Diversity, antimicrobial and antioxidant activities of endophytic fungi in *Chloris barbata* and *Eleusine indica* of *Poaceae* subfamily *Chloridoideae*

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Abstract: Endophytic fungi from grass species are known to possess antimicrobial, insecticidal and antiherbivoral activities. Endophytes associated with grass species of subfamily *Chloridoideae* have not been studied for the diversity, and antimicrobial and antioxidant properties. In the present study, the aerial regions of *Chloris barbata* and *Eleusine indica* were incubated by the moist-blotter, potato dextrose agar (PDA) and malt extract agar (MEA) methods to determine the occurrence, diversity and evenness of endophytic mycoflora. Culture filtrate (CF) and mycelial extracts of certain endophytic fungi were studied for antimicrobial and antioxidant activities in vitro. Thirty one species of 20 genera and 25 species of 16 genera of endophytic fungi were documented in *C. barbata* and *E. indica*, respectively. The species richness of endophytic fungi was high on PDA followed by MEA in *C. barbata* and *E. indica*. The diversity and evenness indices varied depending on the grass and incubation method. Most selected endophytes showed moderate antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* and antifungal activity against *Candida albicans*. The metabolites of *Phoma herbarum* showed antioxidant activity comparable to the standard. The present study indicated that grass species harbour diverse endophytic fungi that could be exploited for antimicrobial and antioxidant activities.

Zusammenfassung: Endophytische Pilze aus Grasarten sind dafür bekannt, antimikrobielle, insektizide und antiherbivorale Aktivitäten aufzuweisen. Endophyten aus Grasarten der Unterfamilie *Chloridoideae* sind noch nicht nicht für auf ihre Diversität und antimikrobielle und antioxidative Eigenschaften hin untersucht worden. In der vorliegenden Studie wurden oberirdische Pflanzenteile von *Chloris barbata* und *Eleusine indica* durch Feuchte-Kammer, Kartoffel-Dextrose-Agar (PDA) und Malzextrakt-Agar (MEA) Kulturmethoden inkubiert, um das Auftreten, die Vielfalt und Verteilung der endophytischen Funga zu bestimmen. Kulturfiltrate (CF) und Myzelextrakte bestimmter endophytischer Pilze wurden auf antimikrobielle und antioxidative Aktivitäten untersucht. Einunddreißig Arten aus 20 Gattungen und 25 Arten aus 16 Gattungen von endophytischen Pilzen wurden in *C. barbata* und *E. indica* dokumentiert. Der Artenreichtum der endophytischen Pilze war hoch auf PDA, gefolgt

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von MEA in *C. barbata* und *E. indica*. Die Diversitäts- und die Verteilungs-Indizes variierten je nach Grasart und Inkubationsverfahren. Die meisten der ausgewählten Endophyten zeigten moderate antibakterielle Aktivität gegen *Staphylococcus aureus* und *Enterococcus faecalis* und antimykotische Aktivität gegen *Candida albicans*. Die Metaboliten von *Phoma herbarum* zeigten antioxidative Aktivität vergleichbar mit dem Standard. Die vorliegende Studie zeigt, dass Grasarten diverse endophytische Pilze beherbergen, die für antimikrobielle und antioxidative Aktivitäten ausgenutzt werden könnten.

Plants protect and feed endophytes that produce plant growth-regulatory, antimicrobial, antiviral or anti insect substances to enhance their growth and competitiveness in nature (CARROLL 1988). Endophytic fungi in grasses have been implicated in the herbicidal and insecticidal activities (SCHARDL & al. 2004). Endophytic fungi have also been studied for their disease resistance activities (BACON & WHITE 2000). As many as 100 endophytic communities are reported to be harbored in a plant species (STONE & al. 2004).

Most studies on grass species and endophytes are restricted to *Epichloe* (anamorph *Neotyphodium*) species in grasses of genera *Lolium* and *Festuca* (JIMMY & al. 2001). A survey of literature indicated limited information availability on endophytes in other grass species (SAIKKONEN & al. 2006). Some of the abundantly occurring perennial grass species in the Western Ghats region of India like *Chloris barbata* SW. and *Eleusine indica* L. of sub-family *Chloridoideae* have been studied for association of fungal flora in their roots (SHIVANNA & VASANTHAKUMARI 2011). In vitro antagonism and root colonization ability in chilli has also been investigated (VASANTHAKUMARI & SHIVANNA 2013). However, whether similar endophytic fungal communities occur in the aerial regions of the above species is not documented.

