



Influence of seasons on endophytic fungal assemblage in *Alloteropsis cimicina* (L.) Stapf. and *Heteropogon contortus* (L.) P. Beauv. of the sub-family panicoideae

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Abstract

Endophytic fungi occur symbiotically in grasses inhabiting diverse environmental and geographical conditions. Certain perennial grasses are studied for the associated endophytic fungi in shoot and root regions. However, endophytic fungal assemblages in aerial regions of grass species *Alloteropsis cimicina* and *Heteropogon contortus* of sub-family panicoideae in the Western Ghats of Karnataka is not documented. Aerial regions of above grass species were determined for endophytic fungal occurrence and diversity PDA, MEA and moist-blotter (MB) methods during rainy, winter and summer seasons for two years. Results of the study revealed the occurrence of 95 fungal species of 38 genera from *A. cimicina* and 76 species of 32 genera from *H. contortus*. These were grouped into 57 and 47 asexual ascomycetes and 29 and 22 sexual ascomycetes, respectively, in *A. cimicina* and *H. contortus*. Certain taxa were media-specific or common to all media tested. Endophytic fungal assemblage was more in rainy in case of *A. cimicina*, while it was more in winter season in case of *H. contortus*. More asexual ascomycetes occurred than sexual ascomycetes. Colonization frequency of fungal species was more in PDA than in other methods. Inflorescence, among aerial regions, harboured increased number of endophytic fungi. This work highlights the importance of incubation methods, plant regions and seasons in determining endophytic fungal assemblage in these two perennial grass species. Richness and distribution of endophytic fungi in plant regions, as well as in grass species, explains beta diversity of endophytic fungi in these perennial grass species.

Key words – Endophytic fungal diversity – isolation methods – panicoideae – seasonal variation

Introduction

The endophytic fungi, residing in tissues of the plant species without causing symptoms of the disease (Petri et al. 1993), exhibit mutualistic association and provide protection against abiotic and biotic stresses and also enhance the growth of plants (Clay 1987, Cheplick & Clay 1988, Backman & Sikora 2008). The fungal endophytes have been isolated from several host plants occurring in various environmental conditions (Mishra et al. 2012, Udayaprakash et al. 2018, Parmar et al. 2018, Pieterse et al. 2018). Hence they are studied for their ability to enhance the

fitness in crop plants (Rajamani et al. 2018, Rodriguez & Redman 2018). Further more, studies have also shown the well-known role of endophytic fungi in the production of secondary metabolites in medicinal plants (Jeewon et al. 2008, Gonda et al. 2010, Porrás-Alfaro & Bayaman 2011). There are few reports on the diversity and bioprospecting of fungal endophytes occurring in grasses of warm climates (Marquez et al. 2007).

Previous reports documented the occurrence of fungal species in the shoot and root regions of certain grass species of sub-family chloridoideae (Shivanna & Vasanthakumari 2011, Rekha & Shivanna 2014). Certain candidate fungal isolates from root and aerial regions of the above grasses have been identified with antimicrobial and antioxidant (Rekha & Shivanna 2014), biocontrol activities and plant growth (Vasanthakumari & Shivanna 2013), as well. There are no studies on the diversity of endophytic fungal communities occurring in the above-ground parts of *A. cymicina* and *H. contortus* of panicoideae growing in Bhadra Wildlife Sanctuary of Karnataka, India. This study is aimed at documentation, characterization and diversity of endophytic fungal species of perennial grasses – *A. cymicina* and *H. contortus* of sub-family panicoideae growing in Lakkavalli region of Bhadra Wildlife Sanctuary in Karnataka, India. The above grass species are important since they are used as ethnomedicine by folk communities (Katewa et al. 2001, Vardhana 2008, Vasanthakumari et al. 2010, Quattrocchi 2016) and they form a good perennial source of fodder for animals in wild.

Materials and methods

Selection of study site and grass species

The Lakkavalli forest region of Bhadra Wildlife Sanctuary, Karnataka, India was selected for the study as it supported abundant species of perennial grasses that also included *Alloteropsis cymicina* and *Heteropogon contortus* of sub-family panicoideae. Three study sites were selected in the sanctuary region (Table 1). Samples were drawn at an interval of 30 days during rainy (June–Sep), winter (Oct–Jan) and summer (Feb–May) seasons, for two years (2016–2017 and 2017–2018).

Table 1 Latitude and longitude details of study sites

Co-ordinates	<i>A. cymicina</i>			<i>H. contortus</i>		
	Location 1	Location 2	Location 3	Location 1	Location 2	Location 3
Latitude	13°72'76.876"	13°73'32.768"	13°71'40.12"	13°71'37.76"	13°73'49.926"	13°73'31.355"
Longitude	75°62'62.608"	75°63'23.196"	75°63'04.001"	75°62'97.309"	75°63'33.476"	75°62'33.862"

Isolation and characterization of endophytic fungi

Apparently healthy plant samples (inflorescence, culm and leaf) were collected in sterilized polypropylene covers contained in cool boxes and brought to the laboratory. Samples were washed in running tap water and then in sterile distilled water and surface-disinfected successively in hydrogen peroxide (3%, 3 min.), ethanol (70%, 3 min.) and in sodium hypochlorite (2%, 3 min.) (Shivanna & Vasanthakumari 2011) and fragmented (1-cm-length). The effectiveness of surface-disinfection was confirmed (Schulz et al. 1998). Fragments of samples were aseptically inoculated and incubated on potato dextrose agar, malt extract agar (PDA and MEA, Himedia Laboratories, Mumbai) amended previously with chloramphenicol (100 mgL⁻¹)/ciprofloxin (500 mgL⁻¹) (Rekha & Shivanna 2014) or moist blotters (Shivanna et al. 2013) under 12/12h light/nUV light (350-400 nm) regime at 23±2°C for 5 to 7 days (Achar & Shivanna 2013) and observed for the occurrence of endophytic fungi. The fungal species that expressed on the incubated segments were identified based on the morphological characteristics (Barnett 1972, Ellis 1976, Sutton 1980, Arx 1981). The hyphae of the fungal species were also tested microscopically for septation, clamp-connection or croziers. The anamorphic fungal endophytes as well as those failing to produce any reproductive propagule were cultured on water agar or autoclaved grass leaf segments on moist blotters and

incubated, as described previously. The endophytic fungi that failed to sporulate were considered as morpho-types. The species nomenclature of fungal isolates was confirmed by visiting Index Fungorum (www.indexfungorum.org). The DNA was extracted from the mycelial mat of fungal endophytes by CTAB method (Wu et al. 2001). Amplification of the isolated DNA was carried out using the Eppendorf Nexus gradient PCR machine and it was carried out with the 25 µl reaction mixture containing 2.5 µl of 10x PCR buffer, 16.8 µl of PCR H₂O, 1 µl of 200 m MNTTP, 0.2 µl of *Taq* polymerase, 1 µl of each primer (ITS1 and ITS4) and 2.5 µl fungal DNA (González-Teuber et al. 2017). The sequence data were submitted to NCBI Gene bank.

