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Research Article

Endophytic fungi from *Dichrocephala integrifolia*: Diversity, antifungal properties, enzymatic activities, and plant growth promotion

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ABSTRACT: *Dichrocephala integrifolia* is a wild medicinal plant utilised in traditional healing and Ayurveda to cope up with several health issues by various groups of people around the world. Medicinal plants are associated with diverse fungal endophytes with potential bioactive properties. In this investigation, 26 fungal endophytes were isolated from *D. integrifolia* and three sterile forms using the Petri plate culture method. The endophytic isolation rate was highest for inflorescence (35.97%) and lowest for stem (15.61%). The highest colonization frequency was shown by *F. solani* (8.37%) and the lowest by Sterile morphotype 3 (1.36%). Out of the four plant parts, inflorescence was found to be highly infected, displaying an infection rate of 93.43% and the least infection occurred in the stem with 45%. The maximum number of isolated endophytic fungi belongs to the class Sordariomycetes, with a relative occurrence (%) of 71.72%. The Simpson's diversity index reveals that the leaf endophytes were more diverse (0.94). Qualitative antifungal activity of the sporulating isolates against *Curvularia lunata* has shown that the maximum number of endophytes possessed Class 3 antagonism. Four isolates were selected based on screening of their antagonistic activity and their antifungal inhibition was calculated against nine fungal phytopathogens. Maximum inhibition (100%) was shown by *Trichoderma* sp. 2 (S2B2) against *Alternaria alternata*, *A. brassicicola*, *Colletotrichum capsici*, *C. lunata*, and *Ustilaginoidea virens* and least inhibition by *Gliocladium* sp. 1 (19.78%) against *C. lunata*. The four isolates were found to produce protease, lipase, amylase and cellulase enzymes. The isolates produced ammonia and hydrogen cyanide, but none of the isolates could solubilize phosphate. Potent biocontrol agents are much needed to replace synthetic chemicals and restore soil microflora.

KEYWORDS: Antagonism, biocontrol, *Dichrocephala integrifolia*, endophytic fungi, phytopathogens

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INTRODUCTION

Dichrocephala integrifolia (L.f.) Kuntze, an annual herb in the family Asteraceae, possesses various therapeutic properties, including analgesic, anti-inflammatory, and antibiotic effects, making it a valuable plant in traditional medicine (Wabo *et al*., 2013; Joshi *et al*., 2020). While medicinal plants offer a rich source of bioactive compounds, the overexploitation of these plants and the extensive use of synthetic chemicals in agriculture have led to significant environmental and health concerns. Biologically active compounds are obtained naturally from plants, animals, and microorganisms, of which fungi are widely studied for their role in the generation of novel substances (Sanchez & Demain, 2017). Endophytic fungi refer to those groups of fungi found associated with the host plant intercellularly or intracellularly that do not show any harmful effect on the host plant. They are safe, eco-friendly, economical, require less time consumption, and are easily mass-cultivable in artificial culture media. Medicinal plants have been known to harbour endophytic fungi, which produce several important bioactive compounds. Previous investigations have shown bioactive antifungal compounds, namely, peptaibols (Grigoletto *et al*., 2020); 2-methoxy-4-vinylphenol, 3,4-dimethoxy styrol, and caryophyllene (Yang *et al*., 2021); isobenzofuranones (Sánchez-Fernández *et al*., 2020); and fusaisocoumarin, emodin, and abscisic acid (Ebrahim *et al*., 2020) have shown prominent antifungal activity. Pathogenic fungi pose serious infections not only to plants but also to humans and animals (Gnat *et al*., 2021), and thus endophytic fungi serve as an important source of antifungal agents. As estimated by Petrini (1991), there could potentially exist over a million undiscovered species of endophytic fungi. Tan and Zou (2001) first reported the presence of fungal endophyte in *Lolium temulentum*. According to Ghosh *et al.* (2020), fungal endophytes were found to be associated with almost all the parts of the host plant. Fang *et al.* (2019) observed that endophytic fungi are also host-specific.

A maximum number of endophytic fungi isolated belong to ascomycetous and anamorphic types and represent a polyphyletic group of highly diverse fungal species

(Rodriguez *et al.*, 2009). After the "Green Revolution," the application of synthetic compounds in the field as fertilizers, fungicides, herbicides, and insecticides has drastically increased (Srivastava *et al.*, 2020). These chemical compounds not only destroy natural soil structures and microhabitats but also pose a serious health hazard to animals, especially humans, through the food chain. In the last few years, organic farming has been emphasized as an important alternative to conventional agricultural practices. Rodriguez *et al.* (2009), suggested that fungal endophytes exhibit specificity towards tissues and organs, resulting in variations across different parts of a host plant like leaves, inflorescences, stems, and roots. Bernardi-Wenzel *et al.* (2010) observed that certain host species often exhibit a predominance of one or two endophyte species, whereas other isolates are rare. In organic farming, biocontrol agents play a major role. Till now, there are few effective biocontrol agents on the market, and the search for biocontrol agents with wide applications is highly needed. Several researchers have shown the application of endophytic fungi as biocontrol agents (Latz *et al.*, 2018; De Silva *et al.*, 2019).

Endophytic fungi occupying a distinct habitat produce many extracellular enzymes and plant growth-promoting substances. These enzymes are used to absorb nutrients from the host cells as well as defend the plant from the attack of pathogens (Fontana *et al.*, 2021). Enzymes of microbial origin are widely used in industries, viz., textiles, food, cosmetics, pharmaceuticals, leather, paper, and detergent (Singh *et al.*, 2016). Many endophytic fungi were reported to produce ammonia, hydrogen cyanide, and phytohormones and also increase the uptake and mobilization of essential elements by the host plant. Zhao *et al.* (2010) investigated the capacity of endophytic fungi to produce growth-promoting compounds that are analogous to those synthesized by their respective host plants but in greater amounts. Khan *et al.* (2015) observed that fungal endophytes possessed the capability to synthesize phytohormones, mainly gibberellins (GAs), which can stimulate the growth and development of crops and mitigate the adverse impacts of abiotic stresses. These fungi also serve a pivotal role in promoting the growth of host plants by initiating the solubilization of inorganic phosphate, potassium, and zinc (Devi *et al.*, 2020). Additionally, these fungi facilitate the release of ammonia and mobilize plant nutrients through diverse mechanisms, including acidification, organic acid secretion, and chelation (Ma *et al.*, 2016). Manipur, being located in the Indo-Burma hotspot zone offers huge scope for fungal diversity. This study aims to explore the diversity of endophytic fungi in *Dichrocephala integrifolia*, evaluate their antifungal and enzymatic activities, and assess their potential in promoting plant growth and biocontrol applications.

