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ORIGINAL ARTICLE

Antimicrobial activity and metabolite profiling of endophytic fungi in *Digitaria bicornis* (Lam) Roem. and Schult. and *Paspalidium flavidum* (Retz.) A. Camus.

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Abstract
Endophytic fungal occurrences were studied in aerial regions of *Digitaria bicornis* and *Paspalidium flavidum* by three isolation methods: potato dextrose agar (PDA), malt extract agar (MEA), and moist blotters. Seventy species of 29 genera of endophytic fungi in *D. bicornis* and 71 species of 30 genera in *P. flavidum* were documented. Endophytic fungal communities were grouped into 40 and 43 anamorphic ascomycetes (21 and 23 genera) and 20 teleomorphic ascomycetes (6 and 7 genera) in *D. bicornis* and *P. flavidum,* respectively. PDA supported the expression of larger number of fungal communities than MEA and MB; and *P. flavidum* hosted more number of endophytic fungi than *D. bicornis*. Seasons played an important role in supporting the assemblage of fungal endophytes. Endophytic fungal species richness and assemblages in plant regions were determined for alpha, beta, and gamma diversities. The ethyl acetate followed by methanolic extracts of certain fungal species showed good antagonistic and antibacterial activities. Among fungal endophytes, *Curvularia protuberata* and *Penicillium citrinum* exhibited high antagonistic and antibacterial activities. The high-resolution orbitrap liquid chromatography–mass spectrometry of ethyl acetate crude extracts of *C. protuberata* and *P. citrinum* revealed the presence of antifungal and antimicrobial, besides a host of compounds in the extracts. The present study indicated that grass endophytes are the sources of compounds with antimicrobial and other pharmacological activities.

Keywords Grass endophytic fungal diversity · Antagonistic assay · Antibacterial assay · Bioactive compounds · OHR LC–MS

Introduction

Endophytic microbes are symbiotically associated with plants (Fisher and Petrini 1988). The fungal symbiotic association ranging from mutualism to antagonism has been well studied in the case of *Epichlöe*/*Neotyphodium* species endophytic in *Lolium*/*Festuca* grass species (Konig et al. 2018). These endophytic fungi colonize plants systemically and increase host plant fitness by resisting the colonization ability of pathogenic fungi (Schulthess and Faeth 1998) and producing metabolites against abiotic and biotic stresses in plants (Kuldau and Bacon 2008). Presently, research work on the endophytic fungi has been intensified by researchers; consequently, there are several reports on the distribution and diversity of fungal endophytes in a variety of host plant systems (Rodriguez et al. 2009; Marquez et al. 2010) and the ability of endophytic fungi in the production of bioactive metabolites (Suryanarayanan and Johnson 2014; Shweta et al. 2015). Fungal endophytes and their host plants are the hot-spots of secondary metabolites and are also the well-known sources for the production of antibacterial and antifungal compounds (Supaphon et al. 2014). A survey of literature revealed that both host plants and their endophytic fungi produce a variety of compounds that find application in agricultural, pharmaceutical, and other industries (Raviraja et al. 2006; Adam et al. 2009). Hence, the endophytic fungal and host metabolites are the preferred sources of novel pharmaceutical agents, specifically multidrug-resistant agents used in combination with antibiotics (Nisa et al. 2020).

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Previous work from this laboratory documented the occurrence of fungal species in the rhizosphere and root (below-ground) and shoot (above-ground) regions of panicoideae grasses (Vasanthakumari and Shivanna 2009; Nischitha and Shivanna 2020). A thorough review of the literature indicated that the assemblages of endophytic fungi in the above-ground regions and the diversity of bioactive metabolites of *D. bicornis* and *P. flavidum* were not studied. Hence, an attempt was made to document assemblages of the endophytic fungi occurring in the aerial regions of perennial grass species—*D. bicornis* and *P. flavidum* of subfamily panicoideae growing in Bhadra Wildlife Sanctuary of Karnataka, India, in different seasons by suitable incubation methods. Certain endophytic fungi were also investigated for antagonistic and antibacterial properties. Those fungal endophytes producing compounds with prominent antimicrobial activity were subjected to chemo-profiling by OHR LC–MS (Kour et al. 2008).