Further, the study was taken up to provide a comparative account of the diversity of endophytic fungal assemblages in the aerial regions in this study with those in the root and rhizosphere fungal species of the same grass species documented in the previous study (Vasanthakumari & Shivanna 2009).

Statistical analyses:

The data of two years (replicate trails) were determined for homogeneity by ANOVA (*A. cimicina* 0.051 and *H. Contortus* 0.005). The colonization frequency and relative abundance of endophytic fungi were determined (Suryanarayanan et al. 2000). The data of endophytic fungal occurrence were subjected to Simpson and Shannon diversity and evenness indices, and species richness with a bootstrap of 9999 at 95% confidence interval. Other statistical analyses employed were rarefaction indices (95% confidence interval), principal component analysis (PCA) correlation and phylogenetic analysis (Neighbour-joining method) (PAST ver. 2.17; MEGA 7.0, Friedrich et al. 2005).

Results

Endophytic fungal diversity

A total of 4,293 isolates of 95 endophytic fungal species of 38 genera of 22 families along with seven isolates of uncertain placement (*Incertae sedis*) were isolated by three incubation methods from 8,100 segments of *A. cimicina*. The total colonization frequency of endophytic fungi in *A. cimicina* was 53.3%. The documented fungal isolates were grouped into 57 asexual ascomycetes of 28 genera, 29 sexual ascomycetes of 10 genera and 9 morphotypes (Table 2). Among 95 fungal isolates, 53 that were common to all three incubation methods tested belonged to 27 genera of 11 families. The species of fungi and their families in each of the isolation methods are detailed in table 3. The endophytic fungal incidence in *A. cimicina* was high on PDA (1811) followed by MEA (1523) and MB (958) (Fig. 1).

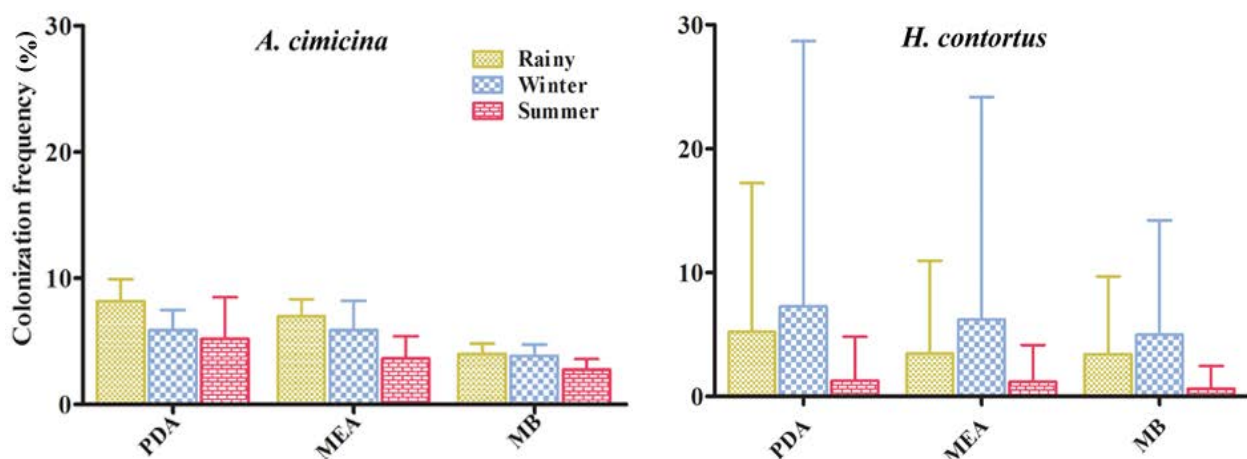


Fig. 1 – The colonization frequency of endophytic fungal assemblages in the aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus* by three incubation methods in three seasons.

Table 2 The occurrence of endophytic fungal species in the aerial regions of *Alloteropsis cimicina* by potato dextrose agar (PDA), malt extract agar (MEA) and moist blotter (MB) methods.

Fungal species	Frequency of occurrence (%) ¹		Incubation methods ²
	PDA	MEA	MB
Anamorphic ascomycetes			
<i>Acremonium</i> sp.	0.95	0.48	0.75
<i>Acrophialophora fusispora</i>	0.15	0.18	0.41
<i>Alternaria</i> spp. ³	0.27	1.41(2) ⁴	0.58(2)
<i>Aspergillus</i> spp. ⁵	9.44(9)	5.98(7)	2.46(8)
<i>Cladosporium</i> spp. ⁶	5.17 (2)	4.28 (2)	0.62
<i>Colletotrichum</i> spp. ⁷	0.43	0.95(3)	1.18 (3)
<i>Exserohilum</i> spp. ⁸	0.07(2)	0.10(2)	0.02
<i>Fusarium</i> spp. ⁹	3.63(5)	4.32(5)	3.34(4)
<i>Gliocladium roseum</i>	1.95	0.75	1.30
<i>Memnoniella echinata</i>	1.90	0.02	0.31
<i>Myrothecium roridum</i>	0.68	1.32	1.93
<i>Penicillium</i> spp. ¹⁰	10.35(11)	11.08(9)	3.26(5)
<i>Pestalotiopsis guepinii</i>	0.07	0.18	0.10
<i>Phoma</i> spp. ¹¹	0.60	0.40	2.39(3)
<i>Purpureocillium lilacinum</i>	0.18	0.33	0
<i>Pyricularia</i> sp.	0.07	0.05	0.31
<i>Stachybotrys chartarum</i>	0.71	0.45	0.26
<i>Trichoderma</i> spp. ¹²	3.67(2)	1.62(2)	0.43
<i>Verticillium</i> sp.	1.01	1.04	1.05
Total frequency	41.66(50)	36.26(49)	22.02(44)
Teleomorphic ascomycetes			
<i>Chaetomium</i> spp. ¹³	3.62(6)	2.79(5)	9.41(6)
<i>Cochliobolus</i> spp. ¹⁴	17.48(10)	19.07(9)	16.09(9)
<i>Dictyodopsis berberidis</i>	0.26	0.63	0
<i>Didymosphaeria spinosa</i>	0.24	0.24	0
<i>Helminthosporium</i> spp. ¹⁵	2.57(2)	4.15(2)	3.84(2)
<i>Khuskia oryzae</i>	2.80	2.81	0.26
<i>Spegazzinia lobulata</i>	0.34	0.52	1.18
<i>Thielavia sepedonium</i>	4.03	2.77	4.31
Total frequency	31.58 (24)	33.28 (22)	35.12(20)
Morphotypes¹⁶	45.03 (9)	27.97(7)	5.17(5)