The presence of secondary metabolites in certain grass species resulted in their importance as herbal medicinal plants (FERGUSON 2001). *Chloris barbata*, traditionally used for antidiabetic, antibacterial, and antioxidant activities and for relieving painful and inflammatory conditions (LI & al. 2003, ALGESBOOPATHI 2009), has been scientifically exploited for secondary metabolites and their therapeutic purposes (SWATHY & al. 2010). *Eleusine indica*, also a traditional medicinal plant, is used for treating influenza, hypertension, oliguria and urinary complaints (SIONIT & al. 1987, LEACH & al. 1995); it also contained certain secondary metabolites with antibacterial activities (BALANGCOD & al. 2012). The above grass species are used as fodder species, and particularly, grains are consumed by domestic fowl and humans (DATTA & BANERJEE 1978, MORTON 1981). The present study was aimed to document the occurrence and diversity of endophytic fungal communities in the aerial regions of *C. barbata* and *E. indica* and to determine the *in vitro* antimicrobial and antioxidant activities exhibited by metabolites of certain endophytic fungal species.

Materials and methods

Selection of grass species and study area:

Chloris barbata and *E. indica* occurring in Bhadra Wildlife Sanctuary were selected for studying the endophytic fungal communities present in their aerial regions at the flowering stage. Lakkavalli forest region located (13° 34'–13° 39' N latitude, 75° 30'–75° 39' E longitude) in the sanctuary of the Central Western Ghats region of Karnataka was selected as the study area. The study region had red loam soil (pH 5.32) and received rainfall of 150–250 cm per annum and temperature of 14.5–31.5 °C. In the study area, two study sites were determined, each with three quadrats (1×1 m). The selected grass species were collected at flowering stage and identified (BHAT & NAGENDRAN 2001, VASANTHA-

KUMARI & al. 2010) and confirmed by comparison with those in standard manuals and flora (BOR 1960, YOGANARASIMHAN & al. 1982, SREEKUMAR & NAIR 1991).

Isolation and characterization of endophytic fungal species:
Samples like leaf, inflorescence and pseudo-stem of the selected grass species were collected, washed in running tap water, surface disinfected (sodium hypochlorite 0.5%, 2 min and 70% ethanol, 2 min, standardised for optimum expression), and rinsed with sterile distilled water. Samples were segmented (1 cm long) under aseptic condition and incubated on the moist-blotter, potato dextrose agar or malt extract agar (PDA/MEA Himedia Laboratories, Mumbai) media (supplemented with chloramphenicol, 100 mgl^{-1}) contained in Petri dishes (9 cm diam.) under 12/12h light/nUV light regime at 21±2 °C for 7 to 12 days (ACHAR & SHIVANNA 2013).

The fungal species occurring on incubated samples were identified based on the characteristics of fruiting bodies and spores (BARNETT 1960, ELLIS 1976, VON ARX 1978). The identification of fungal species was confirmed by visiting *Index Fungorum* (www. index fungorum.org). Certain fungal isolates that failed to sporulate by the above methods were characterized by polymerase chain reaction (PCR) and sequencing of internal transcribed spacer (ITS 1 and ITS 2) regions of 18S rDNA genes (WHITE & al. 1990). The PCR amplicon sequences were BLAST searched against non-redundant nucleotide sequence database at NCBI and submitted to obtain the accession number.

The fungal isolates occurring in plant samples were determined for colonization frequency (SURYANARAYANAN & al. 2000). The data of the occurrence of endophytic fungal isolates were subjected to species richness, SIMPSON and SHANNON diversity and evenness indices (PAST ver. 1.00, HAMMER & al. 2001).

