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Distribution, Diversity and Biochemical Analysis of Endophytic Fungi Associated with *Chromolaena odorata*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Chromolaena odorata is a medicinal plant that possesses several properties, including antibacterial, antifungal, anti-inflammatory, anticancer, antioxidant, etc., and has been used in traditional medicine in various parts of the world. Medicinal plants are associated with endophytic fungi that have potential biological activities as well as protect the plant from biotic and abiotic agents. In this study, endophytic fungi were isolated from the leaves, stems, roots, and inflorescence of *C. odorata* and identified morphologically. Nineteen sporulating endophytic fungi belonging to 9 genera, 8 families and 4 classes, and three sterile forms were obtained. The isolation data recorded were used to calculate Isolation rate, Colonization frequency, Infection rate and Relative occurrence of different groups of fungi. The highest Isolation rate was found in the stem part (34.75%); the highest overall Colonization frequency (%) was shown by *Chaetomium globosum* (8.51%); the maximum Infection rate was found in root segments (77.86%); and the maximum isolate belongs to Sordariomycetes, showing 43.50%. Simpson's dominance index, Simpson's diversity index, Species richness, Shannon-Wiener index and Evenness (E) index were calculated to reveal

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diversity indices. The maximum diversity was recorded in the leaf part (0.99), and the maximum number of endophytic fungal species was observed in stem segments. The production of biochemicals was analyzed qualitatively, and it was observed that all the isolates produce flavonoids and phenols. This indicates that endophytic fungi are a storehouse of natural compounds and can be applied in agriculture, medicine, and pharmaceutical industries.

Keywords: Chromolaena odorata; endophytic fungi; diversity; colonization; flavonoids.

1. INTRODUCTION

Natural products are the major sources of bioactive compounds with vast applications in medicine, pharmaceuticals, agriculture, food industry, textile industry, and cosmetic industry [1]. From time immemorial, medicinal plants have been used to treat many ailments in traditional medicine practice, including allopathy, homoeopathy and Ayurveda [2]. Plants grown in wild or natural habitats and having ethnobotanical importance are given higher priority to exploit and obtain the active ingredients for treating different diseases. In India, there are about 45,000 plant species, out of which only 700 species are generally used in making phytomedicines [3]. Overharvesting medicinal plants for drugs causes biodiversity loss, ecosystem imbalance, and limited plant resources because of things like small fields, plants' ability to thrive in certain environments, seasonal differences, slow growth rates, and the presence of endemic and endangered species. This makes it even more important to find new, more reliable, long-lasting, and cost-effective sources, and endophytic fungi play a big role in this.

The term ‘endophytic fungi’ may be defined as “those groups of fungi that live within photosynthetic plant tissue by forming symbiotic relationships with the host and have no harmful effect on the host plant” [4]. The extraction of bioactive compounds, the detoxification of xenobiotic pollutants, and the utilization of endophytes as biocontrol agents are the current areas of interest in the field of endophytic research [5]. Petrini [6] estimated that there may be more than a million undiscovered species of endophytic fungi. Strobel and Daisy [7] also estimated that there are approximately 300,000 plant species on our planet, with each plant serving as a habitat for one or more endophytes. Endophytic fungi are mostly ascomycetous and anamorphic fungi. They are a polyphyletic group of very different fungal species [8]. India is extremely rich in biodiversity with varied natural vegetation and offers great scope for fungal endophytes. Some endophytic fungi may have a broad range of host plant species, and some may have different relative frequencies in every host, while others are specific to a particular host plant [9]. The life cycle of endophytic fungal species exhibits a combination of symbiotic, saprophytic, and latent pathogenic characteristics that are dependent upon the presence or absence of host tissue or organs [10].

Endophytic fungi have been found to synthesize a diverse array of bioactive compounds through secondary metabolism. Anthones, enniatines, depsipeptides, furandiones, isocumarines, peptides, polyketones, phenols, quinols, terpenoids, tetralones, and xanthones are some of the compounds that belong to this group [11,12]. These compounds have been reported to show antibacterial, antifungal, antiviral, anticancer, antioxidant, antimalarial, insecticidal, anti-inflammatory, and anti-astherosclerotic activities to a large extent, providing a valuable therapeutic agent [13]. *Chromolaena odorata* (L.) R.M.King & H.Rob. is a perennial herb and has been used in traditional medicine to treat wounds, burns, skin infections, and dyspepsia [14,15]. Additionally, it has been reported to have antispasmodic, antiprotozoal, antitrypanosomal, antibacterial, antifungal, antihypertensive, anti-inflammatory, anticancer, antioxidant, astringent, diuretic, and anti-hepatotoxic properties [16,17]. In search of therapeutic and eco-friendly agricultural practices, endophytic fungi might occupy an important place as an alternative to medicinal plants.