Note: ¹Colonization frequency of fungal endophyte occurrence was calculated using formula; ²Data is an average over three replicates (average over seasons, locations and aerial regions), each with 75 samples; ³*Alternaria* spp.: *A. alternata* (0.16,0.81,0.30), *A. tenuissima* (0.01,0.03); ⁴Figure in parenthesis indicate total number of species of genera which may vary in different media; ⁵*Aspergillus* spp.: *A. aculeatus* (2.11,1.39,0.25), *A. candidus* (0.18,0.08,0.17), *A. duricaulis* (0.07,0.02), *A. flavus* (0.58,0.83,0.20), *A. fumigatus* (0.13,0.05,0.23), *A. nidulans* (0.09,0.01), *A. niger* (2.21,1.01,0.41), *A. ochraceus* (0.08,0.10,0.12), *A. versicolor* (0.01); ⁶*Cladosporium* spp.: *C. cladosporioides* (2.73,2.37,0.36), *C. herbarum* (0.28,0.12), *C. oxysporum* (1.15,0.07,0.03); ⁷*Colletotrichum* spp.: *C. australe* (0.28,0.10), *C. boninense* (0.10,0.18), *C. graminicola* (0.25,0.17,0.39); ⁸*Exserohilum* spp.: *E. rostratum* (0.03,0.01,0.01), *E. turcicum* (0.01,0.04); ⁹*Fusarium* species: *F. chlamydosporum* (0.51,0.24,0.74), *F. equiseti* (0.02,0.48,0.02), *F. moniliforme* (0.04,0.03,0.01), *F. oxysporum* (1.49,1.54,1.16), *F. pallidoroseum* (0.04,0.20); ¹⁰*Penicillium* species: *P. citrinum* (0.50,0.35,1.18), *Penicillium* sp. (1) (4.73,5.27,0.62), *Penicillium* sp. (2) (0.25,0.50,0.01), *Penicillium* sp. (3) (0.03,0.19,0.05), *Penicillium* sp. (D) (0.003,0.01), *Penicillium* sp. (GL) (0.01,0.01), *Penicillium* sp. (GY) (0.01), *Penicillium* sp. (LG) (0.79,0.02,0.63),

Penicillium sp. (LM) (0.03,0.02), *Penicillium* sp. (O) (0.01), *Penicillium* sp. (R) (0.27,0.01), *Penicillium* sp. (SS) (0.17,0.06,0.01); ¹¹*Phoma* spp.: *P. enigma* (0.35,0.23,0.35), *P. longicolla* (0.15), *Phoma* sp. (0.88); ¹²*Trichoderma* species: *T. harzianum* (0.93,0.54), *T. viride* (1.21,0.40,0.25); ¹³*Chaetomium* species: *C. cupreum* (0.14,0.11,0.28), *C. globosum* (0.24,0.12,0.16), *C. indicum* (0.04), *C. reflexum* (0.02,0.03,1.06), *C. robustum* (0.30,0.37,0.13), *C. spirochaete* (0.09), *C. tenue* (0.09,0.01,0.46); ¹⁴*Cochliobolus* species: *C. affinis* (0.35,0.46,0.39), *C. clavata* (0.04,1.38), *C. eragrostidis* (0.008,0.06,0.008), *C. fallax* (1.44,0.31,1.41), *C. geniculatus* (0.43,0.60,0.11), *C. harveyi* (0.09,0.33), *C. lunata* (0.56,1.08,0.91), *C. ovoidea* (0.01), *C. pallescens* (1.07,0.44,0.56), *C. spicifer* (0.11,0.031,0.02), *C. trifolii* (0.06), *C. tubercula* (0.01); ¹⁵*Helminthosporium* spp.: *H. halodes* (0.38,0.18,0.70), *H. maydis* (0.21,0.79,0.20); ¹⁶Morphotypes: Non sporulating fungi: NSF (Black) (0.03,1.29), NSF (Brown) (0.01,0.01), NSF (Grey) (0.98,0.27,0.02), NSF (Orange) (0.23,0.04,0.01), NSF (Pink) (0.16,0.05,0.21), NSF (Thick milky white) (0.03,0.02), NSF (White) (1.77,1.40,0.92), NSF (Whitish pink) (0.03,0.01), NSF (Yellow) (7.21,4.69), NSF (Yellowish orange) (0.01). Species of *Arthrographis kalrae*, *Cylindrocladium* sp., *Diaporthe* sp., *Dinemasporium* sp., *Ophiostoma denticulatum*, *Pithomyces* sp., *Pseudonectria* sp., *Septofusidium* sp., *Stilbella* sp. and *Graphium* sp. occurred below 0.3%.

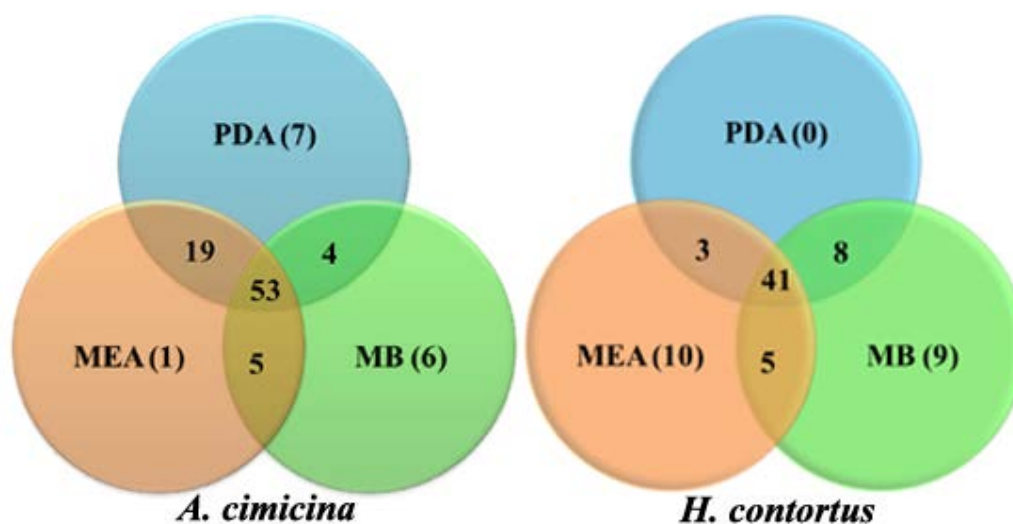


Fig. 2 – Effect of isolation methods (PDA – Potato dextrose agar; MEA – Malt extract agar; MB – Moist blotter method) on the occurrence of endophytic fungi in *Alloteropsis cimicina* and *Heteropogon contortus*.

Some of the fungal species with high incidence on PDA are *Cladosporium cladosporioides* (117.4) and *Penicillium* sp. (203.2) followed by *Aspergillus aculeatus* (90), *A. niger* (95) and *Cochliobolus fallax* (62.2) and those on malt extract agar are *Alternaria alternata* (34.9), *Cochliobolus clavata* (59.6), *C. lunatus* (46.5), *Helminthosporium maydis* (34.2) and *Penicillium* sp. strain 1 (200.2). On the other hand, high incidence of *Chaetomium reflexum* (45.9), *C. fallax* (60.7), *Colletotrichum graminicola* (16.9), *Fusarium chlamydosporum* (32), *Myrothecium roridum* (48.5), *Penicillium citrinum* (50.9), *Phoma* sp. (37.9) and *Thielavia sepedonium* (43.7) was documented on MB. The occurrence of morphotypes was high on PDA as compared to that on MEA or MB (Table 2). Certain fungal isolates expressed exclusively on PDA, MEA or MB method. On the other hand, certain other isolates expressed in more than one method (Fig. 2). Examples of certain species that expressed in all three methods included *Alternaria*, *Aspergillus*, *Chaetomium*, *Penicillium* and *Cochliobolus*. However, fungal species like *Aspergillus versicolor*, *Chaetomium indicum*, *Cochliobolus ovoidea*, *C. trifolii* and *Penicillium* sp. (2 strains) and a morphotype isolate, occurred exclusively in PDA, while a species of *Monographella* sp. occurred exclusively on MEA. On the other hand, *Chaetomium spirochaete*, *Cochliobolus tubercula* and species of *Phoma*, *Pseudonectria* and *Stilbella* occurred only on MB.

