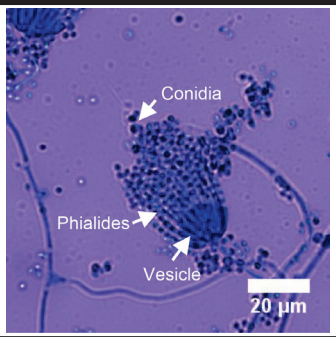
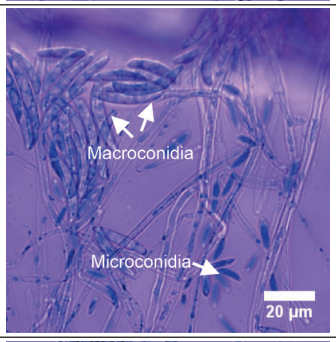
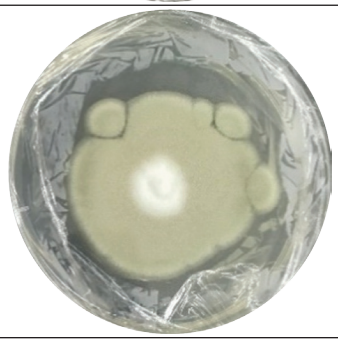
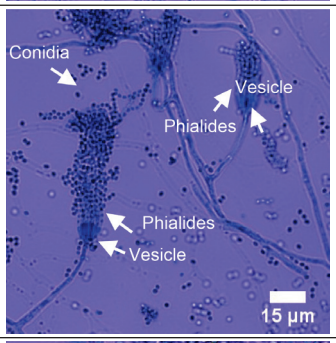
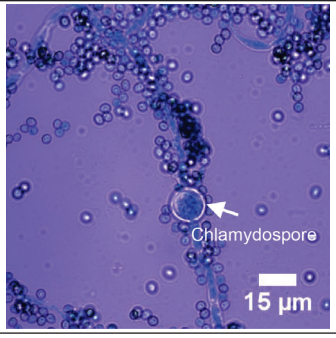
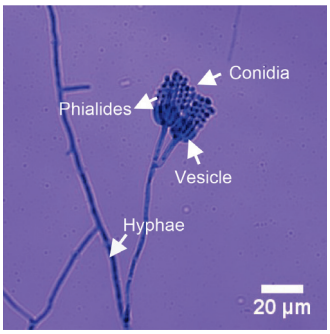
Isolate code	Source	Culture	Structures	Phenotypic Identification
FCLBU1	*Christella* sp. (endophytic)			*Aspergillus* sp.
FCLBU2	*Christella* sp. (endophytic)			*Fusarium* sp.
FCLBU3	*Christella* sp. (endophytic)			*Penicillium* sp.
FCLDU1	*Christella* sp. (endophytic)			*Trichoderma* sp.
FCRU1	*Christella* sp. (rhizospheric)			*Penicillium* sp.