Materials and methods

Grass species and study site

Digitaria bicornis and *P. flavidum* growing in Bhadra Wildlife Sanctuary (BWS) of the Western Ghats region of Karnataka were characterized based on their morphological characteristics (Bhat and Nagendran 2001) and selected for the study. Three study sites 1: (13º73′ N—75º62′ E; 13º72′ N—75º62′ E, site 2: 13º73′ N—75º63′ E; 13º73′ N—75º63′ E, and site 3: 13º71′ N—75º65′ E; 13º71′ N—75º62′ E) located in the Lakkavalli forest region of BWS in Karnataka, India, with abundant growth of grasses were selected for the collection of samples (located at approximately 1.5 km away from each other). The sampling was drawn at an interval of 30 days in three seasons—rainy (Jun–Sep), winter (Oct–Jan), and summer (Feb–May), for 2 years (2016–2017 and 2017–2018).

Isolation and characterization of endophytic fungi

Samples (apparently healthy mature inflorescence, culm, and leaf) were collected in sterile polypropylene bags contained in cool boxes and then surface-disinfected (Shivanna and Vasanthakumari 2011) and fragmented (1-cm length). The effectiveness of the surface-disinfection regime was confirmed (Schulz et al. 1998). The surface-disinfected segments were placed aseptically on chloramphenicol (100 mg L^{-1}) amended potato dextrose agar or malt extract agar medium (PDA and MEA, HiMedia Laboratories, Mumbai) (Nischitha and Shivanna 2020) or moist blotters (Shivanna et al. 2013) and incubated under 12/12-h light/nUv light (350–400 nm) regime at 23 ± 2 °C for 5–7 days (Achar and Shivanna 2013). The incubated segments were observed for the occurrence of endophytic fungi that were identified based on the morphological characteristics detailed in the identification manuals (Barnett 1972; Seifert et al. 2011). Certain slow-growing fungal endophytes failing to produce any reproductive propagules were cultured on autoclaved grass leaf blades placed on moist blotters/water agar and incubated, as described previously. Those fungal isolates failing to sporulate upon incubation are designated as morphotypes. The species nomenclature of fungal isolates was confirmed by visiting *Index Fungorum* (www.indexfungorum.org).

Certain endophytic fungal species with high biological activity were subjected to molecular characterization by the method of Wu et al. (2001) using the internal transcribed spacer (ITS) regions of rDNA—ITS1: 5′-TCCGTAGGT GAACCTGCGC-3′ and ITS4: 5′- TCCTCCGCTTATTGA TATGC-3′. The fungal sequences were BLAST searched for the homology at the National Center for Biotechnology Information (NCBI) database and submitted to the Genbank to obtain the accession number.

Antagonism in vitro

The test bacterial isolates like *Staphylococcus aureus* (MTCC-902) and *Enterococcus faecalis* (MTCC-439)—Gram-positive, and *Escherichia coli* (MTCC-1559), *Salmonella enterica* (MTCC-738), *Salmonella typhi* (MTCC-734), *Xanthomonas campestris* (MTCC-2286), *Pseudomonas syringae* (MTCC-1604), *Klebsiella pneumoniae* (MTCC-7028), and *Pseudomonas aeruginosa* (MTCC-4734)—Gram-negative were obtained from the Institute of Microbial Technology (IMTECH, Chandigarh, India). The plant pathogenic fungi selected were *Alternaria alternata, Fusarium oxysporum,* and *Sclerotium rolfsii* (from field infected tomato, chickpea, and chilli plants, respectively). Selected fungal endophytes of *P. flavidum* were used to determine the in vitro antagonistic activity against the test bacterial and fungal strains by dual-plate culture technique (Talapatra et al. 2017). The plates co-cultured with fungal endophytes and test bacterial and fungal strains were incubated at 28 °C for 5–7 days. The inhibition (%) of colony culture growth was determined after 5 or 7 days (Talapatra et al. 2017).