Preparation of crude extracts for phytochemical and biological assays:
The endophytic fungal isolates: *Cladosporium cladosporioides, Clonastachys rosea, Cochliobolus oryzae, Colletotrichum dematium, Macrophomina phaseolina, Penicillium commune, Pestalotiopsis mangiferae, Phoma herbarum, Trichoderma harzianum,* NSF isolate 21, and NSF isolate 62 from *C. barbata* and *E. indica* were selected for determining their biological activities. Culture discs (5 mm diam.) obtained from the actively growing margin of colony culture on PDA were inoculated into PD broth (300 ml, pH 5.6) contained in Erlenmeyer flasks (500 ml). The inoculated broth was incubated in dark (21±2 °C, 8–10 days) with intermittent shaking. The culture broth was filtered through three-layered muslin cloth to separate out the mycelial mat (MM) and culture filtrate (CF). The culture filtrate was passed through three layered Whatman no.1 filter paper discs. Equal volumes of ethyl acetate (Himedia, Mumbai) and CF were taken and, mixed well for 10 min and allowed to form two clear immiscible layers. The ethyl acetate containing the extracted compounds was extracted repeatedly and the pooled CF fractions were evaporated to dryness at room temperature (Rotary Flash evaporator, Heidolph, Germany). The MM was dried in an oven (34 °C, 24 h) which was then ground into a fine powder with a pestle and mortar using liquid nitrogen. The MM powder and methanol (Himedia, Mumbai) contained in a vial was shaken in a water bath (60 °C, 3–4 h) and filtered. The filtrate obtained was evaporated to dryness using the rotary evaporator. The extracts were dissolved separately in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored at 4 °C, until use.

Samples of the selected grass species were collected from the study areas, shade-dried, ground and extracted separately in methanol. All the crude extracts were evaporated to dryness and subjected to phytochemical, antimicrobial and antioxidant assays in vitro.

Phytochemical assay:
The crude extracts of fungal endophytes and grass species were tested for the presence of certain secondary metabolites like alkaloids, phenols, flavonoids, glycosides, lignans, saponins, tannins and triterpenoids (HARBORNE 1984).

Antimicrobial assay in vitro:
The stock solution of the crude extract of CF or MM from endophytic fungal species was obtained by adding 1 ml of DMSO to 100 mg of crude extract. A 100 µl of stock solution was mixed with 900 µl of DMSO to obtain a concentration of 1000 µg ml^{-1}; from this, concentrations of 500 µg ml^{-1} and 250 µg ml^{-1} were derived from each of the above concentration, 20 µl (20, 10 or 5 µg ml^{-1}) was used for determining the antimicrobial activity (modified method of NATH & al. 2012). The following test

Table 1. Colonization frequency of endophytic fungal species occurring in the aerial parts of *Chloris barbata* and *Eleusine indica* by moist-blotter, potato dextrose agar (PDA) or malt extracts agar (MEA) methods.