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

The plant samples were collected from Heingang Hill in the Imphal East district (latitude 24° 52' 42.0672'' N; longitude 93° 57' 1.6236'' E; altitude 621 m a.s.l.) during the month of February. The mean monthly rainfall was about 0.2 mm, the mean minimum temperature was found to be 9.8°C, the mean maximum temperature was 24.6

°C, the average relative humidity (%) was recorded to be 57%, and the soil pH was recorded to be 5.68. The collected plants were processed for isolation within 24 hours of collection.

2.2 Isolation and Identification of Endophytic Fungi

Inflorescences, leaves, stems, and roots were used for the isolation of endophytic fungi by following the isolation protocol given by Hallmann et al. [18] with minor modifications. The plant parts were surface sterilized by passing through 70% ethanol (3 minutes), followed by a 4% sodium hypochlorite (NaOCl) solution (3 minutes), and finally 70% ethanol for 60 seconds. The plant parts were cut to a size of 0.5 cm and aseptically transferred to Petriplates containing PDA media supplemented with streptomycin sulphate (500 mg/L). A total of 560 segments, with 140 segments for each plant part, were used. Each Petriplate contains 7 plant segments and 20 replicates were used for each plant part, accounting for a total of 80 replicates. The Petriplates were sealed with parafilm and incubated at 28±1 °C for 5 to 7 days, and they were regularly checked for the emergence of fungal hyphae around the plant segments. Those emerged hyphae were aseptically transferred into fresh Petriplates containing PDA medium without antibiotics for obtaining pure culture and incubated for another 7–10 days at 28±1 °C. The isolates were identified based on morphological characteristics such as colony colour on both sides, texture, margin, growth rate, pigmentation, fungal spores, and spore-bearing structures by consulting fungal identification books [19,20]. The endophytic fungal isolates were deposited, and accession numbers were obtained from the National Fungal Culture Collection of India (NFCCI), Agharkar Institute, Pune, India.

2.3 Distribution of Endophytic Fungi

The data obtained were used to calculate the Percentages of Isolation Rate (I_sR%) [21], Colonizing Frequency (CF%), Endophytic Infection Rate (I_fR%) [22] and Relative Occurrence (RO%) of different groups of endophytic fungi [23].

Isolation Rate (I_sR %):

IsR % = (Total number of isolates recovered from each plant part) / (Overall total number of isolates obtained) × 100

Colonization Frequency (CF %):

CF % = (Number of segments colonized by a single fungus) / (Total number of segments observed) × 100

Infection Rate (I_fR %):

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

Relative Occurrence (RO %):

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

2.4 Diversity of Endophytic Fungi

The diversity of the endophytic fungal isolates between different parts of the plant was estimated by using the diversity indices: Simpson's dominance index (D), Simpson's diversity index (1-D), Species richness (S), Shannon-Wiener index (H), and Evenness (E) index [24,25,26]. The diversity indices were calculated using the statistical software PAST.

$$\text{Simpson's dominance index } (\mathrm{D}) = \frac{\sum \mathrm{n}(\mathrm{n}-1)}{\mathrm{N}(\mathrm{N}-1)}$$

Where,

n= total number of organisms of a particular species
N= total number of organisms of all species

$$\text{Simpson's diversity index } (1-\mathrm{D}) = 1 - \frac{\sum n(n-1)}{\mathrm{N}(\mathrm{N}-1)}$$

Where,

n= total number of organisms of a particular species
N= total number of organisms of all species

$$\text{Species richness index (S)} = \sum n$$

Where, n = Number of fungal species

$$\text{Shannon-Wiener index (H)} = -\sum (pi \log pi)$$

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

$$\text{Evenness index (E)} = H/H_{max}$$

Where, H= Shannon-Wiener index; $H_{max} = \ln (N)$, N= Number of species

2.5 Secondary Metabolite Production

The mycelial plug (0.5 cm diameter) of the endophytic fungi was cut off from a 7 days old culture and inoculated on 500 mL Potato Dextrose Broth (PDB) for 15 days. After filtering, the filtrate was extracted three times with equal amounts of ethyl acetate. The mixture was then dried at 40 °C to get rid of the ethyl acetate, leaving behind the dried crude extract [27].

2.6 Biochemical Analysis of Endophytic Fungi

All the endophytic fungal isolates were analyzed for qualitative production of production of alkaloids, flavonoids, terpenoids, tannins, saponins, steroids and phenols [28,29].

2.6.1 Test for Alkaloids

The crude extract of each endophytic fungal isolate was dissolved in a solution of 2N hydrochloric acid (HCl). The mixture was treated with a small quantity of Mayer's reagent, which consisted of a solution of 3 mL of potassium iodide (KI) combined with 2 mL of mercuric chloride ($HgCl_2$). The formation of a cream-coloured precipitate can be considered an indication of the presence of alkaloids.