In case of *H. contortus*, the incubation of plant segments (6,075) by three isolation methods yielded 2,589 endophytic fungal isolates belonging to 76 species of 32 genera of 17 families and 5 isolates of uncertain placement. Fungal isolates were clustered into 47 anamorphic ascomycetes of 28 genera, 22 teleomorphic ascomycetes of 6 genera and seven morphotype isolates (Table 3 and 4). The colonization frequency of fungal isolates in *H. contortus* was 42.7%. The abundance of anamorphic and teleomorphic ascomycetes and morphotypes were 60, 29 and 10%, respectively. The endophytic fungal incidence in *H. contortus* was high on PDA (1,064) followed by MEA (828) and MB (696) (Fig. 1). For example, *Aspergillus aculeatus* (45.6), *A. niger* (66.9), *C. cladosporioides* (142.5), *Penicillium* sp. (215.23) and certain morphotypes (89.1) occurred better on PDA than MEA and MB. On the other hand, *Alternaria longipes* (12.4), *Aspergillus flavus* (18.9), *Chaetomium cupreum* (60.9), *C. clavata* (121.4), *T. sepedonium* (169.6) and *Verticillium* sp. occurred increasingly on MEA than on PDA and MB. However, *Acremonium* sp. (24.9), *A. niger* (35.9), *C. affinis* (28.9), *Gliocladium roseum* (24.5) and *Penicillium* sp. (107.9) expressed highly on MB. In the present study, morphotypes expressed very well on PDA followed by MEA and MB (Table 3). In this study, 41 fungal endophytes were detected by all three isolation methods, while 10 and 9 were specific to MEA and MB (Fig. 2), respectively. The species specifically occurring on PDA included *Colletotrichum boninen* and on MEA includes *Chaetomium indicum*, *Chaetomium reflexum*, *Fusarium moniliforme* and *Gnomonia comari* and certain other species like *Chaetomium tenue*, *Ophiostoma denticulatum*, *Septofusidium* sp. and *Tetraplophaeria* sp. exclusively on MB.

Table 3 Family-wise species diversity of endophytic fungi in aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus*.

	Species	Genera	Family	Morphotypes
<i>A. cimicina</i>				
PDA	83	34	22	9
MEA	78	27	20	7
MB	72	32	18	5
DNA sequence	9	8	7	0
<i>H. contortus</i>				
PDA	61	23	13	7
MEA	50	19	11	5
MB	61	30	15	3
DNA sequence*	4	4	4	0

Note: Endophytic fungal species and their associated families as influenced by the host plants – *A. cimicina* and *H. contortus* and isolation methods (PDA – potato dextrose agar, MEA- malt extract agar and MB – moist blotter); *DNA sequencing done for confirming the identification of some of the fungal strains not distinguishable by their morphological characteristics.

Table 4 Colonization frequency of endophytic fungal species occurring in the aerial regions of *Heteropogon contortus* by potato dextrose agar (PDA), malt extract agar (MEA) and moist blotter (MB) methods.

Fungal species	Frequency of occurrence (%) ¹		Incubation methods ²
	PDA	MEA	MB
Anamorphic ascomycetes			
<i>Acremonium</i> sp.	0.03	0.24	1.66
<i>Acrophialophora fusispora</i>	0.04	0.0	0.7
<i>Alternaria</i> spp. ³	0.38(2) ⁴	1.59(4)	1.11(4)
<i>Aspergillus</i> spp. ⁵	9.76(7)	2.33(6)	4.19(7)
<i>Cladosporium</i> spp. ⁶	12.16(3)	1.26(3)	1.70(3)

Table 4 Continued.

Fungal species	Frequency of occurrence (%) ¹		Incubation methods ²
	PDA	MEA	MB
<i>Colletotrichum</i> spp. ⁷	0	1.69(2)	0.26
<i>Fusarium</i> spp. ⁹	0.79(3)	0.48	0.39(2)
<i>Gliocladium roseum</i>	1.48	0.13	1.62
<i>Memnoniella echinata</i>	0	0	0.2
<i>Myrothecium roridum</i>	0.45	0.05	1.38
<i>Nigrosabulum globosum</i>	0	0	1.29
<i>Penicillium</i> spp. ¹⁰	0	0	10.10(3)
<i>Pestalotiopsis guepinii</i>	0.11	0.36	0.08
<i>Phoma</i> spp. ¹¹	0.35	1.59	0.43
<i>Pseudonectria</i> sp.	0.16	0	0.01
<i>Pyricularia</i> sp.	0.04	0	0
<i>Stachybotrys chartarum</i>	0.0	2.45	1.5
<i>Trichoderma</i> spp. ¹²	1.73(2)	1.08	0.93(2)
<i>Verticillium</i> sp.	0.33	2.52	0.71
Total frequency	49.31(36)	20.92(30)	29.73(40)
Teleomorphic ascomycetes			
<i>Chaetomium</i> spp. ¹³	3.27(5)	7.62(3)	3.52(4)
<i>Cochliobolus</i> spp. ¹⁴	12.72(9)	25.74(9)	16.13(9)
<i>Helminthosporium</i> spp. ¹⁵	0.67(2)	0.12	1.01(2)
<i>Khuskia oryzae</i>	3.74	0.51	1.57
<i>Thielavia sepedonium</i>	0.90	19.56	2.69
Total frequency	21.32(18)	53.57(15)	25.09(18)
Morphotypes¹⁶	13.72(7)	4.93(5)	3.15(3)