Fungal isolates	Frequency of occurrence (%)[1] / Incubation methods					
	Chloris barbata			*Eleusine indica*		
	Moist-Blotter	PDA	MEA	Moist-Blotter	PDA	MEA
Anamorphic ascomycetes						
Alternaria alternata (FR.) KEISSL.	0.0	0.37[2]	0.0	0.55	0.0	0.0
Aspergillus spp.[3]	0.18(1)[4]	2.76(4)	0.73(2)	0.0	2.58(4)	1.66(2)
Cephalosporium acremonium CORDA	0.37	0.55	0.0	0.0	0.0	0.0
Cladosporium spp.[5]	2.96(1)	32.21(2)	23.88(1)	5.92(1)	25.55(1)	5.92(1)
Clonastachys rosea BAINIER	0.0	0.0	1.48	0.0	0.0	0.0
Colletotrichum dematium (PERS.) GROVE	0.37	0.0	1.11	0.0	0.37	5.92
Fusarium spp.[6]	1.11(1)	1.11(2)	3.11(2)	1.29(1)	1.29(2)	2.03(2)
Macrophomina phaseolina (TASSI) GOID	0.92	0.74	1.48	0.0	0.74	5.55
*Myrothecium roridum** TODE	0.55	0.0	0.0	0.18	0.0	0.0
*Periconia byssoides** PERS.	0.0	1.11	0.55	0.0	0.0	0.0
Penicillium spp.[7]	0.18(1)	1.48(1)	2.77(1)	0.0	1.47(2)	3.32(2)
Pestalotiopsis mangiferae (HENN.) STEYAERT	0.0	0.18	0.55	0.0	0.18	8.3
Pyricularia oryzae CAVARA	0.00	0.0	0.0	0.55	0.0	0.0
Robillarda sessilis SACC.	0.37	0.0	0.0	0.0	0.0	0.0
*Stachybotrys chartarum** (EHRENB.) S. HUGHES	3.88	0.0	0.0	0.0	0.0	0.0
Trichoderma harzianum RIFAI	0.0	0.55	1.11	0.0	1.25	4.07
Total frequency	**10.89**	**41.06**	**36.77**	**8.49**	**33.43**	**36.77**
Teleomorphic ascomycetes						
Chaetomium globosum KUNZE	1.85	2.03	0.18	1.66	0.00	0.18
Cochliobolus spp.[8]	15.9(5)	3.32(4)	12.4(1)	13.68(3)	4.06(3)	12.01(4)
Khuskia oryzae H.J. HUDS	2.22	17.4	14.62	0.0	3.14	4.62
Phoma spp.*[9]	10.73(1)	2.4(3)	1.85(3)	11.28(3)	4.12(3)	2.21(2)
*Pyrenochaeta terrestris** (H.N.HANSEN) GORENZ, J.C. WALKER & LARSON	0.55	0.0	0.0	12.03	0.0	0.0
Total frequency	**31.25**	**25.15**	**29.05**	**38.65**	**11.32**	**19.02**

Notes: [1]Frequency of fungal endophyte occurrence was calculated based on the number of segments colonized by each fungus over the total number of segments studied and represented as percentage ; [2]Data is an average of three replicates, each with 90 samples; [3]*Aspergillus* species = *A brasiliensis* sensu auct. pro parte, pre (0.55–1.11), *A. flavus* LINK (0.18–1.29), *A. fumigatus* FRESEN (0.0–0.37), *A. ochraceus* G. WILH. (0.0–0.18), *A. terreus* THOM (0.18–1.11); [4]Figures in parenthesis indicate total number of species of genera which may vary in different media; [5]*Cladosporium* species = *C. cladosporioides* (FRESEN.) G.A. DE VRIES (2.96–25.55), *C. herbarum* (PERS.) LINK (0.0–10.74); [6]*Fusarium* species = *F. oxysporum* E.F. SM. & SWINGLE (1.29–1.11), *F. oxysporum* NSF isolate 71 (0.55–2.0) ; [7]*Penicillium* species = *P. citrinum* THOM (0.0–0.18), *P. commune* THOM (0.18–1.48); [8]*Cochliobolus* species = *Cochliobolus hawaiiensis* ALCORN (0.18–10.92), *Cochliobolus lunatus* R.R. NELSON & F.A. HAASIS (0.18–6.29), *Cochliobolus spicifer* R.R. NELSON (0.0–0.18), *Cochliobolus oryzae-sativae* (S. ITO & KURIB.) DRECHSLER ex DASTUR (0.37–3.51) and *C. geniculatus* R.R. NELSON NSF isolate CB 28 (1.66– 12.4); [9]*Phoma* species = *Phoma* sp. (0.37–10.73), *P. herbarum* WESTEND NSF isolate 21 (0.37–2.22), *P. herbarum* NSF isolate 62 (0.18–1.66).*Incertae sedis

organisms were obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh, India: two Gram-positive bacteria: *Staphylococcus aureus* (MTCC 3160) and *Enterococcus faecalis* (MTCC 439), three Gram-negative bacteria: *Escherichia coli* (MTCC 723), *Pseudomonas fluorescens* (MTCC 9768) and *Salmonella enterica* (MTCC 734), one clinical fungal pathogen: *Candida albicans* (MTCC 3017) and three other fungal species: *Aspergillus flavus* (MTCC 2813), *Fusarium oxysporum* (MTCC 2485) and *Trichoderma harzianum* (MTCC 936).