2.6.2 Test for Flavonoids

In the test tubes, 1 mL of each fungal crude extract solution was supplemented with a small quantity of a 20% sodium hydroxide (NaOH) solution. A change in color from yellow to colorless upon the addition of dilute hydrochloric acid (6N) illustrates the presence of flavonoids.

2.6.3 Test for Phenols

Few drops of a neutral 5% ferric chloride ($FeCl_3$) solution had been added to the 5 mL of each fungal crude extract solution. The observation of a dark green colour indicates the presence of phenolic compounds.

2.6.4 Test for Tannins

The fungal crude extract solution of each endophyte was treated with an alcoholic $FeCl_3$ reagent. The appearance of a bluish-black colour, which disappears with the addition of dilute H_2SO_4 followed by the formation of a yellowish-brown precipitate indicates the presence of tannins.

2.6.5 Test for Terpenoids

The 1 mL of each fungal extract solution in the test tube was mixed with 2 mL of chloroform. Then, 3 mL of concentrated sulfuric acid (H_2SO_4) was added to the mixture to form a layer. The formation of a reddish-brown precipitate at the interface indicated the presence of terpenoids.

2.6.6 Test for Saponins

The dried crude extract of each endophyte was subjected to a frothing assay with the addition of water. The formation of intense and persistent foam suggests the presence of saponins. Subsequently, the foam was combined with a small quantity of olive oil and the generation of an emulsion confirmed the presence of saponins.

2.6.7 Test for Steroids

To the 0.5 mL of crude extracts of endophytic fungi, 2 ml of acetic anhydride were added with 2 ml of H_2SO_4. The colour change from violet to blue or green in samples indicates the presence of steroids.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Endophytic Fungi

This study reveals the first report on the diversity of endophytic fungi isolated from *C. odorata* belonging to the family Asteraceae. There were 423 isolates total, with stem (147), root (117), leaf (92) and inflorescence (67) having the most isolates. From *C. odorata*, 18 endophytic fungal species were identified, belonging to 9 genera, 8 families, and 4 classes, along with 3 sterile mycelia morphotypes (Table 1, Figs. 1–2). Several researchers have claimed that endophytic fungi are a very diverse and understudied group of organisms [30,31].

3.2 Distribution of Endophytic Fungi

All the parts of *C. odorata* were found to be associated with endophytic fungi. The isolate *Cladosporium cladosporioides* was found only at the leaf and *Mucor* sp. at the root part. None of the *Penicillium* spp., *Trichoderma* spp., or *Mucor* sp. were obtained from the inflorescence part. The isolates of *Alternaria* sp. and *Chaetomium globosum* were confined to the stem and root

regions only. The most commonly isolated species was found to be *Chaetomium globosum*, and the least isolated species was *Aspergillus niger* (Table 2). The stem part (34.75%), the root (27.66%), the leaf (21.75%), and the inflorescence (15.84%) all had the highest isolation rates. The highest overall colonization frequency (%) was shown by *Chaetomium globosum* (8.51%) and least by *Aspergillus niger* (0.94%). Similar colonization frequency (%) were shown by *Arthrinium* sp. and *Penicillium* sp. 1 (3.07%), *Aspergillus* sp. 1 and *Trichoderma* sp. 1 (4.73%), *Aspergillus* sp. 2 and *Aspergillus* sp. 5 (8.04%), *Cladosporium cladosporioides*, and Sterile mycelia morphotype 2 (Fig. 3). *Fusarium* sp. 2 colonized the leaf the most (9.28%), followed by *Chaetomium globosum* in the stem (10.71%) and root (15.00%). *Fusarium oxysporum* and *Fusarium* sp. 3 colonized the inflorescence the least (7.12%). The maximum endophyte infection was found in root segments, and the minimum infection was found in leaf segments (Fig. 4). The isolated endophytic fungi were grouped into five major groups, viz., Euromycetes,Dothideomycetes, Mucoromycetes, Sordariomycetes, and Sterile mycelia, and the maximum isolate belongs to Sordariomycetes showing 43.50% (Fig. 5). In a previous study, [32] reported that certain endophytic fungi are host and tissue specific. In similar endophyte studies conducted by Nguyen et al. [33] and Uzma 34], most of the fungal isolates belong to Ascomycota. Yu et al. [35] revealed that the number of endophytic fungi associated with the medicinal plants *Hedychium coronarium*, *Hedychium flavescens*, *Piper longum*, *Piper nigrum*, *Tinospora cordifolia*, and *Zingiber officinale* was higher in the leaves in comparison to other plant parts.

