



Distribution of endophytic fungi associated with *Meriandra bengalensis* Benth. and assessment of their bioactive potential in vitro

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Abstract

Fungal endophytes are known to be diverse and establish a mutualistic relationship with the host plant and protect them from the soil borne pathogens. In this study, we isolated a total of 283 fungal strains belonging to 8 different genera and 16 morphotypes from 450 tissue segments (Leaf, stems and flowers) of benge sage (*Meriandra bengalensis* Benth.) plants during different (winter, summer and rainy) seasons, grown under natural conditions of Manipur and identified them on the basis of cultural morphology. The percentage of isolation rate (IR %) and colonization rates (CR %) of endophytic fungal communities were high in leaf segments (51% and 66%) and winter season (47% and 75%), respectively. The highest relative abundance (RA %) and isolation frequency (IF %) were recorded with *Colletotrichum* sp.2 and diversity indices of fungal endophytes were also calculated. The bioactive potential of *Phoma* sp. MBS13 was evaluated in vitro by dual culture tests against *Aspergillus flavus*, *Aspergillus niger*, *Alternaria solani* and *Fusarium oxysporum* isolates, which showed strong (Class 1 and Class 2) antagonistic activity. In addition, the spectral (FTIR and GC–MS) analysis of ethyl acetate extracted metabolites of *Phoma* sp. MBS13 revealed the presence of various volatile compounds, and showed a good inhibitory activity against some human pathogenic bacteria i.e. *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*. Maximum inhibitory zone (15.3 ± 0.15 mm) was recorded with *E. coli*. Thus, the present findings reveal the importance of endophytic fungi for their role in antimicrobial activity and biocontrol potential against pathogenic microorganisms that could be exploited by the biotechnological and agricultural industries.

Keywords Fungal endophytes · Seasonal variation · Biocontrol · GC–MS analysis · Antibacterial activity

Introduction

Endophytic fungi have either symbiotic or asymbiotic association with the majority of the plant species exist in the environment without causing any discernible infectious symptom (Suryanarayanan et al. 2002). Further, endophytic fungi are beneficial to the host plants by modulating a variety of basic functions like primary productivity, alleviating abiotic stress tolerance, disease resistance and absorption of nutrients by promoting plant growth (Rodriguez et al. 2009). In recent years, the endophytic fungi

have attracted increasing attention due to its potential secondary metabolites productions (Palanichamy et al. 2018) and that can be used as promising substance for antifungal and anticancer activity as a biological control agents in modern medicines, agriculture and industry such as novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds (Strobel and Daisy 2003). Several studies revealed that around 3 lakhs plant species are colonized by endophytic fungi in almost every organ (leaves, stem, flower, root, rhizome and seed) of host plants (Wearn et al. 2012; Gautam 2014), and among them very few were studied to analyze the production of bioactive metabolites (Palanichamy et al. 2018; Kim et al. 2019) from these associated fungi. The diversity and distribution of endophytic fungal communities in medicinal plant species were outlined by different researchers in various ecosystems and niches (Krishnamurthy et al. 2008; Huang et al. 2008; Gautam 2014). However, the seasonal and organ diversity of endophytic fungi in ethanomedicinal

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plants are limited (Göre and Bucak 2007; Mishra et al. 2012; Chowdhary and Kaushik 2015).

Meriandra bengalensis Benth. (Family: Lamiceae) is an aromatic perennial shrub and frequently found in abandoned farm lands in the valley and highlands areas of Manipur, North East (NE) India. The plant (*M. bengalensis*) possesses immense medicinal property. Both fresh leaves and inflorescences extract are used as medicine by local communities to treat colds and stomach ache, tonsillitis, reducing high blood pressure, and also considered as tonic, carminative, astringent and antiseptic etc. (Bruno et al. 1985; Sinha 1996). In addition, *M. bengalensis* plant oil and their essential constituents were documented by GC–MS analysis (Rana and Blazquez 2009). However, their endophytic fungi diversity was not yet explored till date.

Generally, in India, the endophytic fungi associated with common medicinal plant such as *Azadirachta indica* A. Juss. (ver. Neem) was well studied (Rajagopal and Suryanarayann 2000). Whereas, in Lamiaceae family, Gangadevi and Muthumary (2007), Banerjee et al. (2009), Debbab et al. (2009), Jayanthi et al. (2014), Chowdhary and Kaushik (2015), Masaumi et al. (2015), Zafari-Shayan et al. (2016) and Katoch et al. (2017) studied the endophytic fungal diversity in some medicinal plants i.e. *Ocimum sanctum*, *O. bacilicum*, *Leucas aspera*, *Anisomeles malabarica*, *Thymus* sp., *Monarda citriodora*, *Phlomis bruguieri*, *Phlomis olivieri* and *Salvia officinalis*. Besides the enormous diversity of medicinal plant species found in NE India, very few investigations had been made as far as their associated endophytic fungi are concerned (Nirjanta Devi and Singh 2013; Deka and Jha 2020).

However, previous studies have reported that the fungal endophytes isolated from native medicinal plants were the excellent producers of strong bactericidal, cytotoxic, fungicidal and herbicidal metabolites (Wang et al. 2012; Katoch et al. 2017; Kim et al. 2019). There is a need to explore the fungal endophytes from medicinal plants because it has been hypothesized that medicinal plants harbour distinct and rare microbes that mimic the chemistry of their respective hosts and synthesize identical bioactive products (Strobel and Daisy 2003; Nirjanta Devi and Singh 2013). Therefore, an attempt has been made to understand the ecological diversity of endophytic fungi colonized in *M. bengalensis* plants and biological activities of metabolites produced by these fungi. We investigated (1) the occurrence, distribution and species diversity of endophytic fungi during three different seasons and plant tissues associated with this plant and (2) assessed their bioactive potential using extracted fungal metabolites against common plant pathogenic fungi and human pathogenic bacteria.

Materials and methods

Study site and sample collection

The bengle sage (*Meriandra bengalensis* Benth.) a well known perennial medicinal plant, is broadly distributed in tropical and sub-tropical regions of North East India. The study was conducted in abandoned cultivated fields located on Thounaojam village, Bishnupur district (24° 18' 13.1" N; 93° 43' 18.6" E; 754 m a.s.l.), Manipur. The mean annual temperature of the study site ranged between 10.8 to 30.4 °C, and the mean annual rainfall is about 1552 mm. The climate of the study site was tropical humid type.

The present investigation was carried out during October 2015 to September 2016 with three different seasons like, winter (October to January), summer (March to June) and rainy (July–September), and with various plant parts (Leaf, stem and flower). Fifteen randomly selected (five each season), disease free plants were chosen for this study. The different parts of the plant were randomly collected from each season and plant, respectively. These fresh plant materials were immediately placed in poly bags, labeled, and taken to the laboratory for further processing and isolation of fungal endophytes.