Note: ¹Colonization frequency of fungal endophyte occurrence was calculated using formula; ²Data is an average over three replicates (average over seasons, locations and aerial regions), each with 75 samples; ³*Alternaria* spp.: *A. longipes* (0.06,0.47), *A. alternata* (0.16,0.23,0.51), *A. solani* (0.09,0.01), *A. tenuissima* (0.11,0.11); ⁴Figure in parenthesis indicate total number of species of genera which may vary in different media; ⁵*Aspergillus* spp.: *A. aculeatus* (2.11,1.39,0.25), *A. candidus* (0.07,0.18), *A. duricaulis* (0.07,0.02), *A. flavus* (0.14,0.73,0.15), *A. fumigatus* (0.45,0.01,0.23), *A. nidulans* (0.02, 0.19,0.05), *A. niger* (2.58,0.34,1.38), *A. ochraceus* (0.63,0.05,0.10), *A. versicolor* (0.01); ⁶*Cladosporium* spp.: *C. cladosporioides* (5.50,0.54,0.92), *C. herbarum* (0.40,0.11,0.02), *C. oxysporum* (1.15,0.07,0.03); ⁷*Colletotrichum* species: *C. australe* (0.28,0.10), *C. boninense* (0.52), *C. graminicola* (0.46,0.15); ⁸*Exserohilum* species: *E. rostratum* (0.03,0.01,0.01), *E. turcicum* (0.01,0.04); ⁹*Fusarium* spp.: *F. chlamydosporum* (0.07,0.28,0.03), *F. equiseti* (0.02,0.48,0.02), *F. moniliforme* (0.02), *F. oxysporum* (0.36,0.19), *F. pallidoroseum* (0.04,0.20); ¹⁰*Penicillium* species: *P. citrinum* (0.18), *Penicillium* sp. (1) (1.54,1.29,0.35), *Penicillium* sp. (2) (1.52,0.11,0.45), *Penicillium* sp. (3) (0.08,0.57,0.07), *Penicillium* sp. (D) (0.003, 0.01), *Penicillium* sp. (GL) (0.01,0.01), *Penicillium* sp. (GY) (0.01), *Penicillium* sp. (LG) (0.79,0.02,0.63), *Penicillium* sp. (LM) (0.03,0.02), *Penicillium* sp. (O) (0.01), *Penicillium* sp. (R) (0.27,0.01), *Penicillium* sp. (SS) (0.17,0.06,0.01); ¹¹*Phoma* species: *P. enigma* (0.20,0.92,0.25), *P. longicolla* (0.15), *Phoma* sp. (0.88); ¹²*Trichoderma* spp.: *T. harzianum* (0.95,0.63,0.48), *T. viride* (0.05,0.05); ¹³*Chaetomium* species: *C. cupreum* (2.35,0.02), *C. globosum* (0.41,0.04,0.61), *C. indicum* (0.24), *C. reflexum* (0.07), *C. robustum* (0.31,0.15,0.47), *C. spirochaete* (0.03), *C. tenue* (0.05); ¹⁴*Cochliobolus* spp.: *C. affinis* (0.50,0.18,1.11), *C. clavata* (1.39,4.69,0.90), *C. eragrostidis* (0.05,0.10,0.14), *C. fallax* (0.95,0.17,0.75), *C. geniculatus* (0.21,1.10,0.19), *C. harveyi* (0.07,0.76,0.39), *C. lunata* (0.49,0.19,0.93), *C. ovoidea* (0.01), *C. pallescens* (0.40,1.23,0.82), *C. spicifer* (0.16,0.16,0.11), *C. trifolii* (0.06), *C. tubercula* (0.01); ¹⁵*Helminthosporium* spp.: *H. halodes* (0.19,0.04,0.28), *H. maydis* (0.02,0.05); ¹⁶Morphotypes: Non sporulating fungi: NSF

(Black) (0.03,1.29), NSF (Brown) (0.21,0.01), NSF (Grey) (1.05,0.03,0.37), NSF (Orange) (0.23,0.04,0.01), NSF (Pink) (0.20,0.02,0.02), NSF (Thick milky white) (0.03,0.02), NSF (White) (3.44,0.52,0.81), NSF (Whitish pink) (0.05), NSF (Yellow) (0.28), NSF (Yellowish orange) (0.01). Species of *Cylindrocladium* sp., *Diaporthe* sp., *Gnomonia comari*, *Memnoniella echinata*, *Ophiostoma denticulatum*, *Pithomyces* sp., *Pseudonectria* sp., *Septofusidium* sp. and *Tetraplophaeria* sp. occurred below 0.4%.

Certain most commonly occurring endophytic fungal isolates – nine species from *A. cimicina* were characterized by the ITS regions of rDNA. Based on the sequence information, they were characterized into 13 species of 11 genera (Table 5). Analyses of nucleotide sequence for their relatedness by the neighbour-joining method that the isolates could be grouped into distinct clades (Fig. 3).

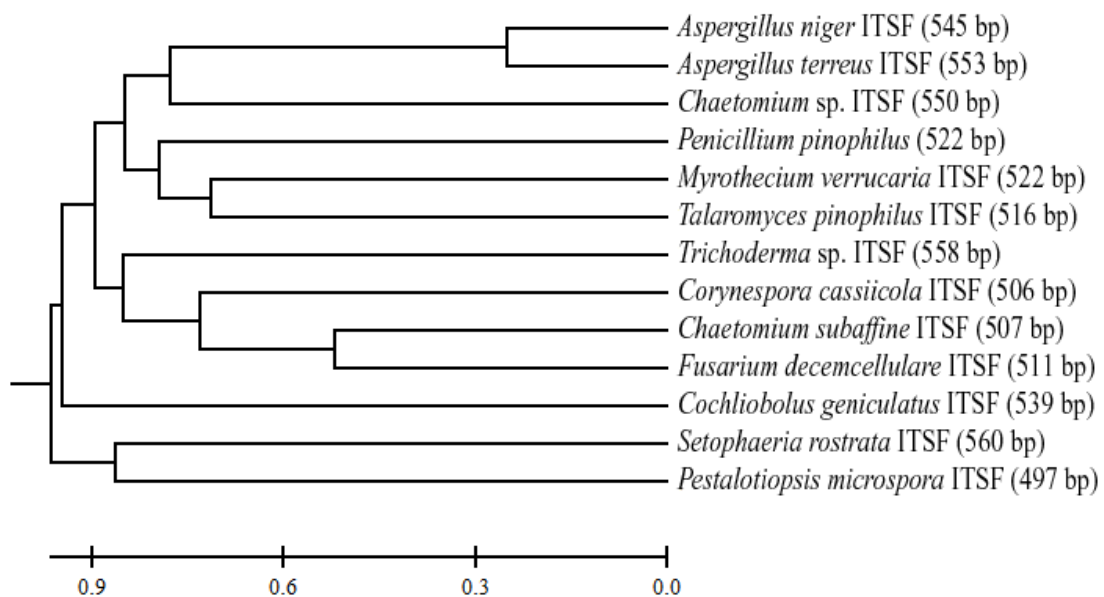


Fig. 3 – Phylogenetic tree based on neighbour-joining analysis of endophytic fungal species (ITS regions) occurring in the aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus*.

Influence of seasons on fungal endophytes

Seasons influenced the assemblage of fungal endophytes in *A. cimicina* and *H. contortus*. The rainy season supported the expression of a large number of endophytic fungal isolates (1782) followed by winter (1443) and summer (1067) in case of *A. cimicina*. The present study showed that *Aspergillus niger*, *C. cladosporioides* and *Penicillium* sp. followed by *Aspergillus aculatus*, *Fusarium oxysporum* and *Thielavia sepedonium* dominated during the rainy season. However, the expression of endophytic fungi in case of *H. contortus* was high during winter (1404) followed by rainy (944) and summer seasons (240). Species fungi like *C. cladosporioides* (85.2), *Cochliobolus clavata* (86.6), *Penicillium* sp. (240.9), *T. sepedonium* (150.5) and *Verticillium* sp. (49.9) were isolated in high number during winter than in rainy and summer and certain endophytic fungal species like *Aspergillus*, *Cochliobolus* and *Penicillium* dominated during the summer season.