Table 3. Endophytic and rhizospheric fungal isolates *(continued)*

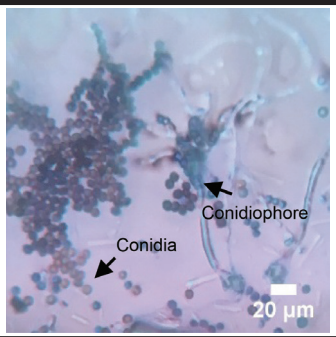
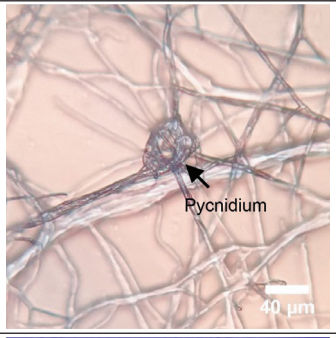
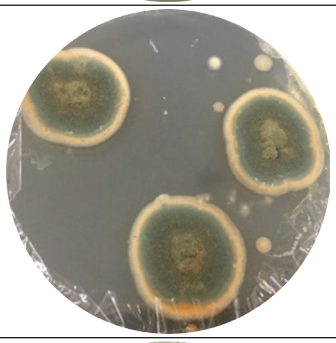
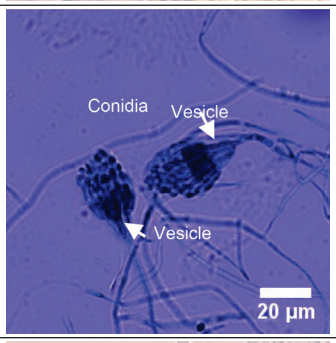
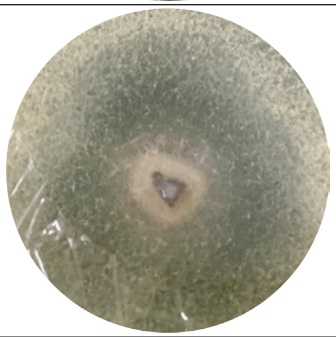
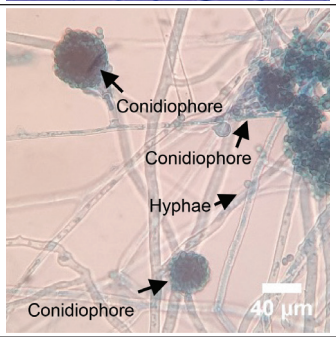
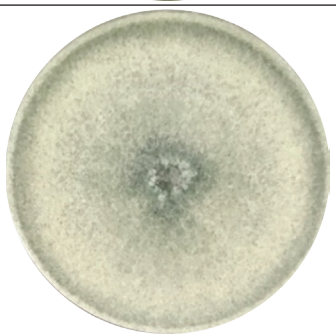
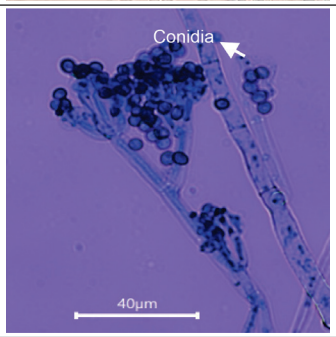
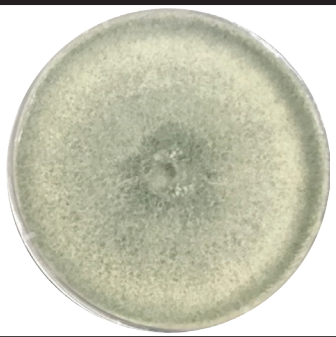
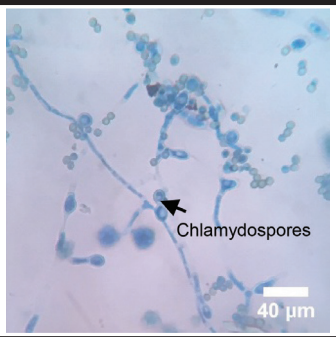
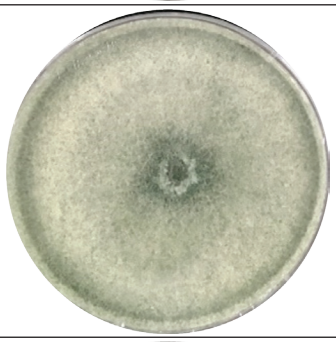
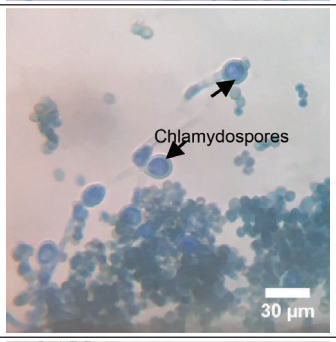
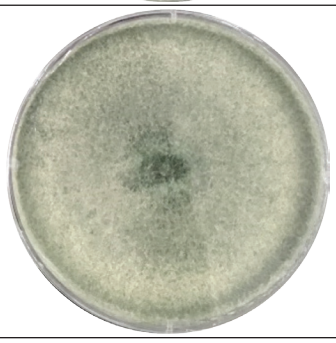
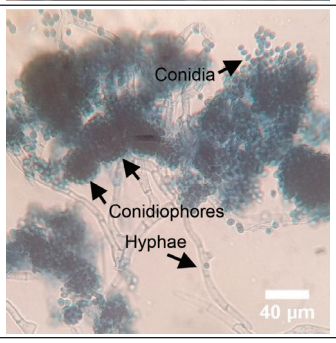
Isolate code	Source	Culture	Structures	Phenotypic Identification
FCRU2	*Christella* sp. (rhizospheric)			*Aspergillus* sp.
FCRU3	*Christella* sp. (rhizospheric)			*Coniothyrium* sp.
FCRU4	*Christella* sp. (rhizospheric)			*Talaromyces* sp.
UL1	*Christella* sp. (endophytic)			*Trichoderma* sp.
UL2	*Christella* sp. (endophytic)			*Trichoderma* sp.

Table 3. Endophytic and rhizospheric fungal isolates *(continued)*

Isolate code	Source	Culture	Structures	Phenotypic Identification
UR1	*Christella* sp. (rhizospheric)			*Trichoderma* sp.
UR2	*Christella* sp. (rhizospheric)			*Trichoderma* sp.
UR3	*Christella* sp. (rhizospheric)			*Trichoderma* sp.