Isolation and identification of endophytic fungi

Symptomless and fresh plant parts (leaf, stem and flower) were initially surface sterilized by the modified method of Paul et al. (2012) as follows, 70% (v/v) ethanol for 2 min, 5% (w/v) sodium hypochlorite solution for 5 min and 70% ethanol for 30 s followed by two rinses in sterile distilled water. The above process was repeated twice. For control, the 1 mL final sterile water rinse was plated and observed post-incubation period. Absence of fungal growth indicate the leaf surface was sterile. The surface-sterilized plant fragments were kept in dry autoclaved plot paper for 10 min to reduce moisture and cut into 0.5–1.0 cm lengths fragments using a sterile surgical blade, and then transferred into Petri dish (90 mm diam.) containing potato dextrose agar (PDA, includes 200 g L⁻¹ fresh potato extract, 20 g L⁻¹ glucose, and 20 g L⁻¹ agar, pH 7.0) that are very common medium used for isolating endophytic fungi. To inhibit bacterial contamination in PDA, streptomycin sulphate antibiotics were added to a final concentration of 150 mg/L⁻¹ (Gangadevi and Muthumary 2007). In total, 450 plant fragments/pieces (includes, leaf (50), stem (50), flower (50) in each season) were used to isolate the associated fungal endophytes from field grown medicinal plants. Generally, maximum 5 pieces of plant segments was accommodate per petri

plates and 10 replicates were used for each plant part and seasons. Further, inoculated plates were incubated at room temperature (28 ± 2 °C) and checked daily for 7 days. The actively growing fungal colony from the leaf, stem and flower tips was removed and transferred into fresh PDA plates. Each colony was transferred at least three more times until a visually uniform culture was obtained. After, the pure form of the isolated fungal cultures was generated on PDA slants, which were maintained at 4 °C. The pure cultures of each fungal endophyte were identified based on morphological features of fungi with the support of National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India.

Evaluation of endophytic fungal diversity

The occurrence and diversity of endophytic fungi communities from different plant parts and seasons were analyzed by Paul et al. (2012) and Yu et al. (2018). Initially, the number of isolates (n) and the absolute frequency (AF) of each fungal taxon was measured. The isolation rate (IR %) was calculated by dividing the total number of isolates recovered from each plant segments by overall total number of endophytic isolates obtained $\times 100$; the relative abundance (RA %) was calculated by dividing the total number of isolates representing a single taxon by the total number of taxa obtained from each plant tissues $\times 100$; the isolation frequency (IF %) was calculated by dividing the total number of isolates representing a each taxon by the total number of plant segments incubated $\times 100$. Moreover, the colonization rate (CR %) of fungal infection was calculated by dividing number of plant segments colonized by fungal species by total number of plant segments used per sample $\times 100$. In addition, to study the similarity of fungal diversity between the different plant parts and seasons were also estimated by ecological diversity indices (Shannon and Weaver 1963; Simpson 1964) such as Species richness (S) which is defined by: $S = \sum n$, where n is number of fungal species associated with bangle sage plant, Shannon–Weaver index (H') was used to show diversity of the endophytic fungal species and was calculated as: $H' = -\sum p_i \ln p_i$. Evenness (E) was calculated as H'/H_{\max} , where $H_{\max} = \ln(S)$, S is the total number of taxa in the subsample. Simpson's index of diversity ($1-D$) was measured as $D = 1 - \sum p_i^2$, where p_i is the proportion of isolates assigned to the i th taxa, that is $P_i = n_i/N$ was calculated to compare the species richness. A Simpson diversity index close to 1 means that the sample is highly diverse. Diversity parameters were calculated for each season, and tissue type using PAST software (Hammer et al. 2001).

Study of antifungal activity by dual culture method

The selected endophytic fungus (*Phoma* sp. MBS13) was screened for their ability to act against the common plant pathogenic fungi species by a dual culture method (Yu et al. 2018). Further, the phytopathogenic fungal isolates i.e. *Aspergillus flavus*, *Aspergillus niger*, *Alternaria solani* and *Fusarium oxysporum* were procured from Department of Plant Pathology, Central Agricultural University (CAU), Imphal. The mycelia discs (5 mm dia.) were removed from the margins of actively growing colonies of the both test pathogens and endophytic fungus placed 5 cm away from each other on opposite sides of 90 mm dia. Petri plate, containing about 20 mL of PDA medium. The paired cultures were incubated at 25 ± 1 °C for 5–7 days and then scored for degree of antagonism on a scale of class 1–5 (Bell et al. 1982). Class 1: Endophytic fungi completely overgrow the pathogen and covered the entire medium surface (highly antagonistic). Class 2: Endophytic fungi overgrow at least two-third of the medium surface (antagonistic). Class 3: Endophytic fungi and the pathogen each colonized approximately one half of the medium surface and neither organism appeared to dominate the other (moderately). Class 4: The pathogen colonized at least two third of the medium surface and appeared to withstand encroachment by the Endophyte fungi (poor antagonist). Class 5: The pathogen completely overgrows the endophytic fungi and occupied the entire medium surface (non antagonist).

Mass cultivation and crude metabolite production

For metabolite extraction, the fungal strain *Phoma* sp. MBS13 was inoculated in 1000 mL of potato dextrose broth (PDB) media and incubated at 25 ± 1 °C for 3–4 weeks under a stationary condition (Deka and Jha 2020). To separate the mycelia, the broth culture was filtered and supernatant was extracted three times with equal volumes of ethyl acetate (EtOAc) and concentrated to dryness by using vacuum evaporator at 40 °C and the brown coloured dried crude extract was stored for further analysis.

Spectral characterization of ethyl acetate extracted *Phoma* sp. MBS13 metabolite

Fourier transform infrared spectroscopy (FTIR) analysis

The crude extract of *Phoma* sp. MBS13 was analyzed by FTIR (THERMO NICOLET iS10 FT-IR SPECTROMETER) to know the different functional groups present in the fungal extracts. The sample was grounded with potassium bromide (KBr) pellet (1:10) and pressed into disks under vacuum using a Spectra Lab Pelletizer. Spectral analysis was performed in the study ranged between $4000\text{--}450\text{ cm}^{-1}$.

Compounds contained in the extract were compared and interpreted for the shifting of functional peaks (Deka and Jha 2020).