3.3 Diversity of Endophytic Fungi

The endophytic fungi isolated from different plant parts of *C. odorata* have shown Simpson's dominance index from 0.08 to 0.12, with the highest dominance recorded from the stem part. An increase in the Simpson's diversity index value represents a corresponding rise in species diversity, and the value ranges from 0 to 1. The maximum Simpson's diversity was recorded from the leaf part, with an index value of 0.99. The maximum number of endophytic fungal species was observed in stem segments with 16 fungal species. The Shannon-Wiener index has shown the most favourable habitat of fungal endophyte in *C. odorata* was the stem part, with an index value of 2.64. On the contrary, the endophytic fungal species were evenly distributed in the inflorescence as compared to other plant parts (Table 2). Shubha and Srinivas [36] isolated endophytic fungi from *Camellia oliefera* and observed that the highest Simpson's and Shannon's diversity indices were observed from the leaves, followed by the barks and fruits. In another study conducted by Nisa et al. [37], it was also reported that Simpson's diversity and Shannon-Wiener indices of endophytic fungi isolated from *Cymbidium aloifolium* have shown maximum values in roots. Studies on endophytic fungi have revealed that diversity varies with the host plant and their habitat.

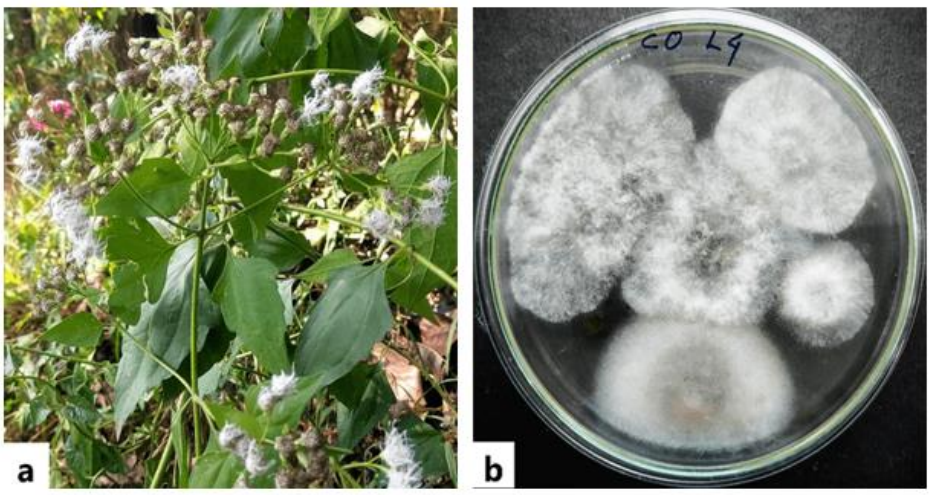

Fig. 1. (a) Chromolaena odorata plant, (b) Emergence of endophytic fungi from plant segments of C. odorata on PDA media

Table 1. List of endophytic fungi isolated from *Chromolaena odorata* along with their NFCCI accession numbers.

Endophytic fungi	NFCCI Accession No.	Family	Class
Alternaria sp.	NFCCI 5524	Pleosporaceae	Dothideomycetes
Arthrinium sp.	NFCCI 5416	Apiosporaceae	Sordariomycetes
Aspergillus niger Tiegh.	NFCCI 5497	Aspergillaceae	Eurotiomycetes
Aspergillus sp. 1	NFCCI 5414	Aspergillaceae	Eurotiomycetes
Aspergillus sp. 2	NFCCI 5418	Aspergillaceae	Eurotiomycetes
Aspergillus sp. 3	NFCCI 5420	Aspergillaceae	Eurotiomycetes
Aspergillus sp. 4	NFCCI 5422	Aspergillaceae	Eurotiomycetes
Aspergillus sp. 5	NFCCI 5521	Aspergillaceae	Eurotiomycetes
Chaetomium globosum Kunze	NFCCI 5423	Chaetomiaceae	Sordariomycetes
Cladosporium cladosporioides (Fresen.) G.A. de Vries	NFCCI 5523	Cladosporiaceae	Dothideomycetes
Fusarium oxysporum Schltdl.	NFCCI 5492	Nectriaceae	Sordariomycetes
Fusarium sp. 1	NFCCI 5421	Nectriaceae	Sordariomycetes
Fusarium sp. 2	NFCCI 5491	Nectriaceae	Sordariomycetes
Fusarium sp. 3	NFCCI 5522	Nectriaceae	Sordariomycetes
Mucor sp.	NFCCI 5412	Mucoraceae	Mucoromycetes
Penicillium sp. 1	NFCCI 5413	Aspergillaceae	Eurotiomycetes
Penicillium sp. 2	NFCCI 5417	Aspergillaceae	Eurotiomycetes
Trichoderma sp. 1	NFCCI 5415	Hypocreaceae	Sordariomycetes
Trichoderma sp. 2	NFCCI 5419	Hypocreaceae	Sordariomycetes
Sterile morphotype 1	*		
Sterile morphotype 2	*		
Sterile morphotype 2	*		