Antibacterial activity of endophytic and rhizospheric fungi

Fifteen (65%) out of 23 endophytic and rhizospheric fungal isolates showed activity against the test bacteria (Table 4). Of the active isolates, 14 produced intracellular compounds which were found to be active against all test bacteria based on agar plug diffusion method. Five (SFR3, FCRU3, UL1, UR1 and UR3) produced extracellular compounds found to be active only against Gram-positive bacteria (*B. subtilis* var. *spizizenii* ATCC 6633 and *S. aureus* ATCC 25923) based on agar well diffusion method. Overall, five isolates (UL1, UL2, UR1, UR2, and UR3) have relatively broad spectrum, inhibiting both Gram-positive and Gram-negative microorganisms.

Minimum inhibitory concentrations (MICs) of most active isolates

Based on the results of agar well and agar plug diffusion methods, the five most active isolates (UL1, UL2, UR1, UR2 and UR3) were determined. The filtrate and mycelial crude extracts of these isolates, grown in static and shaking conditions, were subjected to broth microdilution test to determine the MICs against the test bacteria. Isolates UL1, UL2, UR2 and UR3 have MIC values ranging from 128–256 µg/mL against *B. subtilis* var. *spizizenii* ATCC 6633, *S. aureus* ATCC 25923, and *E. coli* isolates (Table 5). A higher proportion of extract concentrations that were effective in inhibiting the growth of bacteria were recovered from isolates grown in shaking conditions. The highest antibacterial activity was shown by the filtrate extract of UR3, with MIC values ranging from 128–256 µg/mL against all the test bacteria (*B. subtilis* var. *spizizenii* ATCC 6633, *S. aureus* ATCC 25923, and *E. coli* isolates). The MIC breakpoints for *S. aureus* and *E.coli* are 0.5–2 µg/mL and 128 mg/L respectively.[55]

Antifungal activity of endophytic and rhizospheric fungi

Based on the results of dual culture assay, 11 (47.83%) out of 23 isolates inhibited the growth of *C. cladosporioides*

Table 4. Preliminary screening of antibacterial activity of endophytic and rhizospheric fungi

Activity Against	Zone of Inhibition (or ZOI in mm)											
	Agar-well diffusion method						Agar-plug diffusion method					
	B. subtilis	*S. aureus*	*E. coli* (OT11)	*E. coli* (OT16)	*E. coli* (OT18)	*E. coli* (OT22)	*B. subtilis*	*S. aureus*	*E. coli* (OT11)	*E. coli* (OT16)	*E. coli* (OT18)	*E. coli* (OT22)
Blank (-)	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (+)	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++
SFR1	-	-	-	-	-	-	++	+	-	-	-	-
SFR2	-	-	-	-	-	-	+++	+	-	-	-	-
SFR3	+	-	-	-	-	-	++	+	-	-	-	-
SFR4	-	-	-	-	-	-	+	+	-	-	-	-
SFR5	-	-	-	-	-	-	-	-	-	-	-	-
SFR6	-	-	-	-	-	-	+	+	-	-	-	-
SFR7	-	-	-	-	-	-	+	-	-	-	-	-
SFR8	-	-	-	-	-	-	-	-	-	-	-	-
SFR9	-	-	-	-	-	-	-	-	-	-	-	-
SFR10	-	-	-	-	-	-	++	+	-	-	-	-
FCLBU1	-	-	-	-	-	-	-	-	-	-	-	-
FCLBU2	-	-	-	-	-	-	-	-	-	-	-	-
FCLBU3	-	-	-	-	-	-	-	-	-	-	-	-
FCLDU1	-	-	-	-	-	-	-	-	-	-	-	-
FCRU1	-	-	-	-	-	-	++	+	-	-	-	-
FCRU2	-	-	-	-	-	-	-	-	-	-	-	-
FCRU3	-	++	-	-	-	-	-	-	-	-	-	-
FCRU4	-	-	-	-	-	-	+	+	-	-	-	-
UL1	+	++	-	-	-	-	-	+++	++	++	+	++
UL2	-	-	-	-	-	-	+	+++	++	++	++	++
UR1	++	++	-	-	-	-	-	+	+++	++	++	++
UR2	-	-	-	-	-	-	-	+	++	++	+	++
UR3	+	++	-	-	-	-	+	++	+++	+++	++	+++

Activity: - (Inactive, <10 mm ZOI); + (Partially Active, 10-13 mm ZOI); ++ (Active, 14-19 mm ZOI); +++ (Very active, >19 mm ZOI)

Table 5. Minimum inhibitory concentrations (MICs) of fungal isolates tested

Isolate Code	Test Bacteria (MIC, µg/mL)					
	B. subtilis	*S. aureus*	*E.coli* (OT18)	*E.coli* (OT16)	*E.coli* (OT22)	*E.coli* (OT11)
UL1	256[a]	128[a]	256[a]			256[a]
UL2		256[a,b]				
UR2			256[b]			
UR3	128[b] 256[d]	128[b]	256[a,d] 128[b]	256[b]	256[b]	256[b]