Gas chromatography mass spectrometry (GC–MS) analysis

The composition of volatile bioactive compounds from crude extract of *Phoma* sp. MBS13 was determined by GC Clarus 500 Perkin Elmer using software Turbomass 5.2 equipped with mass detector Turbo mass gold Perkin Elmer. 2 μL sample was introduced via an all-glass injector working in the split mode, with He as the carrier gas with a linear velocity of 32 cm s^{-1} . The HP-5 fused silica capillary column (length—30 m; film thickness—25 μm I.D—0.2 mm) was used (Nirjanta Devi and Singh 2013). The identification of components present in the metabolite was accomplished using computer searches on National Institute of Standard and Technology (NIST) Ver.2.1 MS data library and structures of the major compounds obtained through GC–MS analysis have been collected from National Institute of Standards and Technology Chemistry WebBook, US (<https://webbook.nist.gov/chemistry/>).

Antibacterial activity

Antibacterial activity of the fungal metabolite, extracted from *Phoma* sp. MBS13, was tested by modified agar well diffusion method (Deka and Jha 2020) against the human pathogenic bacterial strains i.e. *Bacillus subtilis* and *Staphylococcus aureus* (Gram positive) and *Escherichia coli* and *Salmonella typhi* (Gram negative), obtained from Regional Institute of Medical Sciences (RIMS), Imphal. The respective bacterial isolates were sub-cultured on Nutrient agar (NA) medium and an overnight culture of each bacterial strain (10^7 cells mL^{-1}) was swabbed uniformly onto the individual Muller-Hinton agar (MHA) plates using sterile cotton swabs and different concentration of (20, 40 and 60 $\mu\text{g mL}^{-1}$) of the fungal extract suspension in dimethylsulfoxide (DMSO) was poured into each well on the Petri plates. The antibiotic stranded (Streptomycin Sulphate, 1 mg mL^{-1}) was used as a positive control and DMSO (10 mg mL^{-1}) was used as negative control. After incubation at room temperature for 24 h, the zones of inhibition on the bacterial growth were measured. For each test, three replicates were maintained.

Data analysis

All the experimental results related to antimicrobial activity were expressed as mean \pm SD.

Results

Identification of the endophytic fungi

A total of 283 fungal endophytes (leaf-145, stem-86, flower-52) were isolated from 450 tissue segments of *M. bengalensis* plants during three (winter, summer and rainy) seasons, among which 16 endophytic morphotypes belonging to 8 different genera i.e. *Cladosporium*, *Colletotrichum*, *Fusarium*, *Nigrospora*, *Penicillium*, *Phoma*, *Phomopsis* and *Scytalidium* were identified on the basis of morphological features (Fig. 1) and deposited in NFCCI (Table 1). Out of all, the genera *Colletotrichum* found to highest with 5 species, followed by *Phoma* and *Phomopsis* represented 2 species each. Whereas, the single species, were also recovered from each genera like *Cladosporium*, *Fusarium*, *Nigrospora*, *Penicillium* and *Scytalidium*, respectively. The identified fungal endophytes from *M. bengalensis* as follows, *Cladosporium* sp., *Colletotrichum gleosporioides* 1, *C. gleosporioides* 2, *Colletotrichum* sp.1., *Colletotrichum* sp.2., *Colletotrichum* sp.3., *Fusarium oxysporium*, *Nigrospora oryzae*, *Penicillium* sp., *Phoma* sp.1, *Phoma* sp.2, *Phomopsis* sp.1., *Phomopsis* sp.2., *Scytalidium lignicola* and non sporulating dematiaceous form 1 and 2 (Table S1).

Distribution and diversity of the endophytic fungi

Differences in the fungal isolation rate (IR %) and colonization rate (CR %) in various plant tissues during different seasons were calculated, with general IR (63%) and CR (47.3%), which revealed the medicinal plant were highly colonized by endophyte fungi (Figs. 2, 3). However, the isolation rate (IR %) in three different tissue segments (leaf, stem and flower) and seasons (winter, summer and rainy) of *M. bengalensis* were 51%, 31% and 18% (Fig. 2a) and 47%, 30% and 23% (Fig. 2b), respectively. Similarly, the colonization rate (CR %) were showed as 66%, 44% and 32% (Fig. 3a) and 75%, 39% and 28% (Fig. 3b), respectively. Our results confirmed that the highest IR and CR were recorded in leaf segments and winter seasons. The examined plant tissues and different seasons were found to harbour diverse number of endophytic fungi. The relative abundance (RA) of endophytic fungi in *M. bengalensis* tissues with different season varied from 4.17–60.87% (Table 2). Among all, the endophytic fungi *Colletotrichum* sp.3 found to be dominant with high relative abundance (RA) and while *Colletotrichum* sp.2 in isolation frequency (IF). However, the lowest percent of IF was recovered from the species of *Phomopsis* sp.1. Furthermore, the differences in the species distribution