MATERIALS AND METHODS

Collection of plant sample

Healthy plant samples were collected from the Imphal-East district of Manipur (latitude 24º 47' 0.672'' N and longitude 93º 57' 1.623'' E) in August 2019. The plant samples were brought into the laboratory using sterilized polythene bags, kept in a bucket containing tap water in shade condition, and subjected to endophyte isolation after 16 hours of collection.

Isolation of endophytic fungi

The isolation was conducted following the standard method laid out by Hallmann *et al.* (2006). Plant parts were first washed in water, then surface sterilized using 70% ethanol (3 min) and 4% sodium hypochlorite solution (2 min). The plant segments were cut into small pieces (0.5 cm) and transferred into Petri plates containing solidified Potato Dextrose Agar (PDA) media with added streptomycin sulphate and incubated at 28±1ºC for 3-5 days. The Petri plates were regularly checked for the emergence of fungal hyphae from the plant segments. The fungal hyphae were then pure cultured and proceeded for identification.

Identification of endophytic fungi

Morphological features like colony colour, shape, texture, pigmentation, growth rate, and sporulating structures were used for identification by referring to Barnett and Hunter (1998) and Watanabe (2002) and deposited at the National Fungal Culture Collection of India, Agharkar Institute, Pune, India and obtained accession numbers.

Data analysis of endophytic fungi

The Isolation rate ($I_sR\%$), Colonizing frequency percentage (CF%), Endophytic infection rate (I_fR %), and Relative percentage occurrence (RO%) were calculated for the isolated endophytic fungi (Suryanarayanan *et al.*, 2003; Kumar and Hyde, 2004; Yu *et al.*, 2018). The Isolation rate determines the number of endophytic fungal isolates from each plant; Colonization frequency shows the number of plant segments colonized by an endophytic fungal species; Endophytic infection rate calculates the number of plant segments colonized by the endophytic fungi; and Relative percentage occurrence ascertain the number of endophytic fungal isolates belonging to fungal classes.

$$I_SR\% = \frac{\text{Total number of isolates recovered from each plant part}}{\text{Overall total number of isolates obtained}} \times 100$$

$$CF\% = \frac{\text{Number of segments colonized by a single fungus}}{\text{Total number of segments observed}} \times 100$$

$$I_f\,R\% = \frac{\text{Number of infected segments}}{\text{Total number of segments observed}} \times 100$$

$$RO\% = \frac{\text{Density of colonization of one group}}{\text{Total density of colonization}} \times 100$$

Diversity indices of endophytic fungi

The diversity of the endophytic fungal isolates between different parts of the plant was estimated by using the following diversity indices (Verma *et al.*, 2007; Jena & Tayung, 2013; Sharma *et al.*, 2018). The Simpson's dominance index gives the most common and dominant species, whereas Simpson's diversity index provides the biodiversity of the endophyte species. Both indices give a value range between 0 and 1, where a lower value indicates more dominance and a higher value of higher diversity. The Species richness index provides the total number of different endophytic fungi isolated from the host plant. The Shannon-Wiener index is another diversity index that considers both species richness and evenness. The Evenness index refers to how closely the endophyte species occur in different plant parts. The diversity indices were calculated using the statistical software PAST (version 4.03).

Simpson's Dominance Index $(D) = \frac{\sum n(n-1)}{N(N-1)}$

where, n = total number of organisms of a particular species

N = total number of organisms of all species

Simpson's Diversity Index $(1-D) = 1 - \frac{\sum n(n-1)}{N(N-1)}$

where, n= total number of organisms of a particular species

N= total number of organisms of all species

Species richness index (S) = $\sum n$

Where, n = Number of fungal species

Shannon-Wiener index (H) = $-\sum$ (pi logpi)

where, pi = Number of individuals of species i/ Total number of individuals of all species

Evenness index (E) = H/Hmax

where, H= Shannon-Wiener index; Hmax = ln (N), N= Number of species

Antifungal activity

The sporulating isolates were assessed for antifungal activity by performing the Petri plate dual culture method against the plant pathogen *Curvularia lunata* (Bell *et al.*, 1982). The culture plug of 9 mm size of the endophyte and the pathogen were inoculated into the Petri plate 6 cm apart and incubated at 28±1º C for 10 days. The antagonistic degree was recorded on a scale of class 1 (excellent antagonist), class 2 (strong antagonist), class 3 (good antagonist), class 4 (weak antagonist), and class 5 (non-antagonist). Four isolates that showed prominent antagonistic activity were selected and evaluated for antifungal activity against nine fungal phytopathogens using the Petri plate dual culture technique. The pathogens used were *Alternaria alternata* (ITCC 6778), *Alternaria brassicicola* (ITCC 6193), *Aspergillus flavus* (ITCC 6972), *Aspergillus niger* (ITCC 5406), *Colletotrichum capsici* (ITCC 6078), *Curvularia lunata* (ITCC 7170), *Fusarium oxysporum* (ITCC 4998), *Rhizoctonia solani* (ITCC 4576), and *Ustilaginoidea virens* (ITCC 7046). After seven days of incubation inhibition percentage (I%) were calculated by using the formula, I% = [(r1–r2)/r1] × 100; where r1 = growth of the pathogen on the control plate, r^2 = growth of the pathogen on the dual culture plate (Hajieghrari *et al.*, 2008).

Qualitative extracellular enzyme activities

Protease, lipase, amylase, cellulase, and laccase production was assessed by incubating the endophytic fungi at 28±1°C for five days in an agar medium containing a particular substrate (Rajput *et al.*, 2016; Rao *et al.*, 2019).

Protease activity

Glucose Yeast Peptone Agar (GYPA) media containing skim milk was used for testing protease production. The endophytic fungi were incubated for five days and observed for the appearance of a clear zone around the colony.

Lipase activity

The endophytes were inoculated with Peptone Agar (PA) medium supplemented with Tween 20. The appearance of a clear zone around the colony after incubation shows lipase activity.

Amylase activity

The endophytic fungi were inoculated on a GYPA medium with 1% soluble starch. After incubation, the culture plate was flooded with a 1% iodine solution and observed for the appearance of a clear zone around the colony due to the digestion of starch.