[a]*Filtrate extract, static condition*
[b]*Filtrate extract, shaking condition*
[c]*Mycelial extract, static condition*
[d]*Mycelial extract, shaking condition*

(Table 6). Four different antagonistic interactions were observed: isolate FCRU2 showed a type C interaction, FCLBU2 and FCLBU3 a type E interaction, SFR5 and FCRU3 a type F interaction, and SFR2, SFR3, SFR4, SFR6, FCRU1 and FCRU4, a type D interaction (Figure 1). Ethyl acetate extracts (in 250 ug/mL, 500 ug/mL and 1000 ug/mL concentrations) successfully recovered from FCRU4, did not exhibit activity against the test fungus in the poisoned food technique.

Molecular identification of selected fungal isolates

The nearest phylogenetic affiliations of fungal isolates with higher antimicrobial activity were determined. Isolates UL1, UL2 and UR3 are most phylogenetically affiliated to *Trichoderma virens* (GenBank Accession number LT707586) with 98.29%, 99.22%, and 99.84% sequence similarities respectively based on the internal transcribed spacer (ITS) region (Figure 2).

The rhizospheric fungal isolate FCRU4, which yielded ethyl acetate extracts, is most phylogenetically affiliated to *Talaromyces islandicus* (GenBank Accession number KJ783270) with 99.19% homology based on the internal transcribed spacer (ITS) region (Figure 3).

Table 6. Antifungal activity of endophytic and rhizospheric fungi against *C. cladosporioides*

Isolate Code	Phenotypic Identification	Antifungal activity	Type of Antagonistic Interaction
Blank (-)	N/A	-	N/A
Nystatin (+)	N/A	+	N/A
SFR1	*Aspergillus* sp.	-	N/A
SFR2	*Aspergillus* sp.	+	Type D
SFR3	*Aspergillus* sp.	+	Type D
SFR4	*Aspergillus* sp.	+	Type D
SFR5	*Aspergillus* sp.	+	Type F
SFR6	*Aspergillus* sp.	+	Type D
SFR7	*Aspergillus* sp.	-	N/A
SFR8	*Aspergillus* sp.	-	N/A
SFR9	*Aspergillus* sp.	-	N/A
SFR10	*Aspergillus* sp.	-	N/A
FCLBU1	*Aspergillus* sp.	-	N/A
FCLBU2	*Fusarium* sp.	+	Type E
FCLBU3	*Penicillium* sp.	+	Type E
FCLDU1	*Trichoderma* sp.	-	N/A
FCRU1	*Penicillium* sp.	+	Type D
FCRU2	*Aspergillus* sp.	+	Type C
FCRU3	*Coniothyrium* sp.	+	Type F
FCRU4	*Talaromyces* sp.	+	Type D
UL1	*Trichoderma* sp.	-	N/A
UL2	*Trichoderma* sp.	-	N/A
UR1	*Trichoderma* sp.	-	N/A
UR2	*Trichoderma* sp.	-	N/A
UR3	*Trichoderma* sp.	-	N/A

Antifungal activity:
- = no inhibition
+ = with inhibition
Types of Interaction Observed:
Type C = inhibition of one species on contact, the inhibited species continued to grow at a significantly reduced rate, while the inhibitor species grew at a slightly, reduced rate or unchanged
Type D = mutual inhibition at a distance >2 mm
Type E = inhibition of one species on contact, the inhibitor species continuing to grow at a reduced rate through the inhibited colony
Type F = inhibition of one species on contact or at a distance, the inhibitor species then continuing to grow at an unchanged rate through or over the inhibited colony