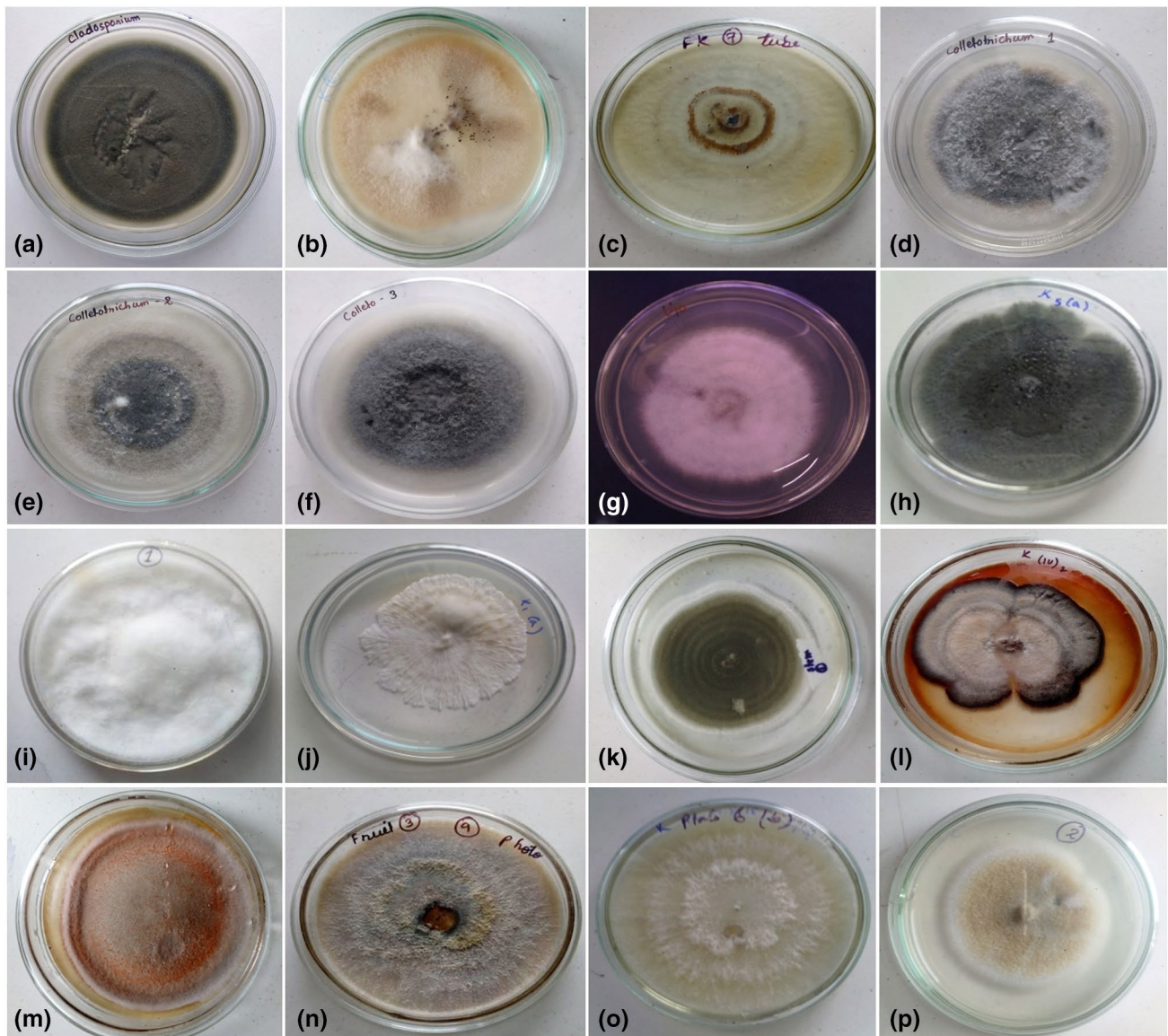


Fig. 1 Colony morphology of isolated fungal endophytes from different plant segments of *Meriandra bengalensis*. **a** *Cladosporium* sp. (MBS1); **b** *Colletotrichum gleosporioides* 1 (MBS2); **c** *C. gleosporioides* 2 (MBS3); **d** *Colletotrichum* sp.1 (MBS4); **e** *Colletotrichum* sp.2 (MBS5); **f** *Colletotrichum* sp.3 (MBS6); **g** *Fusarium* sp.

(MBS7); **h** *Nigrospora oryzae* (MBS8); **i, j** non sporulating dematiaceous form (MBS9) and (MBS10); **k** *Penicillium* sp. (MBS11); **l** *Phoma* sp.1 (MBS12); **m** *Phoma* sp.2 (MBS13); **n** *Phomopsis* sp.1 (MBS14); **o** *Phomopsis* sp.2 (MBS15); **p** *Scytalidium lignicola* (MBS16)

of endophytic fungi isolated from various plant tissues and seasons were also observed. The fungi *Colletotrichum* sp. was commonly recorded with all examined plant tissues i.e. leaf, stem and flower. Whereas, *Fusarium* sp. and *Nigrospora oryzae* were only recovered from leaf and stem tissues. *Penicillium* sp. was found with both leaf and flower, while *Phomopsis* sp. was recorded exclusively from the flowers part of the plant. Likewise, *Phoma* sp. was present only in winter season (Table 2).

To characterize the diversity of the isolated endophytic fungi, we calculated the Species richness (*SR*), Simpson's

index of diversity (*1-D*), Shannon–Wiener index of diversity (*H*), and evenness (*E*) (Table 3). Maximum *SR* of endophytic fungi was recorded with leaf segments (8.33) and winter season (9.33), compared to that of the plant tissues flower (5.33) and rainy (4.67) in *M. bengalensis*. Similarly, Simpson's (*1-D*) and Shannon–Wiener (*H*) index were highest in leaf and winter, 8.40 and 8.67 and 1.960 and 2.115, respectively. However, the evenness (*E*) was maximum in flower and winter season, 0.923 and 0.910, respectively (Table 3).

Table 1 Taxonomic identification of endophytic fungi isolated from *Meriandra bengalensis* Benth.

Family	Endophytes and strain ID	Accession no.
Cladosporiaceae	<i>Cladosporium</i> sp. (MBS1)	NFCCI 4033
Glomerellaceae	<i>Colletotrichum gleosporioides</i> 1 gr. (MBS2)	NFCCI 4036
	<i>Colletotrichum gleosporioides</i> 2 (Penz.) Penz & Sacc. (MBS3)	NFCCI 3973
	<i>Colletotrichum</i> sp.1 (MBS4)	NFCCI 3971
	<i>Colletotrichum</i> sp.2 (MBS5)	NFCCI 3972
	<i>Colletotrichum</i> sp.3 (MBS6)	NFCCI 4029
	<i>Nectriaceae</i>	<i>Fusarium</i> sp. aff. <i>F. oxysporum</i> Schldl (MBS7)
Incertae sedis	<i>Nigrospora oryzae</i> (Berk. & Broome) Petch (MBS8)	NFCCI 4034
	<i>Scytalidium lignicola</i> Pesante (MBS16)	–
	Non sporulating dematiaceous form 1 (MBS9)	–
	Non sporulating dematiaceous form 2 (MBS10)	–
	<i>Trichocomaceae</i>	<i>Penicillium</i> sp. (MBS11)
Didymellaceae	<i>Phoma</i> sp. aff. <i>P. destructiva</i> Plowr. (MBS12)	NFCCI 4037
	<i>Phoma</i> sp. aff. <i>P. herbarum</i> Westend. (MBS13)	NFCCI 4035
Diaporthaceae	<i>Phomopsis</i> sp.1 (MBS14)	NFCCI 4030
	<i>Phomopsis</i> sp.2(MBS15)	NFCCI 4032

Fig. 2 Isolation rate (IR %) of endophytic fungi obtained from studied medicinal plant *M. bengalensis*. **a** Different plant segments (leaf, stem and flower); **b** seasons (summer, rainy and winter)