In both grass species, the increase in the number of endophytic fungal diversity is associated with an increase in the number of isolations from the aerial parts depending on the season is clearly shown by the rarefaction curve. The species richness of fungal endophytes was maximum in *A. cimicina* inflorescence during rainy and winter seasons. However, many endophytic isolates were documented in winter followed by summer in case of *H. contortus* (Fig. 4). The emergence of endophytic fungal species depended on the plant parts, season and the method of incubation

Table 6). The principal component axes drawn to correlate the endophytic fungal assemblage during different seasons explained 77% and 82% of the total variance in case of *A. cimicina* and *H. contortus*, respectively (Fig. 5). This indicated a clear correlation of endophytic fungal assemblage occurrence with three seasons. In case of *A. cimicina*, species of *Cladosporium* and *Penicillium* strongly correlated to all three seasons, while species of *Aspergillus* and *Fusarium* correlated positively to rainy and winter seasons, and species of *Myrothecium* and *Phoma* correlated positively to the winter and summer. In case of *H. contortus*, the incidence of *Cochliobolus* sp. was highly correlated to all seasons and certain species like *Cladosporium* and some morphotype isolates primarily correlated positively to rainy and winter seasons. Species of *Alternaria*, *Colletotrichum*, *Diaporthe* and *Helminthosporium*, on the other hand, correlated to winter and summer seasons (Fig. 5).

Table 5 Blast match of sequences of endophytic fungi from aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus* of the sub-family panicoideae.

Sl. No	Submission No.	Species	Host	Host region	Sequenced region	Query length/ covers	Identity	Gene bank accession No.
1	SUB5437170	<i>Aspergillus niger</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	545/100%	100%	MK775046
2	SUB5437181	<i>Aspergillus terreus</i>	<i>Alloteropsis cimicina</i>	Leaf	ITS	553/100%	100%	MK775114
3	SUB5437231	<i>Chaetomium</i> sp.	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	550/100%	99.6%	MK775138
4	SUB5437229	<i>Chaetomium subaffine</i>	<i>Heteropogon contortus</i>	Leaf	ITS	507/100%	100%	MK775138
5	SUB5437159	<i>Cochliobolus geniculatus</i>	<i>Heteropogon contortus</i>	Inflorescence	ITS	539/100%	100%	MK775037
6	SUB5437207	<i>Corynespora cassicola</i>	<i>Heteropogon contortus</i>	Inflorescence	ITS	560/100%	100%	MK775135
7	SUB5437186	<i>Fusarium decemcellulare</i>	<i>Heteropogon contortus</i>	Inflorescence	ITS	511/100%	99.8%	MK775115
8	SUB5285368	<i>Myrothecium verrucaria</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	522/100%	100%	MK592787
9	SUB5285586	<i>Penicillium pinophilus</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	522/100%	100%	MK592818
10	SUB5437106	<i>Pestalotiopsis microspora</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	497/100%	100%	MK775025
11	SUB5737174	<i>Setphaeria rostrata</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	560/100%	100%	MK775035
12	SUB5437072	<i>Talaromyces pinophilus</i>	<i>Alloteropsis cimicina</i>	Inflorescence	ITS	516/100%	100%	MK774806
13	SUB5436791	<i>Trichoderma</i> sp.	<i>Alloteropsis cimicina</i>	Leaf	ITS	558/100%	100%	MK774866

Endophytic fungal community in plant parts

The inflorescence, among the plant parts, hosted a large number of endophytic fungal isolates (701) followed by leaf (641) and culm (591) in *A. cimicina*. The species richness in different plant parts is detailed in table 7. Some of the endophytes with high incidence in the inflorescence are *Arthrographis kalrae* (58.2), *C. robustum* (108.1), *Fusarium chlamydosporum* (23.9), *T. sepedonium* (63.9) and two isolates of morphotypes (100.9). On the other hand, *Cochliobolus eragrostidis* (20.4), *Dictyodothis berberidis* (50.4) and species of *Graphium* (20.4) and *Penicillium* (210.7) and certain morphotypes (185.7) were in high number in the culm. Leaf region was also found to be associated with the high incidence of *Chaetomium robustum* (55.3), *C. affinis* (47.7), *Exserohilum rostratum* (31.7), *Penicillium* sp. (7.2) and certain morphotypes (225.5).