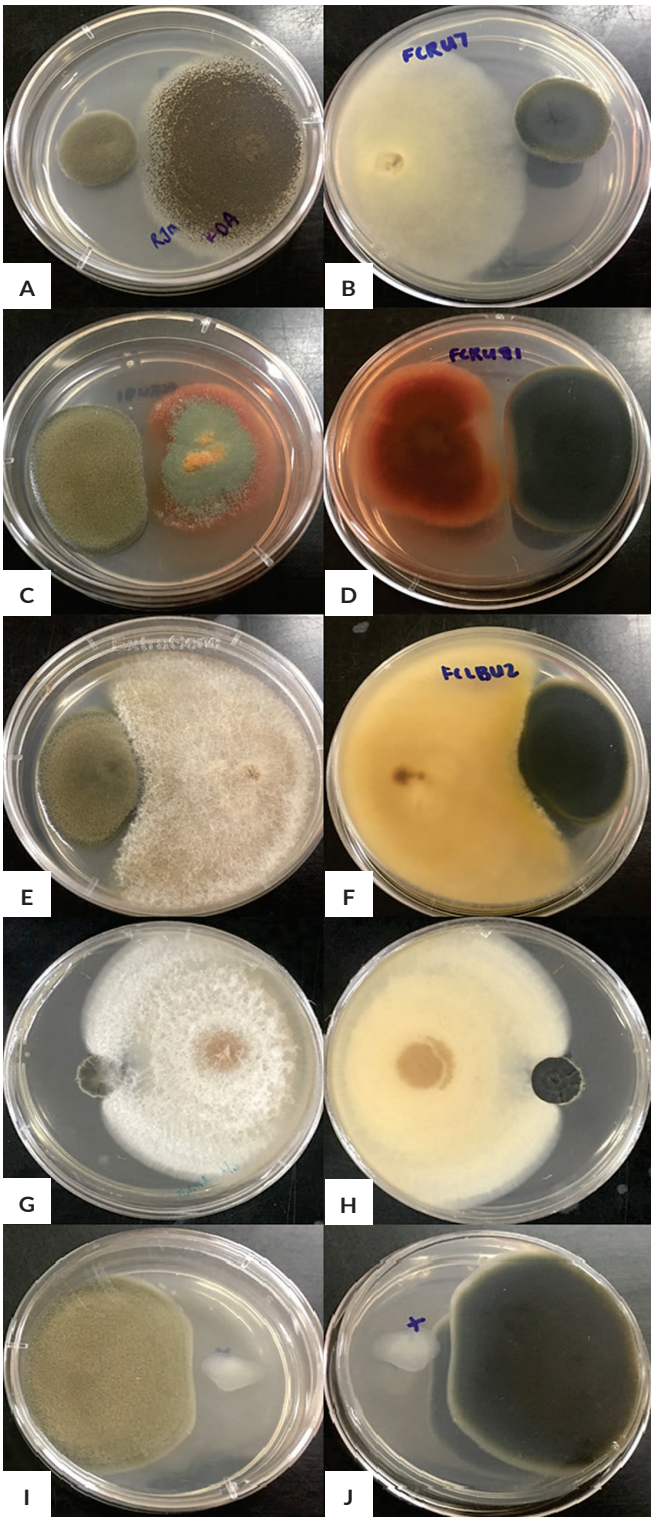


Figure 1. Fungal isolates exhibiting antagonistic interactions against *C. cladosporioides*: **(A,B)** *Aspergillus* sp. (FCRU2) showing type C interaction; **(C,D)** *Talaromyces* sp. (FCRU4) showing type D interaction; **(E,F)** *Fusarium* sp. (FCLBU2) showing type E interaction; **(G,H)** *Coniothyrium* sp. (FCRU3) showing type F interaction; and **(I,J)** control (nystatin) against *C. cladosporioides*.

DISCUSSION

Majority of plant species have not been studied for their associations with fungi, especially plants found in extreme environments. Generally, the diversity of rhizospheric and endophytic fungi varies among plant species, and is affected by several biotic and abiotic factors and stresses.[56-57] Such information is lacking in the case of soft fern (*Christella* sp.) and Cinderella weed (*S. nodiflora*). Ghanta et al. (2012) reported the distribution and diversity of arbuscular mycorrhizal (AM) fungi in the roots of a *Christella* species,