Preparation of crude extract of endophytic fungi

The endophytic fungi from *D. bicornis* and *P. flavidum* (Table 5) were selected for obtaining the culture filtrate (CF) and mycelial mat (MM) fractions. The fungal isolates were initially cultured on PDA and incubated, as described previously (Nischitha and Shivanna 2020). The culture discs (5-mm-diameter) were obtained from the actively growing margin of colony culture on PDA and inoculated into 500 ml

Erlenmeyer flasks containing 300 ml PD broth (pH 5.6). The inoculated broth was incubated in dark at 21 ± 2 °C for 8–11 days under stationary conditions, with intermittent shaking (Nischitha et al. 2020). The culture broth was passed through three-layered muslin cloth followed by three-layered Whatman no.1 filter paper discs to separate MM from the CF. To the filtrate, an equal volume of ethyl acetate was added, mixed well for 10 min, and allowed to settle to obtain clear immiscible layers. The upper ethyl acetate layer-containing compounds were extracted thrice with the same solvent and pooled. The MM was dried in an oven (40 °C, for 24 h), and the dried mat was ground into a fine powder in a sterilized pestle and mortar. The powder was then transferred into a vial-containing methanol and shaken in a water bath at 40 °C for 3–4 h. and filtered with cheese cloth to obtain the filtrate. The ethyl acetate and methanol fractions were evaporated to dryness at ambient conditions using the rotary flash evaporator. The extract residues were dissolved separately in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored at 4 °C, to be used as a stock solution for determining the antibacterial activity (Nischitha et al. 2020).

Antibacterial assay in vitro

The fungal CF and MM extracts were screened for their antibacterial activity by the well-diffusion method (Nath 2012). The sterilized PDA or nutrient agar (NA) medium (20 ml) was poured into sterile Petri dishes and after solidification, 24-h-nutrient broth-grown test microbe cultures were swabbed on the respective agar plates using sterilized cotton swabs. Wells (5-mm-diameter) punched over the medium with a sterile cork borer were placed with 20 µl of the extract of CF or MM dissolved in DMSO (10 µg μl^{-1} and diluted to 100, 50, or 25%) and plates were incubated at 37 °C for 24 h. The experiment was arranged in a randomized complete block design and carried out in triplicate. Commercial antibiotic products like amoxicillin (Amozlin-250), chloramphenicol (Paraxin-250), or ciprofloxacin (Ciplox-250) were used (250 mg l^{-1}) as positive controls and DMSO as a negative control. The test bacterial strains used in the antagonism assay in vitro were also used in this experimentation.

High-resolution orbitrap LC–MS analysis

The metabolite fractions were subjected to orbitrap HR LC–MS analysis**.** The compound separation was achieved by 410 ProStar Binary LC with 500 mass spectrometry photodiode array detectors with methanol as the mobile phase. The compound separation was carried out at a flow rate of 6 ml min^{-2} for over 30 min. Compounds were detected using a UV detector at λ max of 290 nm. The direct infusion was done by both negative and positive ionization modes, and mass (m/z) ranging from 50 to 8000 amu was used (SAIF, IIT, Bombay).

Analyses of endophytic fungal diversity

Data of 2-year trials were subjected to homogeneity by analysis of variance (ANOVA; *D. bicornis*, $p = 0.040$ and *P. flavidum*, $p = 0.02$). Means of antagonistic and antibacterial experiments were compared by using Duncan's Multiple Range Tests (DMRT, $p \leq 0.05$) (Nischitha et al. 2020). The colonization frequency of endophytic fungi and their relative abundance was determined by the method of Suryanarayanan et al. (2000). Shannon and Simpson diversity and evenness indices of endophytic fungal species and their richness (bootstrap value of 9999 at 95% confidence interval) were calculated. Other statistical methods like the rarefaction index (95% confidence interval) and nonmetric multidimensional scaling (NMDS) correlation were also employed (PAST ver. 2.17, Hammer et al. 2001).