** Accession number were not provided to sterile form*

Table 2. Distribution of endophytic fungi isolated from *Chromolaena odorata*

Endophytic fungi	No. of endophytic fungi isolated from plant parts				Total no. of endophytic fungi
	Leaf	Stem	Root	Inflorescence	
Alternaria sp.	-	7	11	-	18
Arthrinium sp.	-	9	-	5	14
Aspergillus niger Tiegh.	-	5	-	-	5
Aspergillus sp. 1	8	9	-	5	22
Aspergillus sp. 2	7	12	10	8	37
Aspergillus sp. 3	-	13	8	5	26
Aspergillus sp. 4	9	5	-	6	20
Aspergillus sp. 5	11	14	11	-	36
Chaetomium globosum Kunze	-	16	23	-	39
Cladosporium cladosporioides (Fresen.) G.A. de Vries	6	-	-	-	6
Fusarium oxysporum Schltdl.	-	-	8	11	19
Fusarium sp. 1	-	-	6	8	14
Fusarium sp. 2	14	-	8	9	31
Fusarium sp. 3	9	-	-	10	19
Mucor sp.	-	-	11	-	11
Penicillium sp. 1	-	7	8	-	15
Penicillium sp. 2	-	14	10	-	24
Trichoderma sp. 1	11	12	-	-	23
Trichoderma sp. 2	10	15	-	-	25
Sterile mycelia morphotype 1	3	4	-	-	7
Sterile mycelia morphotype 2	4	2	-	-	6
Sterile mycelia morphotype 2	-	3	3	-	6
Total	92	147	117	67	423