Antioxidant assay:
The crude ethyl acetate fraction of CF and methanol fractions of MM and grasses were assayed for 2, 2'-Diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, USA) radical scavenging activity. The crude extracts were dissolved in methanol as described previously to obtain the required concentrations and an additional dilution was made to obtain 1.25 mg ml^{-1}. The assay was performed according to SHARMA & BHAT (2009) with slight modification. The reaction mixture was incubated (37 °C, 30 min) and the absorbance (517 nm) was measured with the enzyme-linked immunosorbent assay (ELISA) plate reader (TECAN, Switzerland). Ascorbic acid (Sigma-Aldrich, USA) was used as the standard. The antioxidant activity is given as DPPH scavenging activity (%) using the formula: [(control absorbance – extract absorbance) ÷ (control absorbance) × 100].

Statistical methods:
The frequency of colonization (%) of plant tissues by endophytic fungal isolates was calculated (SURYANARAYANAN & al. 2000). Species richness, Simpson and Shannon diversity and evenness indices of endophytic fungi were analyzed (PAST ver. 1.00, Hammer & al. 2001). The data of antibacterial activity were transformed (arcsine) and subjected to Duncan's multiple range test (DMRT, P=0.05). The data of antifungal and antioxidant activities were analyzed by standard error (± se).

Results and discussion

Chloris barbata and *Eleusine indica* were selected for the study since they occurred throughout the study period and area and were tested previously for the occurence of rhizoisolates with potential antagonistic activity against *Colletotrichum capsici* and *C. graminicola* (VASANTHAKUMARI & SHIVANNA 2013, 2014). The endophytic fungal communities documented in the above-ground parts of grass species were not tissue-specific and hence data were combined and averaged (Table 1). Thirty one species of 20 genera of 12 families and five isolates of uncertain placement, 'Incertae-sedis', occurred in *C. barbata,* while 25 species of 16 genera of 11 families and three isolates 'Incertae-sedis' occurred in *E. indica*. The endophytic fungal species occurrence in plant segments varied depending on the incubation methods. Similar observations were also made in the previous investigation (SCHULZ & al. 1993).

All the fungal species belonged to the group *Ascomycetes*. Anamorphic ascomycetous isolates expressed more on PDA followed by MEA; however teleomorphic ascomycetes were more in moist-blotter than in the other methods. Twenty species of 15 genera and 16 species of 11 genera of anamorphic ascomycetes occurred in *C. barbata* and *E. indica,* respectively. Among them, *Cladosporium cladosporioides* (32.2% on PDA) occurred in high frequency followed by *Fusarium* species (5.92% in MEA) and *Macrophomina phaseolina* (5.55% in MEA) (Table 1). *Cladosporium cladosporioides* and species of *Cochliobolus* and *Phoma* have been reported as endophytes in tissues of *Echinacea purpurea* and *Sapindus saponaria* (LUIZ & al. 2012, GARCIA & al. 2012). Other than the above species, *Aspergillus, Fusarium* and *Penicillium, Macrophomina phaseolina* and *Khuskia oryzae* also occurred in considerable number. More anamorphic

Table 2. Species richness, diversity and evenness indices of endophytic fungal communities occurring in the aerial parts of *Chloris barbata* and *Eleusine indica* by moist-blotter, potato dextrose agar (PDA) or malt extract agar (MEA) methods*

Grass species/ Incubation method	Species richness	Diversity index		Evenness index	
		Shannon (H')	Simpson (D')	Shannon (J')	Simpson (E')
Chloris barbata					
Moist-blotter	19	2.23	6.34	0.75	0.33
PDA	26	2.27	6.16	0.69	0.23
MEA	20	2.03	5.06	0.68	0.26
Eleusine indica					
Moist-blotter	14	2.10	6.52	0.79	0.46
PDA	25	2.03	4.17	0.65	0.18
MEA	20	2.59	11.57	0.86	0.57

Note: * Data is an average of three replicates, each with 90 samples
Species richness, diversity and evenness indices were determined by PAST ver. 1.00 (HAMMER & al. 2001).

ascomycetes rather than the teleomorphic ascomycetes were reported by previous workers (LUIZ & al. 2012, AJAY 2014). Eleven and 10 species of five genera each of teleomorphic ascomycetes were recorded in *C. barbata* and *E. indica*, respectively. *Pyrenochaeta terrestris* and species of *Cochliobolus* and *Phoma* occurred prominently by the moist-blotter method. The latter fungus was also reported to express on blotters in incubated plant materials (MALLIKARJUNASWAMY & SHIVANNA 2008). *Khuskia oryzae* occurred prominently by PDA method (Table 1).