Results

The diverse endophytic fungal associations in the aerial regions of perennial grasses *D. bicornis* and *P. flavidum*, growing abundantly in Lakkavalli forest region of the Western Ghats, were established by the culture-based direct isolation approach. Since the homogeneity analysis of 2-year trails by ANOVA indicated non-significant ($p < 0.05$) variation in the incidence of endophytic fungi in the above grass species in two years, the data were clubbed and averaged and are presented in Tables 1, 2. A total of 3017 endophytic fungal isolates of 70 species of 29 genera belonging to 17 families along with three isolates of *incertae sedis* in *D. bicornis* and 4093 endophytic fungi of 71 species of 30 genera of 16 families and with three isolates under *incertae sedis* of *P. flavidum* were isolated from 8100 segments of each grass species. In the case of *D. bicornis*, the fungal communities were grouped into 40 anamorphic ascomycetes and 20 teleomorphic ascomycetes of 21 and 23 genera, respectively. In *P. flavidum,* endophytic fungi were grouped into 43 anamorphic ascomycetes and 20 teleomorphic ascomycetes of 23 and 6 genera, respectively. There were 10 and 7 morphotype forms in both the grasses species and only one zygomycete *Mycotypha microspora* was documented in *P. flavidum* (Tables 1, 2).

Incubation media and endophytic fungal communities

The endophytic fungal occurrence depended on the isolation method tested. The expression of endophytic fungi of *D. bicornis* was high (1326) on PDA followed by MEA (1047)

Table 1 The occurrence of anamorphic and teleomorphic ascomycetes and morphotype isolates endophytic in the aerial regions of *Digitaria bicornis* by potato dextrose agar (PDA), malt extract agar (MEA), and moist blotter (MB) methods

Fungal species	Colonization frequency (%)/incubation methods[a]		
	PDA	MEA	MB
Anamorphic ascomycetes			
Acrophialophora fusispora (S.B.Saksena) Samson	0.13	0.05	2.07
Alternaria spp.[b]	0.05 (2)[c]	0.23 (2)	0.22
Aspergillus spp.[d]	6.66 (5)	3.83 (5)	1.26 (5)
Cladosporium spp.[e]	3.15 (2)	2.40 (2)	0.19
Gliocladium roseum Bainier	0.45	0.90	0.62
Myrothecium roridum Tode	0.27	0.51	1.55
Penicillium spp.[f]	19.73 (8)	12.29 (3)	2.33 (2)
Phoma spp.[g]	0.02	0.37 (2)	0.99 (3)
Stachybotrys chartarum (Ehrenb.) S. Hughes	0	0	3.56
Trichoderma spp.[h]	0.88 (2)	0.91 (2)	0.09
Total frequency	33.12 (30)	23.00 (25)	14.76 (30)
Teleomorphic ascomycetes			
Chaetomium spp.[i]	1.06 (4)	0.73 (4)	0.42 (4)
Cochliobolus spp.[j]	4.16 (5)	7.14 (4)	4.93 (6)
Khuskia oryzae H.J. Huds	1.56	0.57	0.20
Total frequency	7.18 (13)	8.77 (11)	6.37 (13)
Morphotypes[k]	3.66 (10)	2.94 (8)	0.18 (5)

[a]Data are an average over three replicates (average over seasons, locations, and aerial reions), each with 75 samples

[b]*Alternaria alternata* (Fr.) Keissl. (0.01–0.21), *A. tenuissima* (Kunze) Wiltshire (0.01–0.03)

[c]Figure in parenthesis indicate total number of species vary in different media