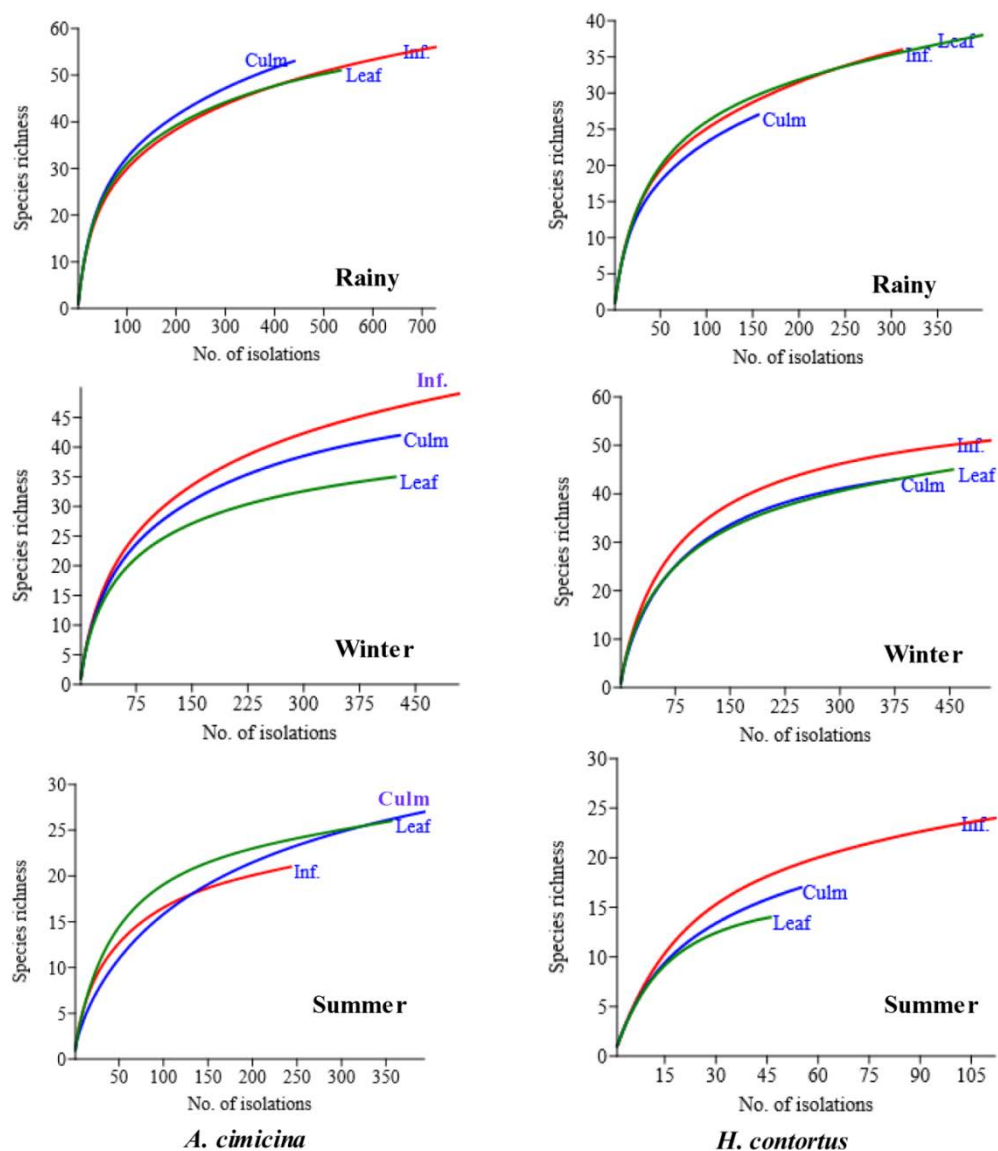


Fig. 4 – The depiction of endophytic fungal species occurring in the aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus* in three seasons by rarefaction curve.

Table 6 Diversity and evenness indices and colonization frequency of endophytic fungal communities occurring in the aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus* of the sub-family Panicoideae.

Grass species/sample units	Diversity index		Evenness index		Colonization frequency (%)
	Shannon (H')	Simpson (D')	Shannon (J')	Simpson (E')	
<i>Alloteropsis cimicina</i>					
PDA	0.94	3.36	0.21	0.04	42.19
MEA	0.94	3.43	0.22	0.04	35.48
MB	0.97	3.71	0.23	0.06	22.31
Rainy	0.96	3.57	0.21	0.04	41.5
Winter	0.95	3.44	0.23	0.06	33.62
Summer	0.88	2.87	0.23	0.06	24.86
Inflorescence	0.97	3.79	0.22	0.04	36.3
Culm	0.95	3.64	0.21	0.04	31.16
Leaf	0.95	3.70	0.21	0.04	32.52

Table 6 Continued.

Grass species/sample units	Diversity index		Evenness index		Colonization frequency (%)
	Shannon (H')	Simpson (D')	Shannon (J')	Simpson (E')	
<i>Heteropogon contortus</i>					
PDA	0.92	3.09	0.22	0.05	41.11
MEA	0.92	3.02	0.23	0.06	32.00
MB	0.95	3.55	0.23	0.06	26.9
Rainy	0.95	3.42	0.24	0.06	36.49
Winter	0.94	3.43	0.22	0.05	54.23
Summer	0.95	3.29	0.24	0.07	9.27
Inflorescence	0.95	3.47	0.22	0.05	38.89
Culm	0.93	3.32	0.23	0.05	24.13
Leaf	0.94	3.30	0.22	0.05	36.97

Note: ¹Data based on the values of three different locations and data are averaged over three replicates; 75 segments/locations/seasons.

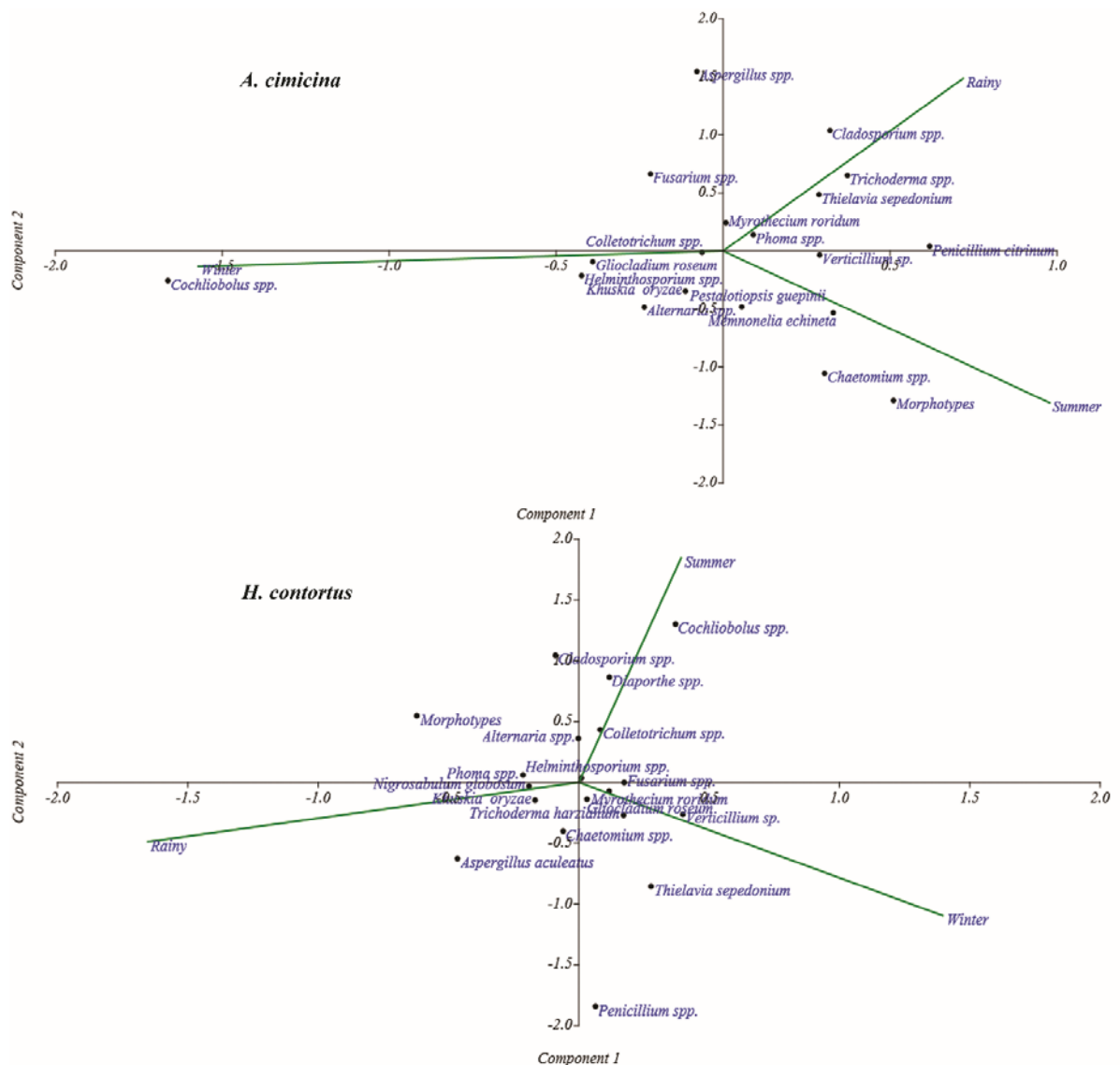


Fig. 5 – Beta diversity of dominating endophytic fungal species in the aerial regions of *Alloteropsis cimicina* and *Heteropogon contortus* in three seasons.