In contrast to the above groups of fungi, certain non-sporulating fungi (NSF) occurred more frequently by MEA than other methods (Table 1). The NSF (isolates CB 28, 21 and 62) neither sporulated under nUV light nor produced clamps or crosiers. However, the ITS 1 and ITS 2 regions of 18S rDNA of the NSF helped in their characterization as teleomorphic ascomycetes. The isolate NSF CB 28 was identified as *Cochliobolus geniculatus* R.R. Nelson (Acc. no. KF590040, length 539 bp, identity 99%, Homologue – HE861840.1). *Cochliobolus* species sporulate profusely upon incubation; however *C. geniculatus* isolate failed to sporulate in any of the incubation methods. On the other hand, the other two isolates with either buff or pink colored mycelia were characterized as *Phoma herbarum* COOKE (Acc. no. KF590039, length 475 bp, identity 100%, Homologue – JX867225.1 and Acc. no. – KC821515, length 503 bp, identity 99%, Homologue – JX867225.1). VASANTHAKUMARI & SHIVANNA (2013) also identified certain NSF isolates occurring in rhizoplane region of grasses as *Phoma putaminum* by PCR based molecular characterization.

The endophytic fungal species richness was high on PDA in *C. barbata* (26) and *E. indica* (25); MEA was next in species richness (20 and 20, respectively). High species richness on PDA was also reported by KUMAR & HYDE (2004). The Shannon and Simpson diversity indices values were high by moist-blotter and PDA methods in case of *C. barbata,* while it was high by MEA in case of *E. indica* (Table 2). Contrary results were obtained by MAHESWARI & RAJAGOPAL (2013) in case of *Kigelia pinnata*. It was the moist-blotter and MEA methods that supported high evenness indices in case of *C. barbata* and *E. indica,* respectively (Table 2). Work on the rhizoplane and

rhizosphere fungi of the above grass species showed that SHANNON and SIMPSON diversity indices were high on PDA and Czapek Dox Agar media (SHIVANNA & VASANTHAKUMARI 2011).

Table 3. Antibacterial activity of solvent extracts of endophytic fungi isolated from *Chloris barbata* and *Eleusine indica.*

Crude extract of fungal isolates	Zone of inhibition (mm)[1]				
	Staphylococcus aureus[2]	*Enterococcus faecalis*[2]	*Salmonella enterica*[3]	*Escherichia coli*[3]	*Pseudomonas fluorescens*[3]
Ethyl acetate extract of culture filtrate					
C. rosea	10a	10 a	0	11 a	10 a
C. oryzae	11 a	10b	11 a	0	10 b
C. dematium	18 a	18 a	15 b	14 b	13 c
M. phaseolina	0	0	0	0	10a
P. herbarum	0	0	0	11a	0
P. herbarum (isolate 21)	11 a	12 a	13 a	13 a	11 b
P. herbarum (isolate 62)	15 a	15 a	11 b	0	11 b
P. commune	20 a	20 a	10 b	12 b	20 a
P. mangiferae	0	0	12a	11a	0
T. harzianum	11c	14 a	13 a	12 b	11 c
Methanol extract of mycelia mat					
C. cladosporioides	0	0	11 a	11 a	11 a
C. rosea	11 a	15 a	10 a	0	10 a
C. oryzae	21 a	23 a	17b	16c	14d
C. dematium	20a	22a	22a	18b	14c
M. phaseolina	0	0	10	0	0
P. herbarum	0	11a	0	10a	0
P. herbarum (isolate 21)	0	10	0	0	0
P. herbarum (isolate 62)	10b	11a	0	10b	11a
P. commune	18b	20a	16c	14d	16c
P. mangiferae	17b	17b	20a	14c	13d
Chloramphenicol[4]	40a	40a	40a	40a	25b

Note: [1]Values were subjected to Arcsine transformation before statistical analysis; Values (mean inhibition zone of three replicates) followed by the same letter(s) in a row are not significantly different (P=0.05) according to DMRT.
[2] Gram positive bacteria;
[3] Gram negative bacteria;
[4] *Phoma herbarum* (isolates 21and 62) are non-sporulating fungi.
[5]Chloramphenicol and crude extracts were used at 20 µg ml^{-1} (20 µl per well).