[d]*Aspergillus aculeatus* Iizuka (0.37–1.80), *A. candidus* Link (0.01–0.02), *A. flavus* Link (0.15–0.45), *A. fumigatus* Fresen. (0.03–0.32), *A. nidulans* (Eidam) G. Winter (0.02), *A. niger* Sensu auct. (0.62–3.97), *A. ochraceus* G. Wilh. (0.04–0.08)

[e]*Cladosporium cladosporioides* (Fresen.) G.A. de Vries (0.19–2.53), *C. herbarum* (Pers.) Link (0.02–0.61)

[f]*Fusarium chlamydosporum* Wollenw. and Reinking (0.03–0.06), *F. oxysporum* Sensu Smith and Swingle (0.86–1.52). *Penicillium commune* Thom (0.56), *Penicillium citrinum* Thom (0.03–11.0)

[g]*Phoma enigma* (0.33–0.41), *P. longicolla* Aveskamp, Gruyter and Verkley (0.02), *Phoma* sp. (0.01–0.13)

[h]*Trichoderma harzianum* Rifai (0.06–0.24), *T. viride* Pers. (0.72–0.81)

[i]*Chaetomium cupreum* L.M. Ames (0.06), *C. globosum* Kunze (0.20–0.83), *C. indicum* Corda (0.03–0.15), *C. robustum* L.M. Ames (0.08–0.10), *C. spirochaete* Palliser (0.06), *C. tenue* X. Wei Wang, Crous and L. Lombard (0.06–0.09)

[j]*Cochliobolus affinis* Boedijn (1.15–1.74), *C. eragrostidis* (Tsuda and Ueyama) Sivan. (0.06–0.08), *C. protuberata* R.R. Nelson and Hodges (2.34–4.49), *C. geniculatus* (Tracy and Earle) Boedijn (0.06), *C. lunatus* (Wakker) Boedijn (0.31–0.76), *C. pallescens* Boedijn (0.08–0.20), *C. spicifer* R.R. Nelson (0.04–0.28)

[k]Morphotypes (0.21–0.49). Species of *Acremonium, Colletotrichum, Cylindrocladium*, *Diaporthe*, *Dinemasporium*, *Diplodia*, *Exserohilum, Helminthosporium, Memnoniella, Pestalotiopsis*, *Pyricularia*, *Septofusidium*, *Thielavia, Torula,* and *Verticillium* < 0.5%

and MB (643) (Table 1), and there was a similar trend in the case of *P. flavidum* on PDA (1594), MEA (1502) and MB methods (996) (Table 1). The relative abundance of endophytic fungal communities was more in *P. flavidum* (51%) than in *D. bicornis* (38%). The expression of anamorphic and teleomorphic ascomycetes in *D. bicornis* was high on both PDA and MB (Table 1). However, in the case of *P. flavidum,* the incidence of anamorphic ascomycetes was high on PDA followed by MEA, whereas the teleomorphic ascomycete expression was high on MEA medium (Table 2).

The predominantly occurring endophytic fungi on PDA in *D. bicornis* included *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Khuskia oryzae,* and *Penicillium* sp. On the other hand, certain fungi like *Cochliobolus eragrostidis, Curvularia protuberata,* and *Cochliobolus lunatus* occurred predominantly on MEA. Fungi like *Acrophialophora fusispora*, *Helminthosporium halodes, Myrothecium roridum,* and *Stachybotrys chartarum* were high on MB (Table 1). *Paspalidium flavidum* also harbored the increased number of endophytic fungi such as *A.*

Table 2 The occurrence of anamorphic and teleomorphic ascomycetes and morphotype isolates endophytic in the aerial regions of *Paspalidium flavidum* by potato dextrose agar (PDA), malt extract agar (MEA), and moist blotter (MB) methods