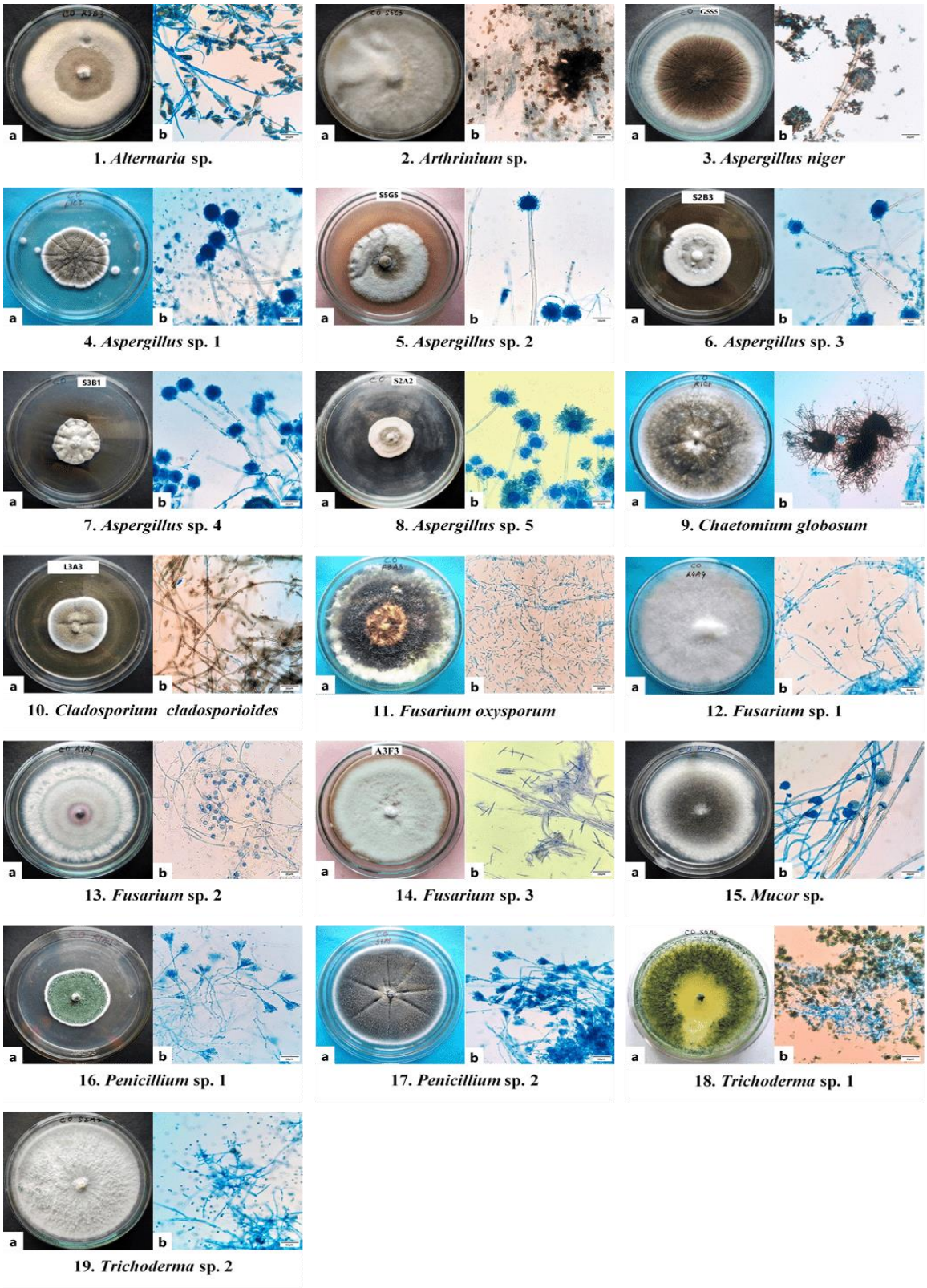

Fig. 2. Morphology of sporulating endophytic fungi isolated from *Chromolaena odorata*. (a) Culture plate morphology, (b) Microscopic images showing spores

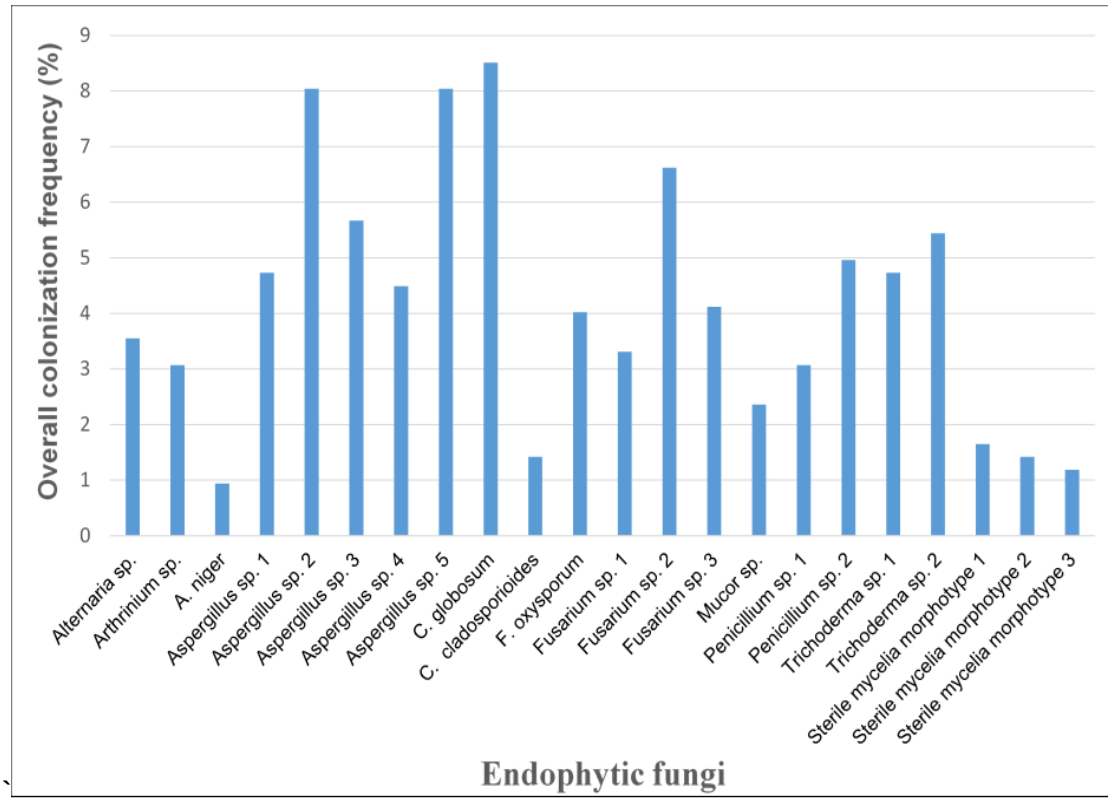

Fig. 3. Overall colonization frequency (%) of endophytic fungi isolated from *Chromolaena odorata*