Cellulase activity

The isolates were inoculated on a GYPA medium containing Carboxymethyl Cellulose (CMC). After incubation, the plates were flooded with Congo red solution and then decolourized with NaCl solution. The observation of a yellow region around the endophyte colony confirms the secretion of the cellulase.

Laccase activity

Laccase production was assessed by inoculating the fungal endophytes on a GYPA medium supplemented with 1-naphthol. After incubation, the colour change of the medium to blue or purple shows laccase activity.

Plant growth promotion activities

Phosphate solubilization and ammonia and hydrogen cyanide production were assessed to examine plant growth promotion abilities. The assays were conducted in triplicates.

Phosphate (PO_4) solubilization activity

The isolates were inoculated on Pikovskaya's agar medium Petri plates with added calcium phosphate, and incubated at 28±1°C for seven days. The appearance of a clear zone around the fungal colony shows phosphate solubilization (Ripa *et al.*, 2019).

Ammonia (NH_3) production assay

The endophytes were inoculated in peptone water and incubated for 72 hrs at 28±1°C. Then 0.5 ml of Nessler's reagent was added to each test tube and observed for colour change into yellow or brown (Mahfooz *et al.*, 2017).

Hydrogen cyanide (HCN) production assay

The fungal isolates were inoculated on test tubes containing Bennett agar media. The filter paper was dissolved in a solution containing picric acid and sodium carbonate, then dried and affixed to the inner wall of the test tube. The test tubes were incubated at 28±1°C for 10 days and observed for colour change from light yellow into brown or red which indicates hydrogen cyanide production (Passari *et al.*, 2016).

RESULTS

Isolation, identification and distribution of endophytic fungi

All plant parts of *Dichrocephala integrifolia* were colonised by endophytic fungi, which were identified based on their morphology. A total of 26 different endophytic fungi, including 3 morphotypes of sterile form, were isolated.

Table 1. Endophytic fungi associated with *Dichrocephala integrifolia* along with their NFCCI accession numbers

Isolate code	Endophytic fungi	NFCCI Accession No.	Family	Class
DB3S1	*Aspergillus* sp. 1	NFCCI 5495	Aspergillaceae	Eurotiomycetes
DB1S3	*Aspergillus* sp. 2	NFCCI 5496	Aspergillaceae	Eurotiomycetes
DC1R1	*Chaetomium* sp. 1	NFCCI 5493	Chaetomiaceae	Sordariomycetes
DS6A6	*Chaetomium* sp. 2	NFCCI 5244	Chaetomiaceae	Sordariomycetes
DF4D4	*Chaetomium* sp. 3	NFCCI 5235	Chaetomiaceae	Sordariomycetes
DF2D2	*Colletotrichum* sp. 1	NFCCI 5626	Glomerellaceae	Sordariomycetes
DF8C8	*Colletotrichum* sp. 2	NFCCI 5518	Glomerellaceae	Sordariomycetes
DF1C1	*Fusarium solani*	NFCCI 5243	Nectriaceae	Sordariomycetes
DF6B6	*Fusarium* sp. 1	NFCCI 5245	Nectriaceae	Sordariomycetes
DF5D5	*Fusarium* sp. 2	NFCCI 5624	Nectriaceae	Sordariomycetes
DF3B3	*Fusarium* sp. 3	NFCCI 5225	Nectriaceae	Sordaroimycetes
DS2A2	*Fusarium* sp. 4	NFCCI 5232	Nectriaceae	Sordariomycetes
DL1A1	*Gliocladium* sp. 1	NFCCI 5627	Hypocreaceae	Sordariomycetes
DR2D2	*Gliocladium* sp. 2	NFCCI 5224	Hypocreaceae	Sordariomycetes
DF3A3	*Gliocladium* sp. 3	NFCCI 5233	Hypocreaceae	Sordariomycetes
DR3B3	*Mucor* sp.	NFCCI 5242	Mucoraceae	Mucoromycetes
DA1S1	*Penicillium* sp. 1	NFCCI 5494	Aspergillaceae	Eurotiomycetes
DR7A7	*Penicillium* sp. 2	NFCCI 5625	Aspergillaceae	Eurotiomycetes
DR2A2	*Penicillium* sp. 3	NFCCI 5234	Aspergillaceae	Eurotiomycetes
DF9A9	*Phoma* sp.	NFCCI 5236	Didymellaceae	Dothideomycetes
DF1D1	*Trichoderma* sp. 1	NFCCI 5628	Hypocreaceae	Sordariomycetes
DS2B2	*Trichoderma* sp. 2	NFCCI 5629	Hypocreaceae	Sordariomycetes
DF10A10	*Trichoderma* sp. 3	NFCCI 5517	Hypocreaceae	Sordariomycetes
DF7E7	Sterile morphotype 1	*		
DL9A9	Sterile morphotype 2	*		
DL7A7	Sterile morphotype 3	*		

*Accession numbers were not provided to sterile forms

The spore-bearing isolates were grouped into 9 genera, 7 families, and 4 classes. The endophytic fungi colonized all parts of the plant. The maximum number of endophytic fungal isolates were isolated from inflorescence, followed by leaf, root, and stem. Leaf and stem parts were colonized by isolates belonging to 6 genera, root by 8 genera, and inflorescence by 7 genera. The fungal isolate *Mucor* sp. was confined to the root part and *Phoma* sp. to the inflorescence part. The isolates *Aspergillus* sp. 2, *Chaetomium* sp. 1 and *Penicillium* sp. 1 were commonly found in leaf, stem, and root parts. The three isolates – *Fusarium solani*, *Fusarium* sp. 3, and *Penicillium* sp. 3 were isolated from all the parts. The most isolated endophytic fungal isolate was observed to be *Fusarium solani*, and *Phoma* sp. was the least isolated (Table 1). Sterile morphotype 1 shows a white cottony colony which covers the entire Petri plate (90 mm) in 8 days; later the colony turns light yellow. Sterile morphotype 2 shows pale grey colouration which covers the culture plate in 5 days of incubation; after the 8th day, the colony turns dark brown colour. Sterile morphotype 3 shows a white colony which covers the entire plate in 10 days; after the 12th day, the colony turns brown colour with white spots.