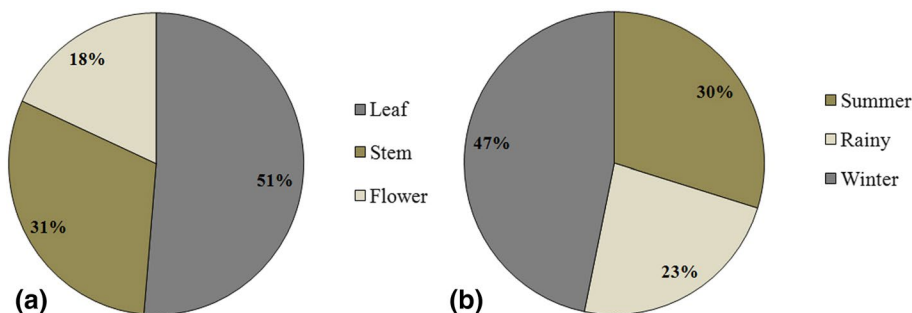
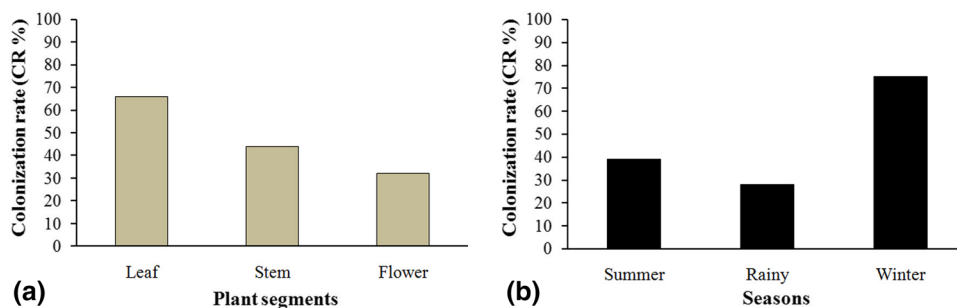


Fig. 3 Colonization rate (CR %) of endophytic fungi from *M. bengalensis* plant. **a** Different plant segments (leaf, stem and flower); **b** seasons (summer, rainy and winter)



Antifungal activity

Dual cultures of endophytic fungus *Phoma* sp. MBS13 from *M. bengalensis* was depicted (Fig. 4) and this exhibited significant potential antifungal activity against studied phytopathogens ranged from class 1 (highly antagonistic) to *A. flavus* and class 2 (antagonistic) to *A. niger*, *A. solani* and *F. oxysporum*.

Metabolite production and spectral analysis

The average yield of crude EtOAc extract (metabolite) in 1L of PDB with *Phoma* sp. MBS13 was recorded in 836 ± 1.16 mg of dry weight.

Table 2 Relative abundance (RA %) and isolation frequency (IF %) of endophytic fungi isolated from leaf, stem and flower tissues of *Meriandra bengalensis* in summer, rainy and winter seasons

Fungal endophytes	<i>Meriandra bengalensis</i>									IF %
	Relative abundance (RA %)									
	Summer			Rainy			Winter			
	Leaf	Stem	Flower	Leaf	Stem	Flower	Leaf	Stem	Flower	
<i>Cladosporium</i> sp. (MBS1)	–	–	–	13.33	–	–	12.50	7.32	–	6.67
<i>C. gleosporioides</i> 1 (MBS2)	–	13.64	–	–	–	–	–	21.95	–	5.56
<i>C. gleosporioides</i> 2 (MBS3)	25.58	–	–	23.33	–	23.08	18.06	–	10.00	15.56
<i>Colletotrichum</i> sp.1 (MBS4)	18.60	13.64	–	20.00	–	–	5.56	7.32	–	15.56
<i>Colletotrichum</i> sp.2 (MBS5)	9.30	–	52.63	16.67	–	–	6.94	–	10.00	16.67
<i>Colletotrichum</i> sp.3 (MBS6)	4.65	36.36	–	–	60.87	–	4.17	12.20	–	13.33
<i>F. oxysporium</i> (MBS7)	–	13.64	–	–	26.09	–	11.11	9.76	–	8.89
<i>N. oryzae</i> (MBS8)	16.28	22.73	–	–	13.04	–	6.94	4.88	–	14.44
<i>S. lignicola</i> (MBS16)	13.95	–	–	–	–	23.08	–	–	10.00	5.56
Non sporulating dematiaceous (MBS9)	6.98	–	–	–	–	–	5.56	4.88	–	7.78
Non sporulating dematiaceous (MBS10)	4.65	–	–	–	–	15.38	4.17	–	20.00	10.00
<i>Penicillium</i> sp. (MBS11)	–	–	15.79	26.67	–	7.69	4.17	–	15.00	12.22
<i>Phoma</i> sp.1 (MBS12)	–	–	–	–	–	–	9.72	19.51	–	7.78
<i>Phoma</i> sp.2 (MBS13)	–	–	–	–	–	–	11.11	12.20	–	6.67
<i>Phomopsis</i> sp.1 (MBS14)	–	–	31.58	–	–	23.08	–	–	15.00	4.44
<i>Phomopsis</i> sp.2 (MBS15)	–	–	–	–	–	7.69	–	–	20.00	4.44
	100	100	100	100	100	100	100	100	100	

‘–’ indicates absence

Table 3 Ecological diversity indices for endophytic fungi isolated from the different plant segments of studied medicinal plants and seasons

Diversity indices ^a	Plant segments			Seasons		
	Leaf	Stem	Flower	Summer	Rainy	Winter
Species richness (<i>S</i>)	8.33 ± 2.03	5.67 ± 1.76	5.33 ± 1.20	5.33 ± 1.45	4.67 ± 0.88	9.33 ± 1.45
Simpson's index of diversity (1- <i>D</i>)	0.840 ± 0.03	0.721 ± 0.09	0.750 ± 0.07	0.731 ± 0.07	0.712 ± 0.08	0.867 ± 0.02
Shannon–Wiener index of diversity (<i>H'</i>)	1.960 ± 0.23	1.502 ± 0.33	1.531 ± 0.27	1.481 ± 0.27	1.399 ± 0.24	2.115 ± 0.14
Evenness (<i>E</i>)	0.907 ± 0.03	0.876 ± 0.02	0.923 ± 0.03	0.890 ± 0.02	0.905 ± 0.03	0.910 ± 0.01

^aMean ± SE

FT-IR analysis

The FT-IR spectrum of EtOAc extracted bioactive metabolite *Phoma* sp. MBS13 showed bands at 2961.79 cm⁻¹, 2923.74 cm⁻¹, 2852.38 cm⁻¹, 1742.02 cm⁻¹, 1463.67 cm⁻¹, 1261.35 cm⁻¹, 1097.79 cm⁻¹, 1022.14 cm⁻¹ and 802.26 cm⁻¹ (Fig. 5). Bands at 2961.79 cm⁻¹, 2923.74 cm⁻¹ and 2852.38 cm⁻¹ that corresponded to the stretching vibrations of alkanes; 1742.02 cm⁻¹ to that of acid anhydride; 1463.67 cm⁻¹ to aromatics; 1261.35 cm⁻¹ to alkyl halide; 1097.79 cm⁻¹ and 1022.14 cm⁻¹ to amine (C–N) and 802.26 cm⁻¹ to alkene.