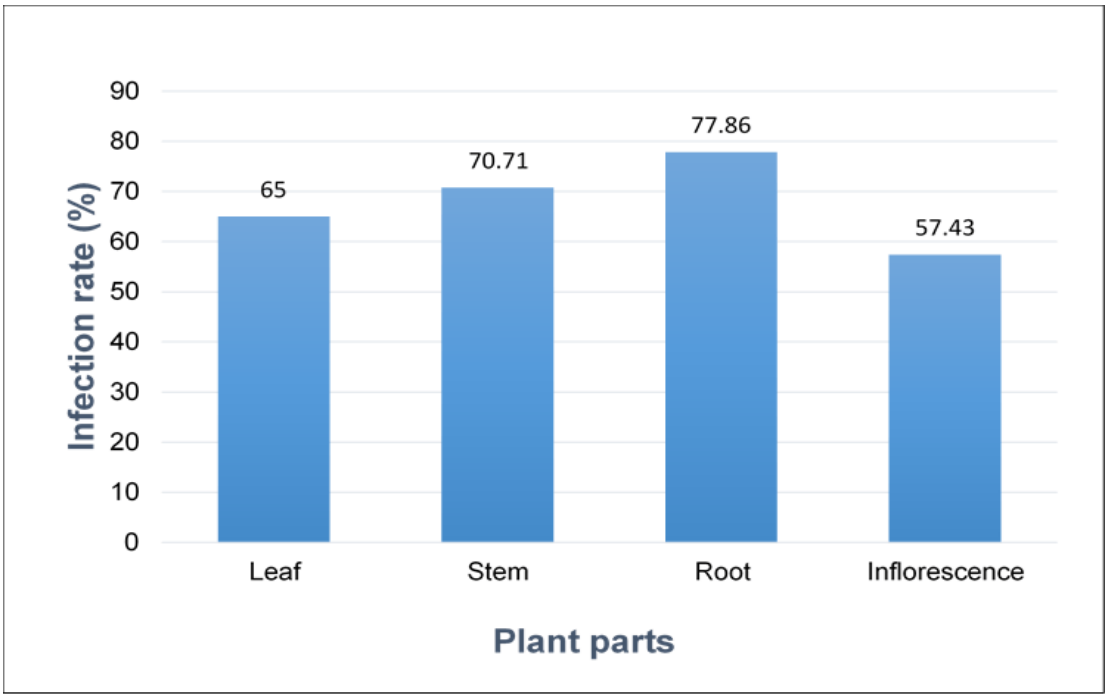

Fig. 4. Infection rate (%) of different parts of *Chromolaena odorata* by their associated endophytic fungi

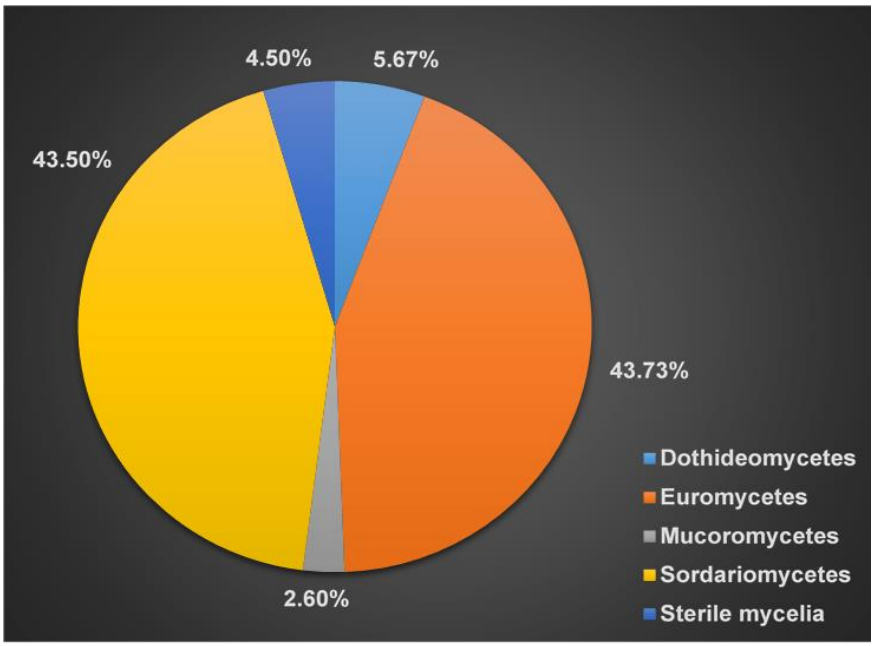

Fig. 5. Relative occurrence (%) of endophytic fungal groups isolated from leaf, stem, root and inflorescence parts of *Chromolaena odorata*

Table 3. Diversity indices of endophytic fungi associated with leaf, stem, root and inflorescence of *Chromolaena odorata*

Diversity indices	Plant parts			
	Leaf	Stem	Root	Inflorescence
Simpson's dominance index (D)	0.10	0.08	0.10	0.12
Simpson's diversity index (1-D)	0.99	0.92	0.90	0.88
Species richness (S)	11	16	12	9
Shannon-Wiener index (H)	2.32	2.64	2.39	2.15
Evenness index (E)	0.97	0.95	0.96	0.98