Data analysis

The isolation rate was highest for inflorescence (35.97%), followed by leaf (28.73%), root (19.68%), and stem (15.61%). The highest overall colonization frequency (%) was exhibited by *Fusarium solani* (8.37%) and the lowest by Sterile morphotype 3 (1.36%). The leaf and stem parts of *D. integrifolia* showed maximum colonization by *Fusarium solani* (7.86% and 6.43%, respectively), root segments by *Penicillium* sp. 2 (7.14%), and inflorescence by *Trichoderma* sp. 3 (10.00%). The highest endophytic infection was found in inflorescence (91.43%). The maximum number of isolates belongs to the class Sordariomycetes (71.72%) (Figures 1-3).

Diversity

The endophytic fungi isolated from leaf parts show more dominance in comparison to other parts. The root and inflorescence parts show a similar dominance value (0.7). The Simpson's diversity index shows that the leaf endophytes were more diverse (0.94), and the least divergence was found in the stem part (0.92) of the plant. Both the leaf and inflorescence parts show a maximum Species richness index value of 18. The Shannon-Wiener index indicates more diversity of endophytic fungi in the inflorescence region (2.97) of the plant. The Evenness index has shown that the isolates were distributed most evenly in the leaf part (0.98) (Table 2).

Antifungal activity

The screening of antifungal activity against the pathogen *C. lunata* has shown that all the isolates possess antifungal properties. *Gliocladium* sp. 1, *Trichoderma* sp. 1, *Trichoderma* sp. 2, and *Trichoderma* sp. 3 have shown Class 1 antagonism. The Class 2 degree of antagonism was observed for *Chaetomium* sp. 3, *Fusarium* sp. 2, *Fusarium* sp. 4, *Gliocladium* sp. 2, *Gliocladium* sp. 3, *Mucor* sp., *Penicillium* sp. 2, *Penicillium* sp. 3, and *Phoma* sp. The maximum number of isolates displayed a Class 3 degree

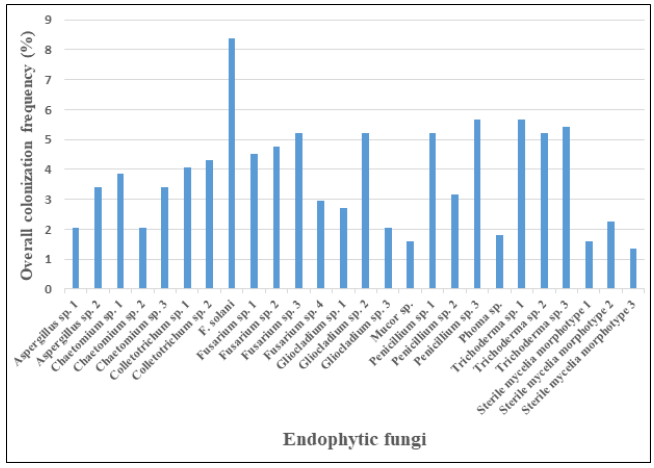

Figure 1. Overall colonization frequency (%) of fungal endophytes isolated from *Dichrocephala integrifolia.*

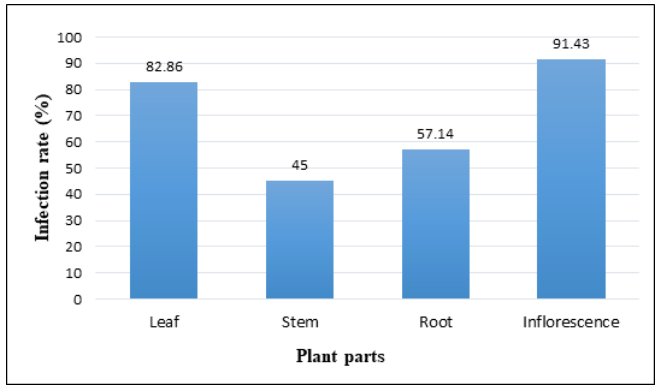

Figure 2. Infection rate (%) of different parts of *Dichrocephala integrifolia* by their associated endophytic fungi.

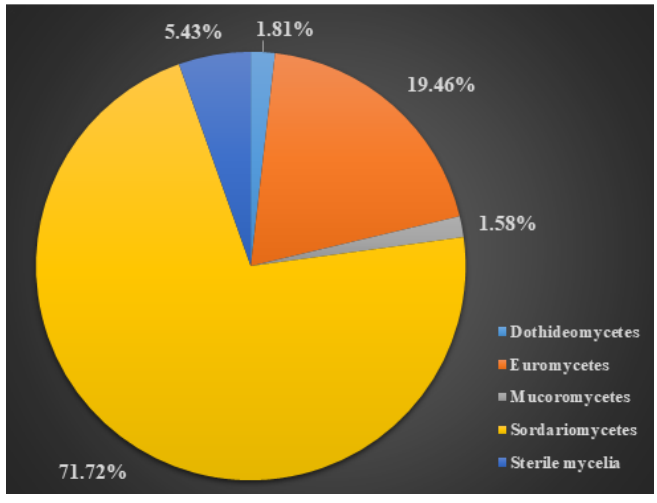

Figure 3. Relative occurrence (%) of endophytic fungal groups obtained from leaf, stem, root, and inflorescence parts of *Dichrocephala integrifolia*.

Table 2. Diversity of fungal endophytes isolated from different parts of *Dichrocephala integrifolia*

Diversity indices	**Plant parts**			
	Leaf	**Stem**	**Root**	**Inflorescence**
Simpson's dominance index (D)	0.06	0.08	0.07	0.07
Simpson's diversity index (1-D)	0.94	0.92	0.93	0.93
Species richness (S)	18	15	16	18
Shannon-Wiener index (H)	2.82	2.56	2.68	2.97
Evenness index (E)	0.98	0.95	0.97	0.97

Table 3. Antagonistic inhibition percentage (I%) of selected endophytic fungi from *Dichrocephala integrifolia* against nine phytopathogens by dual culture method