Fungal species	Colonization frequency(%)[a]/Incubation methods[a]		
	PDA	MEA	MB
Anamorphic ascomycetes			
Acrophialophora fusispora (S.B.Saksena) Samson	0.73	0.24	0.62
Aspergillus spp.[b]	4.36 (7)[c]	3.42 (6)	1.11 (5)
Cladosporium spp.[d]	2.14 (3)	1.75 (2)	0.46
Colletotrichum spp. [e]	0.16	0.18	0.87
Cylindrocladium spp.[f]	0.14 (2)	0.34 (2)	0.30 (2)
Fusarium spp.[g]	1.95 (4)	1.60 (4)	1.83 (4)
Gliocladium roseum Bainier	1.34	1.28	0.31
Myrothecium roridum Tode	0.18	1.17	2.84
Penicillium spp.[h]	11.52 (4)	13.87 (4)	2.86
Phoma spp.[i]	0.35	0.32	0.51
Trichoderma spp.[j]	2.19 (2)	1.36 (2)	0.61 (2)
Total frequency	26.48 (35)	27.12 (32)	15.56 (28)
Teleomorphic ascomycetes			
Chaetomium spp.[k]	0.87 (4)	0.37 (5)	0.91 (4)
Cochliobolus spp.[l]	2.72 (5)	3.61 (8)	3.55 (7)
Spegazzinia lobulata Thrower	0	0	1.20
Total frequency	4.29 (12)	5.08 (16)	6.58 (15)
Morphotypes[m]	8.17 (6)	4.51 (6)	2.19 (4)

[a]Data are an average over three replicates (average over seasons, locations, and aerial reions), each with 75 samples

[b]*Aspergillus aculeatus* Iizuka (0.43–1.18), *A. candidus* Link (0.01–0.14), *A. flavus* Link (0.06–0.18), *A. fumigatus* Fresen. (0.17–0.28), *A. nidulans* (Eidam) G. Winter (0.04–0.13), *A. niger* Sensu auct. (0.16–2.35), *A ochraceus* G. Wilh (0.15–0.28)

[c]Figure in parenthesis indicate total number of species of genera which may vary in different media

[d]*Cladosporium cladosporioides* (Fresen.) G.A. de Vries (0.45–2.00), *C. herbarum* (Pers.) Link (0.03–0.09), *C. oxysporum* Berk. and Curt. (0.03)

[e]*Colletotrichum australe* Damm, P.F. Cannon and Crous (0.09), *C. boninense* Moriwaki, Toy. Sato and Tsukib. (0.12), *C. graminicola* (Ces.) G.W. Wilson (0.08–0.74)

[f]*Cylindrocladium parvum* P.J. Anderson (0.11–0.28), *Cylindrocladium* sp. (0.02–0.18)

[g]*Fusarium chlamydosporum* Wollenw. and Reinking (0.12–0.81), *F. equiseti* (Corda) Sacc. (0.01–0.03), *F. moniliforme* J. Sheld. (0.01), *F. oxysporum* Sensu Smith and Swingle (1.80–1.51), *F. pallidoroseum* (Cooke) Sacc. (0.01–0.11)

[h]*Penicillium commune* Thom (0.01–0.03), *Penicillium* sp. (0.41–12.2)

[i]*Phoma enigma* (0.25–0.38), *Phoma* sp. (0.09–0.12)

[j]*Trichoderma harzianum* Rifai (1.62–1.21), *T. viride* Pers. (0.14–0.57)

[k]*Chaetomium cupreum* L.M. Ames (0.03–0.08), *C. globosum* Kunze (0.37–0.32), *C. reflexum* Skolko and J.W. Groves (0.08–0.07), *C. robustum* L.M. Ames (0.01–0.24), *C. spirochaete* Palliser (0.08–0.25), *C. tenue* X. Wei, Wang, Crous and L. Lombard (0.07)

[l]*Cochliobolus affinis* R.R. Nelson and Hodges (1.22–1.47), *C. clavata* Jain (0.05), *C. eragrostidis* (Tsuda and Ueyama) Sivan. (0.11), *C. protuberata* Boedijn (1.75–1.12), *C. geniculatus* (Tracy and Earle) Boedijn (0.05–0.44), *C. lunatus* (Wakker) Boedijn (0.28–0.46), *C. pallescens* Boedijn (0.05–0.43), *C. spicifer* R.R. Nelson (0.18), *C. trifolii* (Kauffm.) Boedijn (0.01)