GC–MS analysis

GC–MS analysis of EtOAc extracted metabolite produced by *Phoma* sp. MBS13 revealed the presence of volatile bioactive compounds viz., E-11,13-Tetradecadien-1-ol, 2-Trifluoroacetoxytridecane, N-Hexadecanoic acid, Octadecanoic acid, 2-(2-hydroxyethoxy)-ethyl ester, Decane, 1-fluoro-, 3-Heptafluorobutyroxy-tetradecane, Pentafluoropropionic acid-tetradecyl ester, 3-Acetoxytridecane, Oleic acid, Z-8-Methyl-9-tetradecenoic acid, 4-Fluoro-1-methyl-5-carboxylic acid, ethyl(ester), Undecanoic acid, 10-bromo-, Erucic acid, 2-Piperidinone,

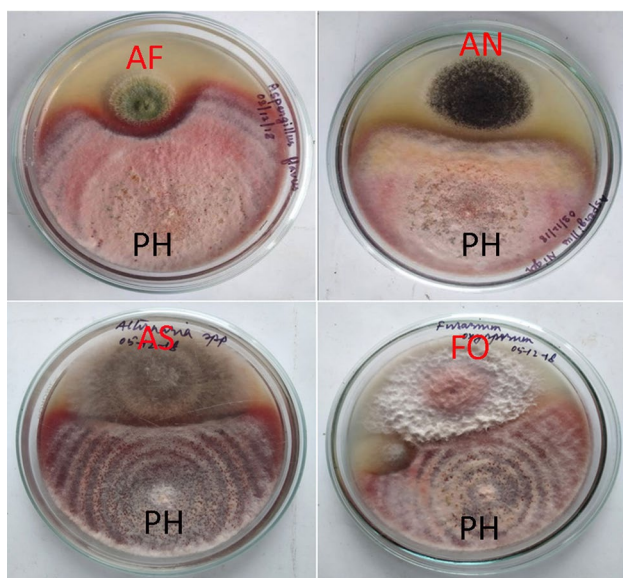


Fig. 4 Endophytic fungus *Phoma* sp. (MBS13) from *M. bengalensis* showing antifungal activity in dual culture antagonistic study against common plant fungal pathogens. PH=*Phoma* sp.; AF=*Aspergillus flavus*; AN=*Aspergillus niger*; AS=*Alternaria solani*; FO=*Fusarium oxysporum*

n-[4-bromo-n-butyl]-, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)-ethyl ester, 1,1,1-Trifluoroheptadecan-2-one, 4-Tetradecanol, 4-Acetoxy-1-decene, 2,6,6-Tri-methyl-bicyclo[3.1.1]hept-3-ylamine, 9-Octadecenal, 3-Decyn-2-ol, Cyclohexane, (1-hexadecylheptadecyl)-, and Sulfurous acid, dodecyl 2-propyl ester. The chemical formula, structure, molecular weight and RT (min)

of these identified compounds have been presented in Table S2 and GC–MS chromatogram is depicted in Fig. 6.

Antibacterial activity

The antibacterial activity of EtOAc extracted metabolite from *Phoma* sp. MBS13 exhibited increased inhibitory activity with increasing concentrations towards both Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative (*E. coli*, *S. typhi*) bacteria (Fig. 7, Table 4). Maximum inhibitory zone (15.3 ± 0.15 mm) was recorded with *E. coli*.

Discussion

There are no reports available regarding to the endophytic fungi diversity in *M. bengalensis* from Manipur. Several studies revealed that the endophytic fungi communities could increase the plant fitness by improving the tolerance to heavy metals and drought and also by reducing the phytopathogen settling (Suryanarayanan et al. 2002) in various environmental conditions. In addition, endophytic fungal communities associated with different kinds of host plants (herb, shrub and trees), plant fragments (leaves, stem, flower, root, rhizomes and seed) and seasons (summer, rainy and winter) from various ecosystems such as tropical, sub-tropical, temperate and Arctic were investigated by earlier workers and found a number of fungi (Suryanarayanan et al. 2002; Göre and Bucak 2007; Rosa et al. 2010; Jayanthi et al. 2014). Rajagopal and Suryanarayann (2000), Gautam (2014), and Chowdhary and Kaushik (2015) investigated the occurrence of endophytic fungi from different tissue

SAMPLE NAME:WSD-K(iv)3

INSTRUMENT: THERMO NICOLET IS10 FT-IR SPECTROMETER (THERMO SCIENTIFIC)

Sat Apr 29 15:24:14 2017 (GMT+05:30)

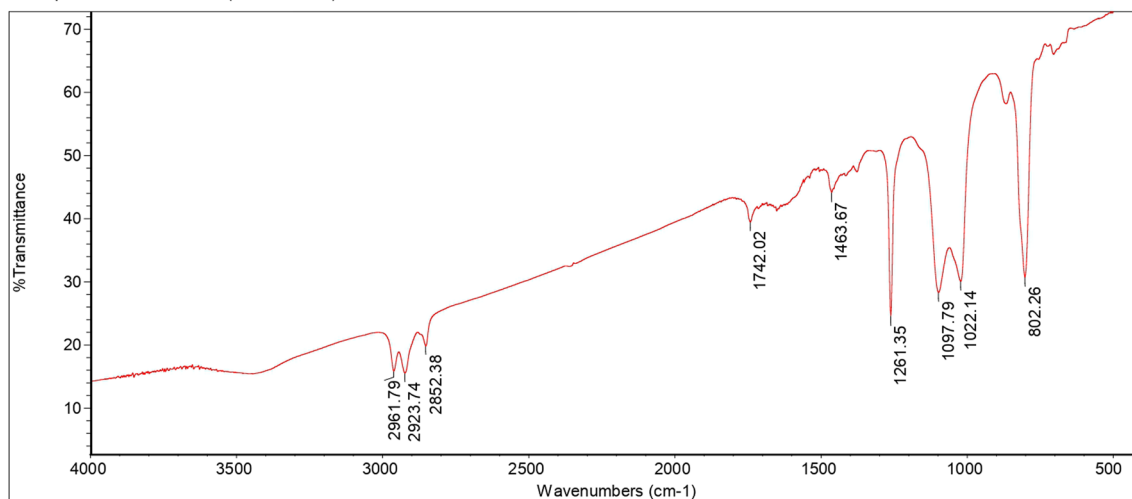


Fig. 5 Characterization of *Phoma* sp. MBS13 fungal crude metabolite by FT-IR spectrum analysis