Fungal pathogens	**Antagonistic inhibition percentage (I%)**			
	***Colletotrichum* sp. 1**	***Gliocladium* sp. 1**	***Penicillium* sp. 3**	***Trichoderma* sp. 2**
Alternaria alternata	42.55±0.09	27.66±0.13	22.34±0.05	100.00±0.00
Alternaria brassicicola	72.86±0.08	69.11±0.05	60.18±0.05	100.00±0.00
Aspergillus flavus	54.91±0.11	44.11±0.05	36.27±0.09	79.41±0.13
Aspergillus niger	59.56±0.15	43.38±0.08	50.73±0.06	89.70±0.13
Colletotrichum capsici	54.03±0.06	47.76±0.04	38.36±0.05	100.00±0.00
Curvularia lunata	34.44±0.05	19.78±0.03	20.65±0.09	100.00±0.00
Fusarium oxysporum	40.62±0.08	24.37±0.05	15.62±0.04	90.21±0.09
Rhizoctonia solani	42.67±0.08	36.93±0.05	35.07±0.09	88.17±0.08
Ustilaginoidea virens	68.63±0.15	61.76±0.09	59.80±0.05	100.00±0.00

Data are means of inhibition percentage (triplicate) ± standard deviation (SD)

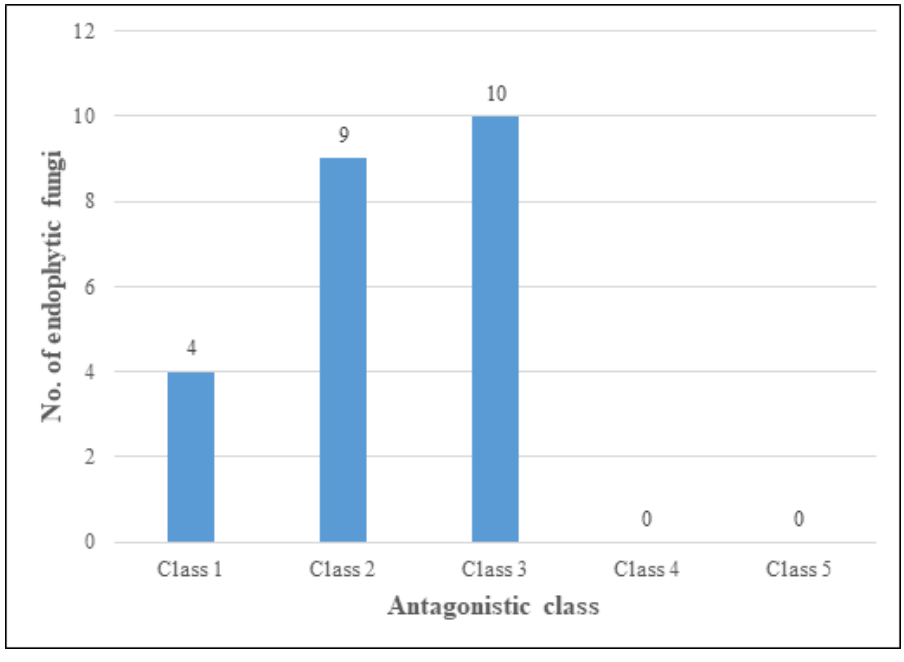

Figure 4. The degree of antagonism shown by the fungal endophytes associated with *Dichrocephala integrifolia* against the plant pathogen *Curvularia lunata*.