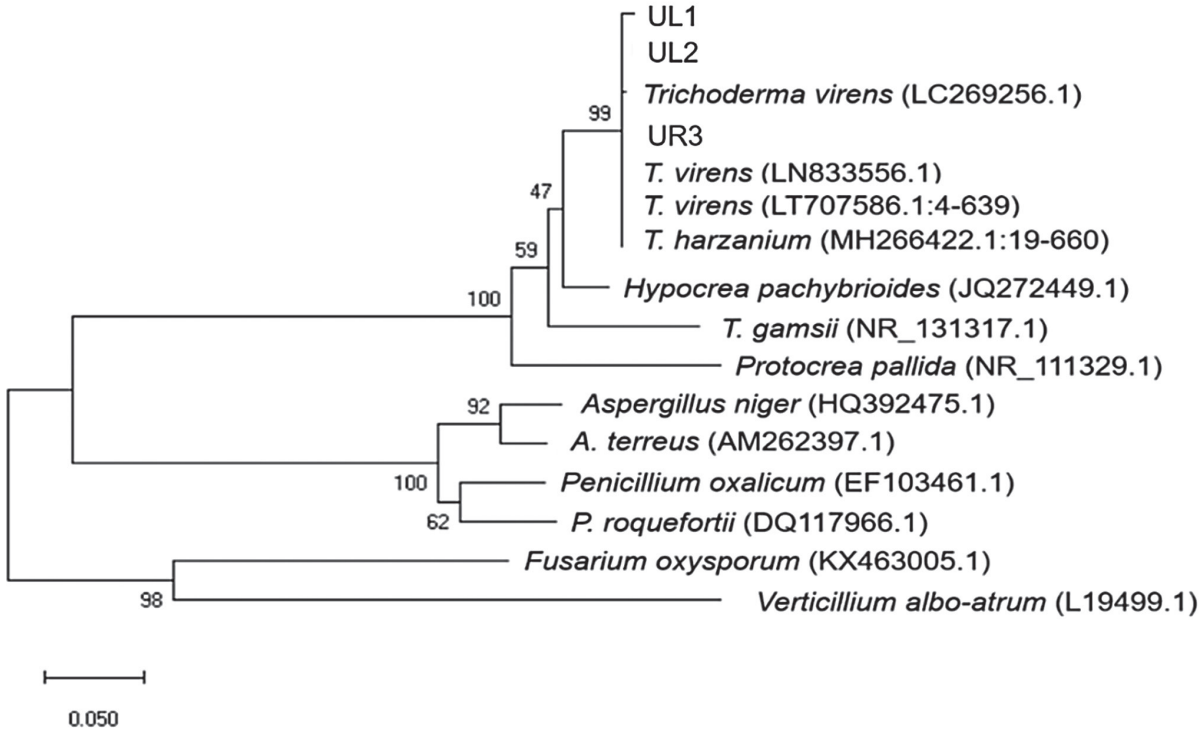


Figure 2. Neighbor-joining tree showing phylogenetic relationships of fungal isolates UL1, UL2, UR3 to other fungi.

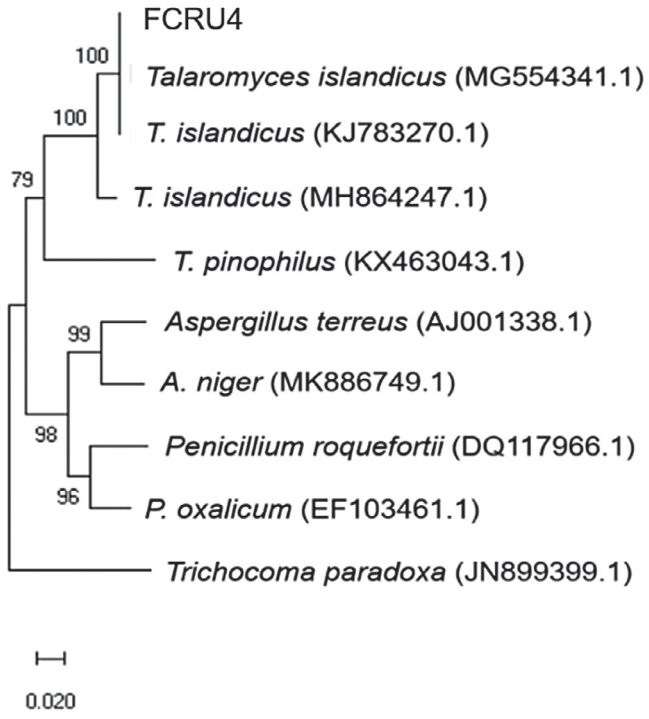


Figure 3. Neighbor-joining tree showing the phylogenetic relationship of FCRU4 to other fungi.

the *C. dentata*,[58] while Seerangan et al. (2014) reported the presence of a dark septate endophyte in *S. nodiflora*.[23]

Several studies investigated the mechanisms of symbiotic interactions between microorganisms and their plant hosts.[59-61] The stresses brought about by thriving in above normal conditions, such as higher temperatures in hot springs, offer selection pressure which could pave the way for novel adaptations such as production of bioactive metabolites (e.g., enzymes, compounds).[53-55,59-62] In this study, rhizospheric fungal isolates (*Aspergillus* spp., *Coniothyrium* sp., *Penicillium* spp., *Talaromyces* sp., and *Trichoderma* sp.) from Cinderella weed (*S. nodiflora*) and soft fern (*Christella* sp.) and endophytic fungal isolates (*Fusarium* sp., *Penicillium* spp., and *Trichoderma* spp.) from the latter were taxonomically identified and screened for antimicrobial activity against a selection of bacteria and a pathogenic fungus from a diseased tomato plant.