The phytochemical assay of crude extracts indicated that alkaloids were not detectable in the selected endophytic fungal cultures and grass species. The study also documented that all the selected endophytic fungal species produced triterpenoids and saponins; all the anamorphic and two teleomorphic isolates were tested positively for flavonoids. Glycosides were produced by three anamorphic isolates (*C. cladosporioides, M. phaseolina* and *P. mangiferae*), while tannins were produced by one each of anamorphic (*P. mangiferae*) and teleomorphic ascomycetes (*C. oryzae*). The *Phoma* species and *Pestalotiopsis mangiferae* were the only two species that produced phe-

nols and it was only the former that produced lignans. This suggested the occurrence of variability in endophytic fungi in producing secondary metabolites.

Table 4. Antifungal activity of solvent extracts of endophytic fungi isolated from *Chloris barbata* and *Eleusine indica.*

Crude extract of fungal isolates	Zone of inhibition (mm)[1] ± se	
	Candida albicans[2]	*Fusarium oxysporum*[3]
Ethyl acetate extract of culture filtrate		
C. rosea	11±0	0
C. oryzae	13±0.66	0
C. dematium	13±0	13±0.66
P. herbarum (NSF isolate 21)	11±0.57	0
P. herbarum (NSF isolate 62)	10±0.88	0
P. commune	10±0	0
P. mangiferae	10±0.33	10±0
T. harzianum	21±5.17	0
Methanol extract of mycelial mat		
C. rosea	14±0.33	0
C. oryzae	12±0.33	0
C. dematium	14±0.33	12±0.57
P. herbarum (NSF isolate 21)	12±0.33	0
P. herbarum (NSF isolate 62)	11±0.57	0
Control [4]	30±0.0 (flucanozole)	30±0.0 (bavistin)

Note: [1]Values are mean inhibition zone of three replicates (n=3); crude extracts were used at 20 µg ml^{-1} (20 µl per well).
[2]Clinical pathogen; [3]Plant pathogen.
[4]Crude extracts did not show antifungal activity to *T. harzianum*. Antifungal compound Mancozeb was used for *T. harzianum* (ZI 28 mm - data not shown)

Among the extractants, both methanol and ethyl acetate were commonly used in the extraction of metabolites with antimicrobial property (SHEKHAWAT & al. 2013, WANG & al. 2013). However, ethyl acetate extract of endophytic fungi showed high antibacterial rather than antifungal activities (BAGYALAKSHMI & al. 2012, PRABAVATHY & VALLI 2013). All the fungal isolates occurring in different aerial regions of grass species were initially screened for their antimicrobial activity against the selected bacterial and fungal test species. However only those with prominent activity to at least one test organism were selected for a detailed study. The ethyl acetate extract of most isolates showed prominent antibacterial activity to all the test bacteria (Table 3). Among them, the CF of *C. dematium* (18 mm), *P. herbarum* (15 mm) and *P. commune* (20 mm) and mycelial extract of *C. oryzae* (21 and 23 mm) and *C. dematium* (20 and 22 mm) showed good activity to *S. aureus* and *E. faecalis.* Moderately high antibacterial activity (>20 mm ZI) was expressed by the methanol extract of *C. oryzae* to *E. faecalis.* The mycelial extract of *C. dematium* also possessed moderate activity to *S. enterica*. This supported the findings of SURENDRA & al. (2012) who reported that *C. dematium* metabolite possessed strong antibacterial effect. While methanol extract of *P. commune* produced moderate activity to *E. faecalis* that of *P. mangiferae* showed similar degree of activity to *S. enterica.* However, moderate antibacterial activity (14–

20 mm ZI) was shown by CF of *C. dematium* and *P. commune,* whereas mycelial extract of *P. commune* and *P. mangiferae* showed activity to all the test bacteria. KAMALRAJ & al. (2013) in their work identified a novel antibacterial and antifungal phenol derivative isolated from an endophytic *P. mangiferae*, effective against human pathogens like *Bacillus subtilis, Klebsiella pneumoniae, Candida albicans, Escherchia coli, Micrococcus luteus* and *Pseudomonas aeruginosa.* In the present study, *Penicillium commune* was also antibacterial to *P. fluorescens,* almost similar to that of control (25 mm).