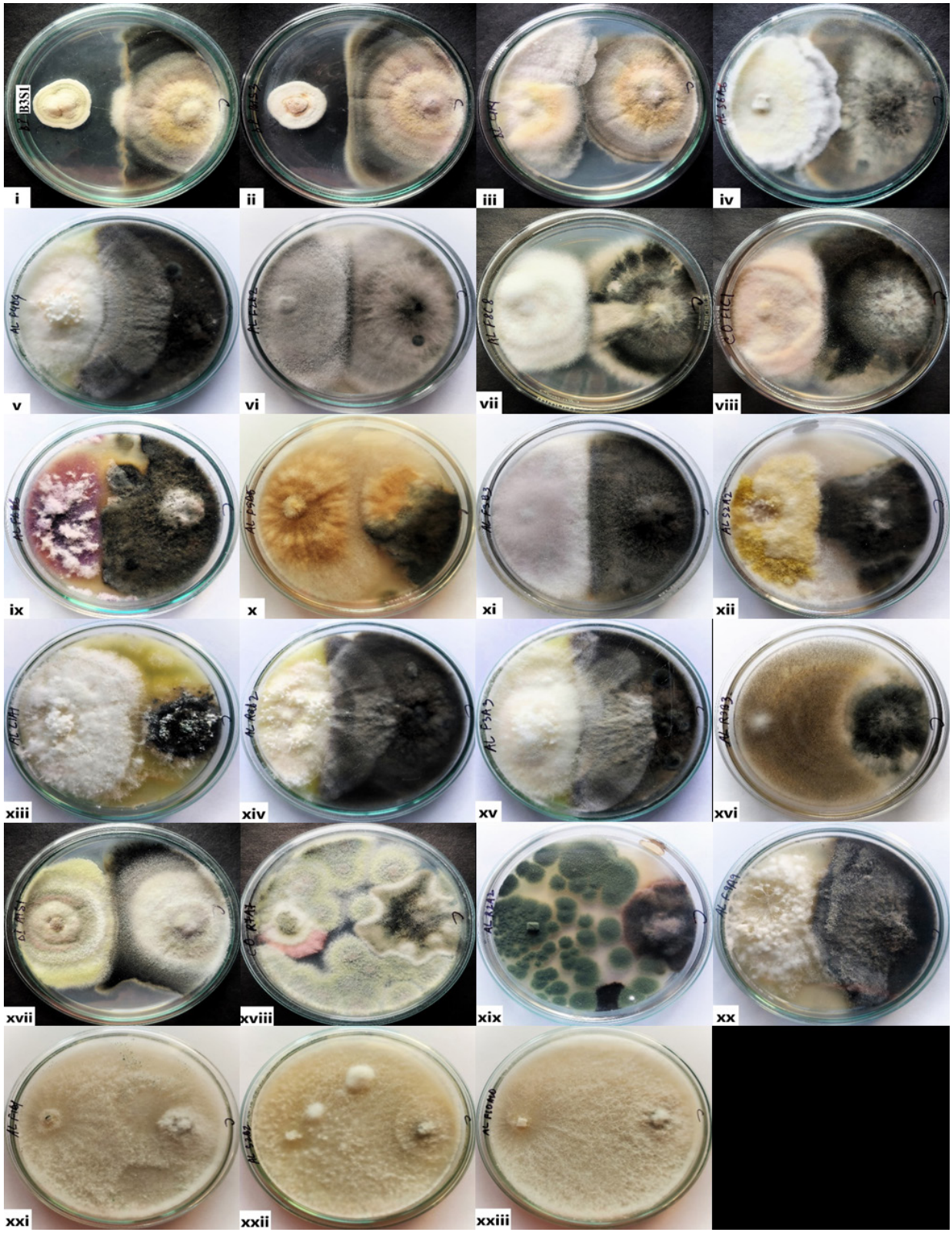

Figure 5. Antagonistic activity of endophytic fungi isolated from *Dichrocephala integrifolia* against the plant pathogen *Curvularia lunata* by dual culture method. The pathogen is inoculated on the right side and the endophytic fungal isolates on the left side of the petriplates. The endophytic fungi inoculated are listed as – (i) *Aspergillus* sp. 1, (ii) *Aspergillus* sp. 2, (iii) *Chaetomium* sp.1, (iv) *Chaetomium* sp. 2, (v) *Chaetomium* sp. 3, (vi) *Colletotrichum* sp. 1, (vii) *Colletotrichum* sp. 2, (viii) *F. solani*, (ix) *Fusarium* sp. 1, (x) *Fusarium* sp. 2, (xi) *Fusarium* sp. 3, (xii) *Fusarium* sp. 4, (xiii) *Gliocladium* sp. 1, (xiv) *Gliocladium* sp. 2, (xv) *Gliocladium* sp. 3, (xvi) *Mucor* sp., (xvii) *Penicillium* sp. 1, (xviii) *Penicillium* sp. 2, (xix) *Penicillium* sp. 3, (xx) *Phoma* sp., (xxi) *Trichoderma* sp. 1, (xxii) *Trichoderma* sp. 2 and (xxiii) *Trichoderma* sp. 3.

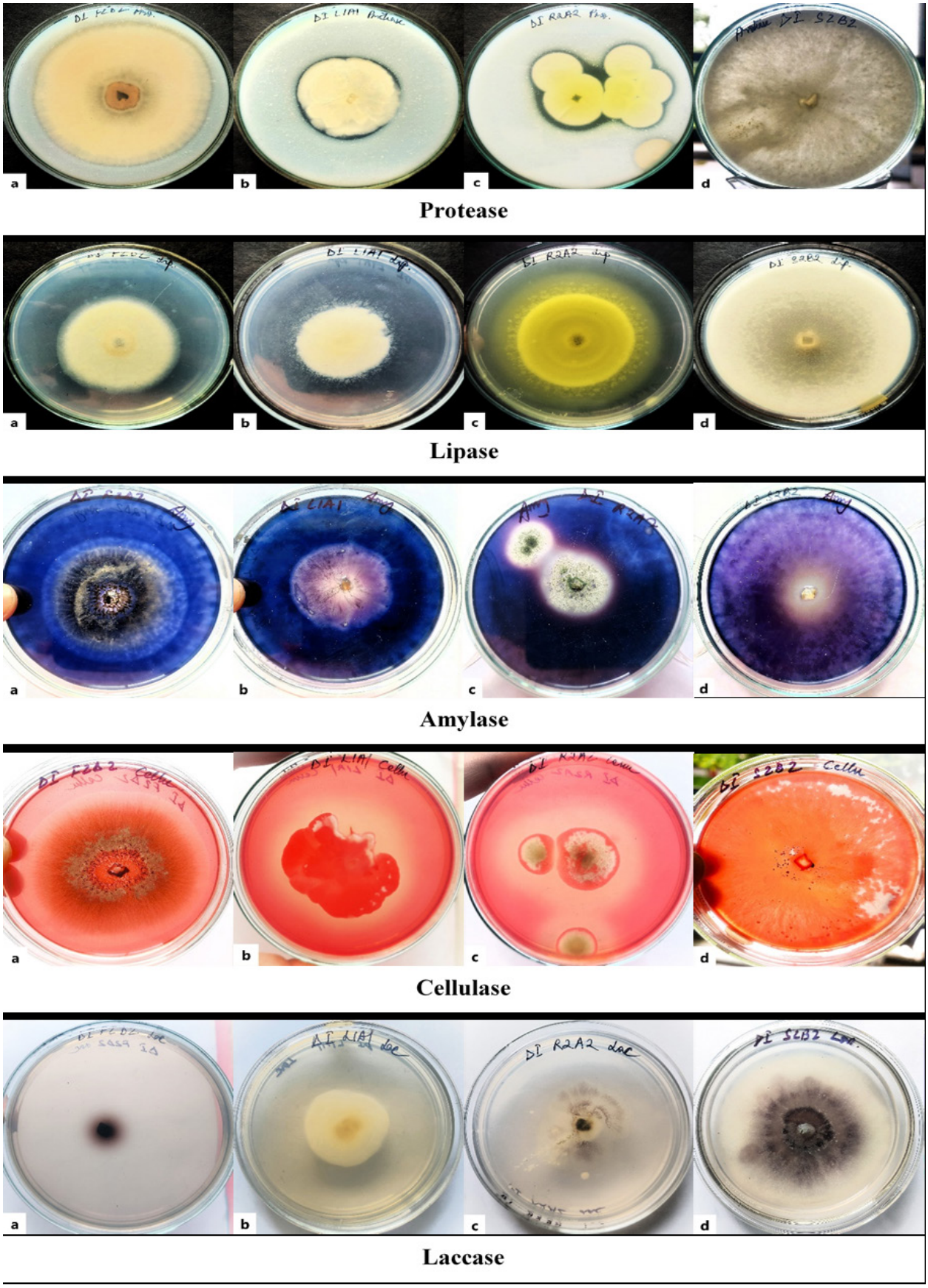

Figure 6. Enzyme activity of endophytic fungi isolated from *Dichrocephala integrifolia*. The inoculated endophytes are (a) *Colletotrichum* sp. 1, (b) *Gliocladium* sp. 1, (c) *Penicillium* sp.3 and (d) *Trichoderma* sp.2.