The endophytic fungal occurrence were also high in the inflorescence (1007) than in the leaf (957) and culm (624) in case of *H. contortus*. Inflorescence followed by foliages hosted high species diversity (Table 7). The foliages harbored endophytic fungi which was also commonly harbored in culm and inflorescence. Certain fungal species that were harbored exclusively in the leaf were *Diaporthe eres*, *D. podocarpi* and *Pyricularia* sp; similarly, the inflorescence also harbored specific fungal species like *Helminthosporium maydis*, *Trichoderma viride*, *Torula* sp. and a morphotype (strain 1). The culm was colonized by *Fusarium moniliforme*.

Table 7 Affinity of endophytic fungal species to different regions of *Alloteropsis cimicina* and *Heteropogon contortus*.

	<i>A. cimicina</i>	<i>H. contortus</i>
Inflorescence	88	70
Culm	88	63
Leaves	91	66
Root	24	26
Rhizosphere	23	26
Shoot	267	199
Shoot and root*	291	225
Shoot, root and rhizosphere*	314	251

Discussion

The ascomycetous fungi dominated the endophytic fungal assemblage followed by morphotypes, and among ascomycetes, the mitosporic ascomycetes occurred in high frequencies than the teleomorphic forms. This work documents a large number of endophytic fungal isolates of diverse species in these two perennial grass species, with some fungal species common to both grasses. The sequential washes of plant parts with disinfectants coupled with fungal growth expression from inner tissues upon prolonged incubation confirmed the effectiveness of surface disinfection. The endophytic fungal communities inhabiting the inflorescence, culm and leaves of *A. cimicina* and *H. contortus* were highly diverse. Grass species have been shown to be associated with high incidences of fungal species in their culm, leaf and root regions (Marquez et al. 2007, Kauhanen et al. 2006). The expression of endophytic fungi appear to depend on the isolation method and the plant part used. The PDA method always supported the maximum expression of fungal species than the other methods tested. Similar observations were also documented by previous works (Vasanthakumari & Shivanna 2009, Sun et al. 2013). The MEA, rather than the PDA method has also been recommended for good endophytic fungal expression (Rosa et al. 2009, Higgins et al. 2011, Kim et al. 2013). The fungal species are also expressed exclusively in specific isolation methods (Higgins et al. 2011, Kim et al. 2013, Tibpromma et al. 2018). This was attributed to either the supplementation of certain nutrients or induction of abiotic or biotic stress conditions on the growth and expression of fungal colonies (Vasanthakumari & Shivanna 2009, Shivanna & Vasanthakumari 2011, Tibpromma et al. 2018). Marquez et al. (2012) opine that most endophytic surveys depended on the fungal detection methods/techniques and in most reports fast-growing species are emphasized more than the slow-growing ones that are often excluded (Hyde & Soyong 2008). The direct isolation methods employed in the study could detect most cultivable fungi including the morphotype that failed to sporulate despite attempts of exposure to nUV light, nutrient stress and extended culture. Such of the isolates were characterized by an indirect method of detection involving the similarity of nucleotide sequences in rDNA. The taxonomic status of the morphotype isolates obtained in the endophytic survey help in understanding their association and interaction with plants.

It is interesting to note that seasons have a direct impact on the expression of fungal communities. As reported earlier (Kim et al. 2013, Singh et al. 2016), the rainy as well as the summer seasons favoured high expression of endophytic fungi (Schulthess et al. 1998, Higgins et

al. 2011). In this study, the rainy season probably helped in facilitating moderate temperature (Kim et al. 2013, Singh et al. 2016) which is favourable for fungal expression. On the other hand, dry spell resulted in nutrient stress thus suppressing the expression of certain fungal species. The seasonal fluctuation and isolation methods are also shown to contribute to the expression of fungal communities in plant parts (Singh et al. 2016). The rarefaction curve indicated that the quantification of fungal species depended on the seasonal fluctuation, and rainy and winter seasons contributed to the species richness besides their isolation frequencies.

The ascomycetous endophytic fungi are ubiquitous and occur dominantly in different plant parts. In this study, inflorescence followed by leaves and culms were colonized in high frequency by certain fungal isolates. Some of them appear to be organ-specific. Shoots have been shown to be associated with endophytic fungi in several plant species (Navratilova et al. 2018). Foliages among the aerial regions have been documented with high frequency of endophytic fungi (Lacerda et al. 2018). The high incidence of diverse endophytic fungi in the inflorescence of grass species is not documented in the literature. Although this part-specificity is not understood, the endophytic fungi are thought to have an affinity to colonize inflorescence for the sole purpose of nutrition. In contrast to this, certain endophytic fungi are documented more in the root than in the shoot (Marquez et al. 2010, Parmar et al. 2018). Earlier work in this laboratory demonstrated that certain fungal isolates in the root (Vasanthakumari & Shivanna 2009) was also documented in the shoot regions of *A. cymicina* and *H. contortus*. The present study also indicated that certain shoot endophytes of the above grass species were different from those associated with roots regions (Vasanthakumari & Shivanna 2009). The above observations suggested that certain endophytic fungal isolates could be associated with both roots as well as shoot regions and still others are localized either to the root or shoot regions. This further supported the fact that certain endophytic fungi have developed life history strategies to associate themselves with the entire plant system and offer several benefits to the host plant. Certain fungi from rhizosphere and rhizplane regions of grasses of sub-family chloridoideae were demonstrated to control anthracnose disease in chilli and sorghum crops in the green house and field (Vasanthakumari & Shivanna 2013). Endophytic fungal isolates from grasses of *Cynodon dactylon* and *Dactyloctenium aegyptium* were also shown to possess antimicrobial and antioxidant properties (Rekha & Shivanna 2014). The literature on the association of the same endophytic fungi in both root and shoot of the plant system is extremely limited (Marquez et al. 2010, Udayaprakash et al. 2018) and hence there is a necessity to study the life history strategies of such endophytic fungi in host plants (Luo et al. 2017, Gomes et al. 2018). The endophytic fungi associated with foliages settle in the soil and might lead a saprophytic mode of life (Unterseher et al. 2013, Guerreiro et al. 2018) via their ability to produce cellulases (Suryanarayanan et al. 2009). These fungal isolates might switch-over to the pathogenic mode to infect root hairs/tissues and once again gain entry into the root and subsequently into the shoot region. The PCA of endophytic fungal assemblages of *A. cymicina* and *H. contortus* indicated the correlation of endophytic fungal occurrence with seasons. Such observations were also made in case of endophytic fungi of *Tectona grandis* (Singh et al. 2016) and *Taxus globosa* (Rivera-Orduna et al. 2011).

In conclusion, the endophytic fungi occur in abundant diversity in the perennial grasses – *A. cymicina* and *H. contortus*. The anamorphic ascomycetes occurred in high frequency rather than the teleomorphic ones and morphotypes despite the employment of three isolation methods. Certain unidentifiable strains and morphotypes were characterized into ascomycetous forms. Certain fungal isolates were specific to the isolation technique and plant region, while others were common. Furthermore, the fungal isolates that were associated shoot regions were also common to roots of the above grass species. This suggested that certain endophytic fungi are common to the above-ground and below-ground regions of the grass species and requires a further detailed study of life history strategies of such endophytic fungi.

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