Table 5. Antioxidant activity expressed by extracts of certain endophytic fungal isolates and grasses *Chloris barbata* and *Eleusine indica.*

Crude extract of fungal isolates/grass species	Radical scavenging activity (%) ± standard error
Ethyl acetate extract of CF	
Cladosporium cladosporioides	68.77 ± 0.0178
Penicillium commune	70.69 ± 0.0210
Phoma herbarum (NSF isolate 21)	75.65 ± 0.0106
P. herbarum (NSF isolate 62)	76.45 ± 0.0107
Methanol extract of MM	
P. herbarum (NSF isolate 21)	72.73 ± 0.0093
P. herbarum (NSF isolate 62)	70.34 ± 0.0078
Grass species	
Chloris barbata	41.56 ± 0.0530
Eleusine indica.	61.18 ± 0.0294
Standard (Ascorbic acid)	78.40 ± 0.0103

Note: The radical scavenging activity of the crude extract was determined by DPPH method; The reaction mixture received 30 µl of crude extract (30 µg ml^{-1}).

Eight isolates out of 10 endophytic fungi expressed low (< 15 mm ZI) to moderate (< 20 mm ZI) antifungal activity in their ethyl acetate and the methanol fractions (Table 4). The CF of eight fungal species and mycelial extract of five fungal species produced low to moderate ZI to *C. albicans*. Several reports are available on the antifungal activity of endophytic fungal extracts to *C. albicans* (JIRAYU & al. 2011, POWTHONG & al. 2012) as compared to a few phytopathogenic fungi (TEJESVI & al. 2007, CAMILA & al. 2011). The CF of *T. harzianum* produced moderate antifungal activity to *C. albicans* in comparison to control (flucanozole); metabolites of other selected fungi were less effective. Similarly, the MM extract of *T. harzianum* had moderate activity (21 mm) to the above pathogen. In comparison to the above, *C. dematium* and *P. mangiferae* metabolites affected the colony growth of *F. oxysporum*. On the other hand, the CF of *C. dematium* and *P. mangiferae* and MM of *C. dematium* showed antifungal activity to *A. flavus*. TEJESVI & al. (2008) reported that *Pestalotiopsis* expressed >75% colony inhibition to *Bacillus subtilis*, *E. coli, P. fluorescens* and *S. aureus*. In the present study, *P. mangiferae* mycelial extract had moderate activity to *S. enterica* and *E. faecalis* and *S. aureus,* as well.

Although the chemical compounds responsible for antimicrobial property of endophyte extracts are not known, the role of the presence of flavonoids, triterpenoids, glycosides and saponins individually and in combination cannot be ruled out. Purification and characterization of crude extracts could help in further understanding of their functionality. The methanol extracts of *C. barbata* and *E. indica* that contained phenolic

and flavonoid compounds failed to exhibit antibacterial or antifungal activity even at 20 μg ml^{-1} of the crude extract. This might suggest that the antimicrobial compounds could have been produced by certain endophytic fungal species in association and not by the grass species alone. The lack of antimicrobial activity in grass extracts could also be due to the very low level of antimicrobial compounds present in the sample of crude extract taken for experimentation.

The CF extract of *P. herbarum* (isolate 62) (76.45 %) showed radical scavenging activity (Table 5) nearly similar to that of the standard ascorbic acid (78.40%). This is followed by CF extract of *P. herbarum* (isolate 21) (75.65 %). The two grass species showed activity very less than the standard. The above grass species have proven utility in traditional systems of medicine (ADEL & al. 2011).

Conclusion: The present study indicated that fungal endophytes of certain *Poaceae* species produced metabolites with antibacterial, antifungal and antioxidant properties.

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