Table 4. Qualitative analysis for the production of extracellular enzymes and plant growth promotion abilities of endophytic fungi

Endophytic fungi	Enzyme production					Growth promotion		
	Protease	Lipase	Amylase	Cellulase	Laccase	NH_4	PO_4	HCN
Colletotrichum sp. 1	+	+	+	+	+	+	-	-
Gliocladium sp. 1	+	+	+	+	-	+	-	+
Penicillium sp. 3	+	+	+	+	+	+	-	+
Trichoderma sp. 2	+	+	+	+	+	+	-	+

'+' indicates presence; '-' indicates absence

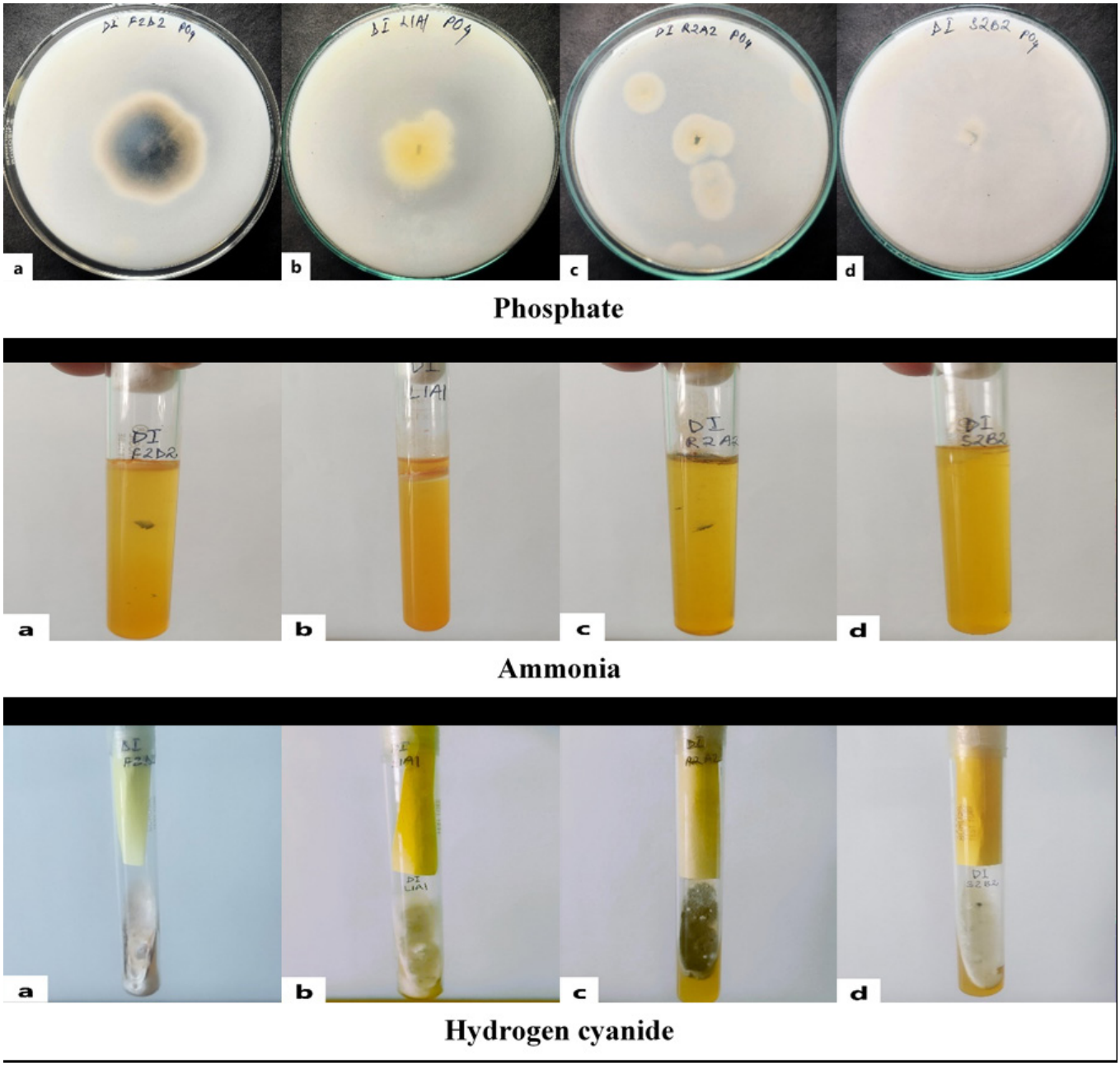

Figure 7. Plant growth promotion activities of endophytic fungi isolated from *Dichrocephala integrifolia*. The inoculated endophyte are listed as (a) *Colletotrichum* sp. 1, (b) *Gliocladium* sp. 1, (c) *Penicillium* sp. 3 and (d) *Trichoderma* sp.2.

of antagonism, namely, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Chaetomium* sp. 1, *Chaetomium* sp. 2, *Colletotrichum* sp. 1, *Colletotrichum* sp. 2, *F. solani*, *Fusarium* sp. 1, *Fusarium* sp. 3, and *Penicillium* sp. 1. None of the isolates displayed Class 4 and 5 antagonistic classes. Four endophytic fungi, namely *Colletotrichum* sp. 1, *Gliocladium* sp. 1, *Penicillium* sp. 3, and *Trichoderma* sp. 2, were selected and further evaluated for antagonistic inhibition percentages (I%) against nine phytopathogens. The endophyte that possessed the strongest antifungal property was found to be *Trichoderma* sp. 2, which showed 100% inhibition against *A. alternata*, *A. brassicicola*, *C. capsici*, *C. lunata*, and *U. virens* (Table 3, Figures 4, 5).

Qualitative enzyme activity and plant growth promotion abilities

The isolates *Colletotrichum* sp. 1, *Penicllium* sp. 3, and *Trichoderma* sp. 2 produce all five enzymes. *Gliocladium* sp.1 was unable to produce laccase. Phosphate solubilization is indicated by the appearance of a clear zone around the colony; ammonia production is indicated by the colour change of peptone water into a yellow colour; and HCN production is indicated by the colour change of filter paper into a brown or reddish colour. All the isolates produce ammonia, and none of them could solubilize inorganic phosphate. Hydrogen cyanide was released by the isolates except for *Colletotrichum* sp. 1 (Table 4, Figures 6 and 7).