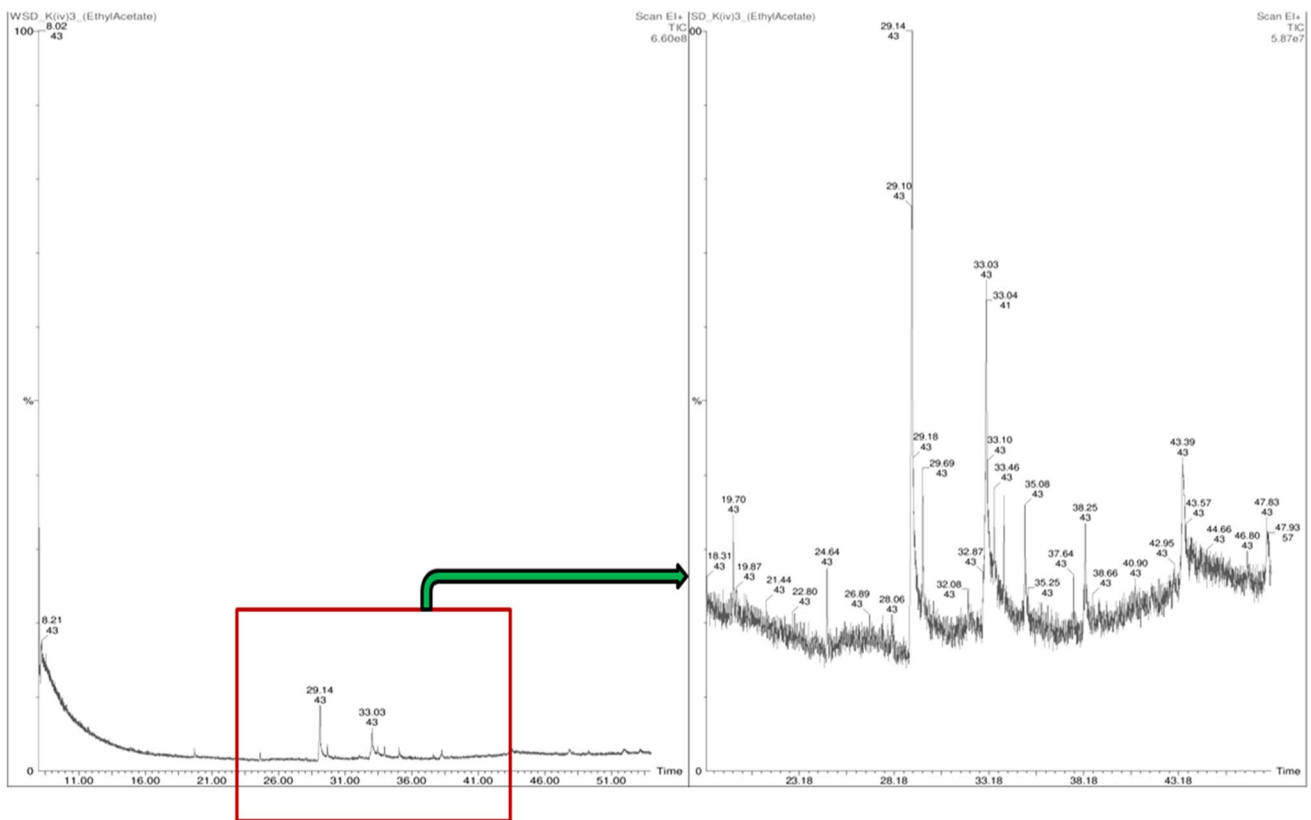


Fig. 6 GC–MS chromatogram of EtOAc extract of bioactive metabolite produced by *Phoma* sp. MBS13

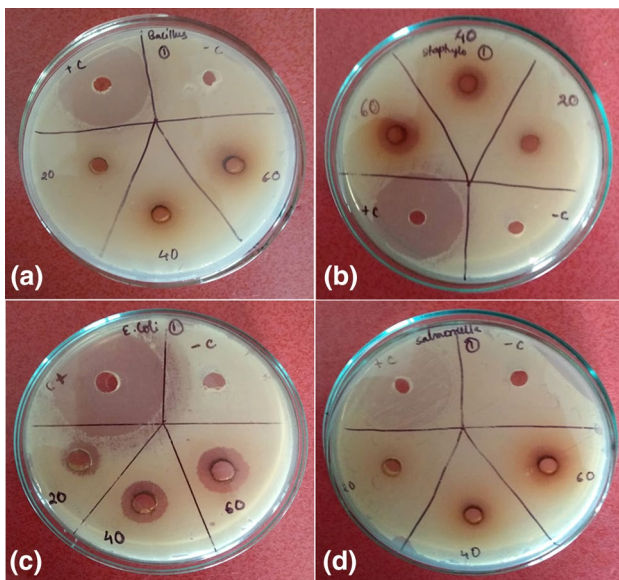


Fig. 7 Antibacterial activity of bioactive crude EtOAc extract of metabolite produced by *Phoma* sp. MBS13 against human bacterial pathogens. **a** *Bacillus subtilis*, **b** *Staphylococcus aureus*, **c** *Escherichia coli*, **d** *Salmonella typhi*. The metabolite concentration was 20, 40 and 60 $\mu\text{g mL}^{-1}$, respectively. –ve: DMSO; +ve: standard antibiotic (Streptomycin sulphate)

parts of Indian medicinal plant species. However, Banerjee et al. (2009) isolated number of endophytic fungi species especially from medicinal plants of Lamiaceae family which were recorded as significant diversity of endophytic fungi. For that reason, we choose the unexplored medicinal plant *M. benglanesis*, for our experiment.

The occurrence and distribution of endophytic fungi in plant-associated habitats may affect the microbial communities that influence them colonize leaves, stem, fruits and roots etc. (Rosa et al. 2010). Previous reports also revealed that endophytes were isolated from different plant tissues/organs or seasons (Göre and Bucak 2007; Paul et al. 2012; Yu et al. 2018). In this study, we isolated endophytic fungi from leaves, stem and flowers of *M. benglanesis* plant during three different seasons. It was a new idea which was not previously investigated and we found a total 283 endophytic strains containing 16 morphospecies. Most endophytic fungal species obtained herein were consistent with previous studies related to other Lamiaceae members (*Ocimum sanctum*, *O. bacilicum* and *Leucas aspera*) by Banerjee et al. (2009) and *Thymus* sp. by Masaumi et al. (2015) and *Anisomeles malabarica* by Jayanthi et al. (2014). Whereas, Gautam (2014) represented only 8 species from the leaves of *Ocimum sanctum* grown in Himachal Pradesh, India, which is comparatively low to our study.