Less diverse rhizospheric fungi (i.e., only *Aspergillus*) were isolated from Cinderella weed (*S. nodiflora*) inhabiting the "stressed" (with the plant's root system submerged in water with a temperature of 41^{0}C) portion compared to the diversity of rhizospheric and endophytic fungi (*Penicillium* sp., *Fusarium* sp., *Trichoderma* sp., *Aspergillus* sp., *Coniothyrium* sp. and *Talaromyces* sp.) isolated from soft fern (*Christella* spp.) inhabiting the "unstressed" (soil temperature of 29.5^{0}C) portion of the hot spring. It was suggested that rhizospheric fungi thriving in higher temperatures primarily function for heat tolerance, with relatively small energy compensation for antagonism.[63-65] Ismail et al. (2018),

reported the potential of *Aspergillus* to secrete plant growth-promoting hormone and mitigate heat stress by releasing phenols and flavonoids.[58,66] Rhizospheric fungi such as *Aspergillus niger, Fusarium oxusporum, Paecilomyces formosis, Penicillium funiculosum, Penicillium corylophilum*, *Phoma* spp., *Rhizopus stolonifera,* and *Trichoderma* spp. were also found to promote host thermotolerance by mediating the regulation of plant endogenous hormones and production of HSP90 (heat shock protein90) inhibitor.[67] In this study, it was observed that the fungi isolated from Cinderella weed (*S. nodiflora*) exhibited lower antimicrobial activity compared to the fungi isolated from soft fern *(Christella* sp.). The rhizospheric fungi associated with Cinderella weed (*S. nodiflora*) could be functioning for heat tolerance, given that the plant was thriving in a stressed site (i.e., higher temperature).

Overall, the endophytic and rhizospheric fungi belonging to *Aspergillus* spp., *Coniothyrium* spp., *Fusarium spp. Penicillium* spp., *Talaromyces* spp. and *Trichoderma* spp. associated with Cinderella weed (*S. nodiflora*) and soft fern (*Christella* sp.) were effective against the tested bacteria and fungus. It can be noted that fungal isolates grown in shaking conditions were more effective in inhibiting the growth of test bacteria. The same observation was reported in the case of *Trichoderma harzianum*, in which cultures grown in shaking condition were more effective in inhibiting the growth of phytopathogenic bacteria *Xanthomonas campestris* and *Clavibacter michiganensis* compared to cultures grown in static condition.[47]

Aspergillus spp. have documented antagonistic activity against pathogens.[68] *A. niger* has the ability to suppress sheath blight caused by *Rhizoctonia solani.*[69] *A. niger, A. flavus,* and *A. terreus* suppressed the colonization of *Fusarium udum,* which causes wilt disease of pigeon-pea.[70] Non-aflatoxigenic strains of *Aspergillus* were used to control aflatoxigenic strains.[71,72]

A species of *Coniothyrium,* is a known biocontrol agent of pathogenic fungi. The mycoparasite *Coniothyrium minitans* is used as biocontrol for *Sclerotinia* spp. (*sclerotium, minor,* and *trifoliorum*).[73-81] A commercial product of the mycoparasite (Constanz) is commercially registered and available in Europe and USA.[74,78,82] The culture filtrates of *C. minitans* can inhibit mycelial growth of *S. sclerotiorum.*[83] Also, *C. minitans* produces antifungal substances, such as macrosphelide A.[82] antifungal substances produced by *C. minitans* strain Chy-1, inhibits growth of mycelia, extension of germ tubes of ascospores, and germination of myceliogenic sclerotia of *S. sclerotium*, and reduces the incidence of rapeseed (*Brassica napus*) leaf blight caused by *S. sclerotium.*[84] Aside from mycoparasitism, the production of antifungal substances can be exploited as another mechanism for antifungal activity of *Coniothyrium* spp.[81] In the present study, it is possible that isolate FCRU3 (*Coniothyrium* sp.) either produces antifungal substances or acts as a mycoparasite to *C. cladosporioides,* as indicated by the antagonistic reaction.

Fusarium spp. are also used as biocontrol agents. Some pathogenic strains of *F. oxysporum,* which cause serious damage on many agricultural crops, are controlled with the use of nonpathogenic *F. oxysporum* that exhibit either direct or indirect antagonism mediated through the host plant.[85,86] The use of nonpathogenic strains of *F. oxysporum* to control *Fusarium* wilt has also been reported in numerous crops and field trials (e.g., banana, strawberry, and tomato).[87-89]

Since the discovery of penicillin, thousands of *Penicillium* isolates have been screened and new metabolites discovered through bioprospecting programs.[90] *Penicillium* species are known to produce numerous bioactive compounds, including mycotoxins, herbicides, antioxidants, insecticides, antibiotics, antifungal substances and anticancer compounds.[91-94] Some of the extracted and characterized compounds from terrestrial *Penicillium* strains are mycophenolic acid, griseofulvin, and 3-O-methylfunicone.[95-98] The antifungal activity of *Penicillium* spp. against *Colletotrichum acutatum, F. oxysporum*, *Fusarium solani, Macrophomina phaseolina, Aspergillus japonicus* var. *aculeatus* and *C. cladosporioides* has been reported.[92,94]