DISCUSSION

The increasing population raises food demand, which brings challenges and difficulties in land use and agricultural management (Pereira, 2017). The global population has been estimated to reach 9.8 billion in 2050 (Falcon *et al*., 2022). The excessive use of chemical fertilizer alters the soil pH, increases acidification, and lowers organic matter content, which damages the microflora as well as hinders plant growth (Pahalvi *et al*., 2021). The adoption of sustainable agricultural practices which are environmentally, economically, and socially acceptable has emerged as an important alternative to face the above issues (Zeweld *et al*., 2017). Medicinal plants have served as a significant reservoir of biologically active substances. However due to certain limitations like ecological imbalance, time for regrowth, endemic plants, and seasonal specificity, other sources of natural compounds are in search, and endophytic fungi, due to their potential biological activities and eco-friendly nature, have become an important alternative. In our study, the maximum number of endophytic fungi were isolated from the leaf and inflorescence of *Dichrocephala integrifolia*, which may be due to the presence of more photosynthetic tissues. Uzma *et al.* (2016) reported that the leaves of six medicinal plants, viz., *Tinospora cordifolia*, *Piper nigrum*, *P. longum*, *Zingiber officinale*, *Hedychium coronarium*, and *H. flavescens*, harbour more endophytic fungi than the other tissues. The isolates *Chaetomium* sp. 2 and *Mucor* sp. were confined only to the root parts; *Chaetomium* sp. 3 was in the stem; and *Phoma* sp. was in inflorescence. This indicates that fungal endophytes are tissue-specific. In a recent study, Fang *et al.* (2019) also claimed that certain endophytic fungi are tissue-specific.

Fusarium solani was observed to be the most abundant endophyte in *D. integrifolia*. Several studies on endophytic fungi reported that the secondary metabolite *F. solani* possessed strong antimicrobial properties (Kyekyeku *et al.*, 2017; Hateet *et al.*, 2014; Tayung *et al.*, 2011). In our study, the Sordariomycetes class was found to be the major group. In a similar study, Duan *et al.* (2019) also found Sordariomycetes as the major class while analyzing the endophytic fungi associated with *Distylium chinense*. In a meta-analysis conducted by Mondelaers *et al.* (2009), it was observed that organic farming is far better than conventional farming, as the former increases the organic content of the soil as well as nurtures microflora. Reliable and widely applicable biocontrol agents are in high demand in organic farming, and till now, only a few commercial biocontrol agents are available on the market. Endophytic fungi show biocontrol activity in the host plant through the mechanisms of induced resistance, antibiosis, parasitism, and competition by the production of antimicrobial compounds and extracellular enzymes (Segaran & Sathiavelu, 2019). Endophytic fungi, being non-pathogenic and producing various secondary metabolites with antimicrobial properties, provide a good option for biocontrol agents. The isolates from *D. integrifolia* were found to possess higher antifungal properties, as none of the isolates showed a Class 4 or Class 5 antagonistic class. The seed treatment of tomato and cotton with the endophytic fungus *Beauveri bassiana* induced resistance against the pathogenic fungi *Pythium myriotylum* and *Rhizoctonia solani* (Ownley *et al.*, 2008). Kim *et al.* (2010) have reported a reduction of powdery mildew as well as aphids in cucumber plants by the endophytic fungus *Lecanicillium longisporum*. Wei *et al.* (2019) reported that *Fusarium solani* isolated from cotton plants could control *Verticillium* wilt in cotton caused by *Verticillium dahliae*. In our case, *Trichoderma* sp. 2 was found to be the strongest antifungal agent, and further extraction of bioactive compounds displaying antifungal properties and *in vivo* experimentation could be an important area of research.

Enzymes produced by endophytic fungi are more advantageous than those produced by other groups of organisms due to being safer, more cost-effective, requiring less time consumption, and easily separating the hyphal mat from the enzyme part (Rana *et al.*, 2019). In our study, all four isolates of endophytic fungus were observed to be

good producers of extracellular enzymes. Quantitative analysis of the produced enzymes will indicate their use in industries. Fungicidal activity of lipases are reported from the extracted endophytes (Alves *et al.,* 2018). The endophyte *Cylindrocephalum* sp. from *Alpinia calcarata* was shown to produce a substantial quantity of amylase, as reported by Sunitha *et al.* (2012). Fungal endophytes improve the uptake of macronutrients from soil and organic matter, increase biomass, and restrict herbivores from eating plants (Vishwakarma *et al.*, 2021). The fungal endophytes enhance the growth of the host plant by producing ammonia, which is then directly transferred to the host cells. This transfer of nitrogen is crucial for the elongation of shoots and roots, as well as the synthesis of biomolecules such as proteins and enzymes. Nitrogen is essential for the growth of shoot and roots, as well as the synthesis of biomolecules such as proteins, enzymes, chlorophylls, and nucleic acids (Suman *et al.*, 2016). The endophytic fungi *Penicillium chrysogenum* and *P. crustosum*, associated with the plant *Teucrium polium*, were reported to produce a high amount of ammonia (Hassan, 2017). Hydrogen cyanide is a bioactive compound produced by endophytic fungi that protects the host plant from invading phytopathogens, herbivores, and insects. It has been reported that HCN produced by the endophytic fungi *Aspergillus alabamensis*, *A. oryzae*, and *A. tubingensis* could suppress the wilt disease of pepper plants (Attia *et al.*, 2022). Endophytic fungi associated with roots of host plant enhances nutrient cycling and soil organic content (Mosaddeghi *et al*., 2021). It has been observed that *Epichloë festucae*, increases the survival of the host plant *Lolium perenne* by improving root growth, and metabolism, increasing biomass, and increasing nutrients in shoot and root regions (Chen *et al*., 2020). In another study, the endophytic fungus *Phomopsis liquidambaris* has been shown to increase the absorption and distribution of N, P, Fe, Mn, Zn, Mo, and Se in rice grains, which significantly increases the yield and carbon content up to 23.8% and 17.3%, respectively (Tang *et al*., 2022).

CONCLUSION

This study attempts to understand the distribution and diversity of fungal endophytes isolated from different parts of the medicinal plant, *Dichrocephala integrifolia*. The endophytic isolates were evaluated for antifungal, enzymatic, and growth promotion activities *in vitro*. From the results, several conclusions can be drawn. The plant *D. integrifolia* rich in endophytic fungi exhibited antifungal activities that could be used as a strong biocontrol agent, especially *Trichoderma* sp. 2 which displayed 100% inhibition against the pathogens *Alternaria alternata*, *Alternaria brassicicola*, *Colletotrichum capsici*, *Curvularia lunata*, and *Ustilaginoidea virens*, and can play a major role in integrated pest management. Due to the high demand for microbial enzymes, all 4 fungal endophytes could be utilized to produce a high amount of enzymes in specialized culture media. The plant growth promotion abilities of the endophytic fungi can be used to promote health to the host plant and increase the yield. Further studies like field trials of the antifungal activity, isolation of the antifungal compounds, and quantitative analysis of the extracellular enzymes will be important aspects of future research.

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