Table 4 Antimicrobial activity on various human pathogens by EtOAc extract of *Phoma* sp. MBS13

EtOAc extract of <i>Phoma</i> sp. MBS13	Zone of inhibition (mm)			
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
20	0.0±0.0	0.0±0.12	10.0±0.65	0.0±0.00
40	8.6±0.73	11.3±0.53	12.3±0.73	5.0±0.23
60	11.3±0.13	14.0±0.06	15.3±0.15	10.0±0.58
S (10 µg mL ⁻¹)	24.0±0.52	23.0±1.45	24.0±1.60	23.0±0.73

Means ± SD (n = 3)

S Standard antibiotic Streptomycin sulphate

Among the isolated endophyte species, the genera *Colletotrichum* represented five different species and commonly distributed to all three seasons and tissue fragments (leaf, stem and flower) with high rate relative abundance (RA %) and isolation frequency (IF %). Similar results were also observed by Paul et al. (2012) in leaves of chili cultivar i.e. *Capsicum annum* from Korea. Whereas, the lowest percent of IF% was recovered from the species of *Phomopsis* sp.1., it was not frequently occurred endophytes. The presence of *Colletotrichum* sp. is common to majority of studies, but not as species diversity (Banerjee et al. 2009; Paul et al. 2012). This results revealed the diversity and species occurrence of particular endophytes is not evenly distributed, it is always influenced by plant species, climate and different age of host plants (Göre and Bucak 2007). Similarly, the association of other fungal endophytes presents in the study, like *Cladosporium* sp., *Fusarium oxysporium*, *Nigrospora oryzae*, *Penicillium* sp., *Phoma* sp., *Phomopsis* sp. and *Scytalidium* sp. were also studied by different authors from time to time (Gangadevi and Muthumary 2007; Mishra et al. 2012; Katoch et al. 2017). Further, association of sterile mycelia (non sporulating dematiaceous form) were also prevalent in most of the endophytic research studies (Paul et al. 2012).

Moreover, the present results revealed that the isolation and distribution of endophytic fungi in *M. benglanesis* plant was organ-specific and season specific. The number of endophytic fungi isolated from leaf segments (IR %) were highest accounting 51% of all isolates; 31% and 18% isolates were obtained from the stem and flower tissues, respectively. Similarly, the endophytic fungi isolated from different seasons, were higher in winter (47%), summer (30%) and rainy (23%), respectively. Masaumi et al. (2015) and Yu et al. (2018) confirmed that the present finding were as similar to earlier reports. The similar trend of results were also recorded in study of colonization frequency (CR %) as well. The species richness (SR), the Simpson's index (*I-D*) and Shannon–Wiener index (*H*) indicated that the highest species diversity was in the leaf and winter season, followed by the stem and summer, accordingly. Yu et al. (2018) also find similar observation in *C. oleifera* species.

Previous results suggesting that these endophytic fungi could play an important role in the plant development by its

potential antifungal activity against phytopathogenic fungi (Paul et al. 2012). In this study, we found that *Phoma* sp. (MBS13) inhibited the mycelia growth activity of pathogenic *Aspergillus flavus*, *Aspergillus niger*, *Alternaria solani* and *Fusarium oxysporum* in dual culture assay. Rajagopal et al. (2008) reported that endophytic *Phoma* sp. isolated from Neem trees could inhibit the growth of plant pathogenic fungal species such as *Aspergillus niger*.

Endophytic fungi are recognized as a potential source of antimicrobial secondary metabolites that could be used in biomedicinal industries (Strobel and Daisy 2003). Different researchers have characterized the compounds present in the EtOAc extracted bioactive metabolites produced by different endophytic fungi (Palanichamy et al. 2018; Deka and Jha 2020), using FT-IR and GC–MS analysis. In the present study, FTIR results of EtOAc extracted metabolite of *Phoma* sp. MBS13 showed bands corresponding to the stretching vibrations of alkanes, acid anhydride, aromatics, alkyl halide, amine (C-N) and alkene. However, Deka and Jha (2018) also observed the (alkanes, acid anhydride, amine etc.) type of compounds present in the EtOAc crude metabolite of an endophytic fungus i.e. *Talaromyces verruculosus* isolated from *Citrus macroptera* plant.

GC–MS analysis revealed the presence volatile compounds in the crude metabolite produced by *Phoma* sp. MBS13 isolated from *M. benglanesis*. The antimicrobial compounds like Sulfurous acid, dodecyl 2-propyl ester, Cyclohexane, (1-hexadecylheptadecyl)- and Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester were identified in the metabolite produced by the potent fungi (*Phoma* sp. MBS13). Similarly, Nirjanta Devi and Singh (2013) and Kanjana et al. (2019) also identified Cyclohexane and Hexadecanoic acid from different endophytic fungal species viz. *Colletotrichum gloeosporioides*, *Chaetomium globosum* and *Cladosporium tenuissimum*. Zafari-Shayan et al. (2016) also identified Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester, Hexadecanoic acid and Z-8-Methyl-9-tetradecenoic acid from leaf extract of *Phlomis bruguieri* (Lamiaceae) plant. Nirjanta Devi and Singh (2013) and Deka and Jha (2020) suggested that the medicinal plant containing potential bioactive compounds may also be produced by the colonized endophytes of the

same. Moreover, the different functional compounds i.e. E-11,13-Tetradecadien-1-ol, 2-Trifluoroacetoxytridecane, 3-Heptafluorobutyroxy-tetradecane, Oleic acid, 4-Fluoro-1-methyl-5-carboxylic acid, ethyl(ester), 2-Piperidinone, n-[4-bromo-n-butyl]-, 1,1,1-Trifluoroheptadecen-2-one, 4-Tetradecanol, 2,6,6-Trimethyl-bicyclo[3.1.1]hept-3-ylamine and 9-Octadecenal present in *Phoma* sp. MBS13 fungus extract showed the important active secondary metabolites for several industrial applications (Wang et al. 2012). In addition, results of the present work suggested that *M. benglanesis* harboured endophytic fungus *Phoma* sp. MBS13 metabolites are capable of producing antimicrobial activity against both gram (+ve) and (–ve) pathogenic bacteria. Similarly, Gomes da Silva et al. (2017) reported that the endophytic *Phoma* sp. URM 7221 extract positively inhibited the growth of *Bacillus subtilis* (35 ± 0.0 mm) and *Staphylococcus aureus* (22 ± 0.4 mm), which is comparatively higher to our study.

Conclusion

The present study concluded that the isolation of endophytic fungi from the different plant segments of studied medicinal plant *M. benglanesis* revealed the presence of diverse species of endophytes. The diversity and species richness of fungal endophytes were also varied with different plant tissues and seasons. The maximum fungal endophytes were recovered from leaf tissue and winter seasons. We showed that the endophytic fungus *Phoma* sp. MBS13 was able to inhibit the growth of common fungal phytopathogens and act as biocontrol. The EtOAc extracted bioactive metabolite of the studied fungus was analyzed by FTIR and GC–MS and it demonstrated the positive antibacterial activity against human pathogenic bacteria. Our study suggests that cultivated medicinal plants could be the interesting niche that harbors many different fungal species and the endophytic isolate *Phoma* sp. MBS13, can produce different compounds having antimicrobial properties.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42535-022-00374-7>.

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Declarations

Conflict of interest The authors do not have any conflict of interest to declare.

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