3.4 Biochemical Analysis of Endophytic Fungi

The qualitative study of ethyl acetate extracts of the endophytic fungi reveals that all the isolates produce flavonoids and phenols (Table 4). Ramesha and Srinivas [38] observed that biochemicals produced by endophytic fungi have beneficial properties in the plant and also protect against invading pathogens. According to Liu et al. [39], alkaloids, flavonoids, phenols, saponins, steroids, tannins, and terpenoids are major phytochemicals produced by endophytic fungi. An alkaloid, Asperfumoid, extracted from *Aspergillus fumigatus* isolated from *Cynodon dactylon* has shown strong antifungal activity against *Candida albicans* [40]. Flavonoids are an important group of secondary metabolites that have antioxidant, anti-ageing, anti-cancer, and anti-inflammatory properties with wide applications in the pharmaceutical and food processing industries [41]. According to Subban and Johnpaul [42], the flavonoid that the endophytic fungus *Alternaria tenuissima* produces exhibits potent antibacterial and antioxidant properties. The phenolic compound (2,4,7-trioxa-bicyclo [4.1.0] hep-tan-3-yl) phenol was extracted from the endophyte *Pestalotiopsis mangiferae* of *Mangifera indica* and displayed potential antimicrobial activities against human pathogens [43]. Saponins are a very important group of compounds owing to their anticancer and anticholesterol activities [44]. The endophytic fungi *Fusarium* sp. and *Aspergillus* sp., isolated from *Panax notoginseng*, yielded saponins (1.061 and 0.583 mg/mL, respectively) that exhibited broad-spectrum antibacterial activity, with MICs ranging from 1.6 to 3.2 mg/mL [45]. Serrano et al. [46] obtained two steroids, Penicisteroids A and B, from the endophytic fungus *Penicillium chrysogenum* associated with marine alga *Laurencia* sp. and reporte

Table 4. Qualitative analysis of biochemical production by the endophytic fungi isolated from Chromolaena odorata.

Endophytic fungi	Biochemical analysis						
	Alkaloids	Flavonoids	Phenols	Saponins	Steroids	Tannins	Terpenoids
Alternaria sp.	+	+	+	-	+	-	+
Arthrinium sp.	-	+	+	-	-	+	-
Aspergillus niger Tiegh.	+	+	+	-	+	+	-
Aspergillus sp. 1	+	+	+	+	-	+	+
Aspergillus sp. 2	+	+	+	-	+	+	
Aspergillus sp. 3	-	+	+	+	-	-	+
Aspergillus sp. 4	+	+	+	-	+	+	+
Aspergillus sp. 5	+	+	+	-	-		
Chaetomium globosum Kunze	+	+	+	-	-	+	+
Cladosporium cladosporioides (Fresen.) G.A. de Vries	+	+	+	-	-	+	-
Fusarium oxysporum Schltdl.	+	+	+	+	+	-	+
Fusarium sp. 1	+	+	+	-	-	+	+
Fusarium sp. 2	-	+	+	+	-	+	-
Fusarium sp. 3	+	+	+	+	+	-	-
Mucor sp.	+	+	+	+	-	+	+
Penicillium sp. 1	+	+	+	-	-	+	-
Penicillium sp. 2	+	+	+	+	-		+
Trichoderma sp. 1	+	+	+	-	+	+	-
Trichoderma sp. 2	+	+	+	-	+	-	-

'+' indicates positive result; '-' indicates negative result

that they have strong antifungal and cytotoxic activity. Tannins are beneficial in providing preventive measures against the development of chronic diseases [47]. Sharma (2019) highlighted the role of tannins in the protection of the plant against pathogens, insects, and herbivores. Isah et al. [48] observed that terpenoids are one of the largest and most structurally varied classes of naturally occurring substances. The terpenoids microdiplodins C and D obtained from the endophyte *Microdiplodia* sp. exhibited anti-inflammatory properties [49].

4. CONCLUSION

This study analyzed the diversity of endophytic fungi in the medicinal plant *C. odorata* and their synthesized compound groups. The interaction of endophytic fungi with plants enhances their overall health and resistance to biotic and abiotic factors. The biochemicals produced by the endophytic fungi have vast applications in agriculture, medicine, and the pharmaceutical industries. Evaluation of biological activities and analysis of secondary mebolites using Gas chromatography–mass spectrometry, Liquid chromatography–mass spectrometry, Fourier-transform infrared spectroscopy, and Nuclear magnetic resonance are important fields of future research that will provide the full potential of endophytic fungi.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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