Talaromyces spp. are *Penicillium* that produces sexual structures, such as cleistothecia, asci, and ascospores), and which also produce a variety of active compounds such as tetraene lactones, diphenyl ether derivatives, epiaustdiol, furanosteroid and anthraquinones.[99-104] Eighty-eight compounds have been recovered from *Talaromyces* spp. and evaluated for cytotoxic, antiproliferative, immunosuppressive, antimicrobial, and enzyme inhibitory activities.[105] Emodin, skyrin, norlichexanthone, secalonic acid A, and stemphyperylenol, extracted from *Talaromyces* sp. ZH-154 endophytic to the mangrove tree (*Kandelia candel* (L.) Druce), were found to be active against *E. coli, S. aureus, Pseudomonas aeruginosa, A. niger* and *Candida albicans.*[106] Additionally, *T. flavus* has demonstrated the ability to reduce the viability and apothecial production of the pathogen *S. sclerotiorum* with its production of hydrogen peroxide as the main bioactivity mechanism.[107,108]

Trichoderma are well known producers of secondary metabolites against phytopathogens.[99,100,109] Some *Trichoderma* spp., usually isolated as endophytes especially in tropical vegetations, have minimal nutritional needs and produce a plethora of secondary metabolites including peptaibols, gliotoxin, gliovirin, polyketides, pyrones, and terpenes that have activity against pathogenic bacteria, yeasts and filamentous fungi.[107-112] The antifungal compound, trichodermin, was isolated from *Trichoderma koningiopsis,* which exhibited strong fungicidal effects against *Aspergillus fumigatus, Pyricularia oryzae* and *Botrytis cinerea.*[109] Antimicrobial activity of *Trichoderma* was reported by Leylaie et al. (2018), which confirmed the antibacterial activity of seven endophytic *Trichoderma* species isolated from (*Vinca* sp.) against *S. aureus, E. coli, Ralstonia solanacearum* and *Clavibacter michiganensis*, and Santos et al. (2018), which documented the eradication of staphylococcal biofilms by

the ethanolic extracts of *Trichoderma asperelloides*.[109,100] In this study, it can be noted that only *Trichoderma* spp. (isolates UL1, UL2, UR1, UR2, and UR3) exhibited activity against the *E. coli* isolates.

This study confirmed the antibacterial activity of the endophytic and rhizospheric fungi in clinically significant bacteria. The *E.coli* isolates that were used in the assays were resistant to ampicillin, cephalothin, ciprofloxacin, ertapenem, erythromycin, moxifloxacin, ofloxacin, tetracycline, imipenem, oxytetracycline and sulfamethoxazole (Obusan et al. 2017, unpublished data). In general, there is increasing trend of *E. coli* resistance against ampicillin, amoxicillin and trimethoprim-sulfamethoxazole.[113-115] The other test bacterium used, *S. aureus*, has resistant strains (e.g., methicillin-resistant *S. aureus* or MRSA) and is a public health concern — causing staphylococcal infections in health care and community settings.[116,117] There is a growing evidence supporting the role of *Bacillus* spp. in the dissemination of antibiotic resistance genes in the food industry. *Bacillus* spp., in commercially available probiotic feeds, were found to be resistant to chloramphenicol, tetracycline, erythromycin, lincomycin, penicillin, and streptomycin.[118-123] This study also confirmed the antagonistic activity of the fungal isolates against *C. cladosporioides*, which was isolated from a diseased tomato plant (Rey, 2019; personal communication). This fungal pathogen is known to affect several plants (e.g., mandarin, grape, wheat and strawberry), causing blight disease.[124-125]

CONCLUSION

Endophytic and rhizospheric fungi associated with Cinderella weed (*S. nodiflora*) and soft fern (*Christella* sp.) from a hot spring in Los Baños, Laguna, Philippines are potential sources of antimicrobials. Four fungal isolates identified as *Trichoderma* spp. inhibited *B. subtilis* var. *spizizenii* ATCC 6633, *S. aureus* ATCC 25923, and multi-antibiotic resistant *E. coli* isolates, with MIC values ranging from 128 - 256 µg/mL. On the other hand, eleven fungal isolates identified as *Aspergillus* spp., *Coniothyrium* spp., *Fusarium* spp., *Penicillium* spp., and *Talaromyces* spp. showed antagonism against the plant phytopathogen *C. cladosporioides*. The nearest phylogenetic affiliations of fungal isolates with promising antimicrobial activity were known: UL1, UL2 and UR3 are most phylogenetically affiliated to *Trichoderma virens* while FCRU4 to *Talaromyces islandicus*. The antimicrobial activity of these fungi must be optimized and the specific mechanisms need to be elucidated for various applications.

Statement of Authorship

All authors have approved the final version of the manuscript.

Author Disclosure

All authors declared no conflicts of interest.

Funding Source

Microbial Ecology of Terrestrial and Aquatic Systems Laboratory

Availability of data and materials

All data generated are included in the article. Other relevant data may be requested through the corresponding author.

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