[m]Morphotype (0.03–3.72). Species of *Acremonium*, *Alternaria, Diaporthe*, *Diplodia*, *Glomerella*, *Helminthosporium, Khuskia, Mycotypha, Pestalotiopsis, Pithomyces, Pyricularia, Septofusidium, Stachybotrys*, *Thielavia,* and *Verticillium* < 0.5%

niger, C. cladosporioides, C. protuberata, Fusarium oxysporum, Gliocladium roseum, and *Pithomyces* sp. on PDA and *Cochliobolus pallescens, C. lunatus, K. oryzae* and species of *Acremonium, Penicillium,* and *Verticillium* on MEA and *Chaetomium spirochaete, Cochliobolus affinis, Colletotrichum graminicola, Fusarium chlamydosporum, M. roridum,* and *Spegazzinia lobulata* on MB (Table 2).

In the present study, out of 70 and 71 fungal endophytes of *D. bicornis* and *P. flavidum*, respectively, 33 and 39 fungal species expressed well in all three isolation methods. Contrary to the above observation, certain species were also found to be exclusively associated with a particular isolation method. For example, in case of *D. bicornis, Exserohilum turcicum*, *Penicillium commune,* and morphotypes expressed exclusively on PDA, while *A. nidulans*, *C. spirochaete,* and *Cladosporium oxysporum* expressed only on MEA; and 11 fungal endophytes expressed exclusively on MB. However, in the case of *P. flavidum, C. oxysporum*, *F. moniliforme, Diaporthe* sp. and a morphotype expressed exclusively on PDA, and *Cochliobolus trifolii, Colletotrichum australe, Chaetomium tenue, C. eragrostidis, Pyricularia grisea, Glomerella* sp., and a morphotype (strain 4) expressed only on MEA, and fungi like *Cochliobolus clavata, Colletotrichum boninense, M. microspora,* and *S. lobulata* expressed only on MB. Two endophytic fungi identified as *Cochliobolus* and *Penicillium* species by their morphological characteristics were confirmed by the molecular method as *Curvularia protuberata* R.R. Nelson & Hodges and *Penicillium citrinum* Thom., and the gene sequences were submitted to the Genbank of NCBI and obtained the accession no. MT799982 and MT775474.

Endophytic fungal communities in plant regions

The inflorescence, among aerial regions, of *D. bicornis* and *P. flavidum* yielded a good number of endophytic fungal isolates (1198 and 1527) followed by culm (904 and 1249) and leaf (915 and 1317) that were isolated from 2700 segments of each plant region (Table 3). Fungal species like *Aspergillus candidus*, *C. cladosporioides*, *C. affinis*, *C. lunatus*, *Phoma enigma*, *S. chartarum,* and a morphotype colonized the inflorescence of *D. bicornis*. On the other hand, the inflorescence of *P. flavidum* harbored

Table 3 The diversity and evenness indices and colonization frequency of endophytic fungal assemblages in the aerial regions of *Digitaria bicornis* and *Paspalidium flavidum*

Grass species/ sample units[a]	Species richness	Diversity index		Evenness index		Colonization frequency (%)
		Shannon (H′)	Simpson (D′)	Shannon (J′)	Simpson (E′)	
Digitaria bicornis						
PDA	54	0.93	3.04	0.24	0.06	43.96
MEA	47	0.90	2.77	0.23	0.06	34.71
MB	50	0.89	2.83	0.22	0.05	21.32
Rainy	62	0.92	3.03	0.22	0.05	54.92
Winter	44	0.87	2.60	0.23	0.06	40.48
Summer	24	0.91	2.77	0.29	0.12	4.50
Paspalidium flavidum						
PDA	56	0.95	3.46	0.24	0.07	38.94
MEA	56	0.87	2.83	0.22	0.05	36.71
MB	53	0.89	2.91	0.22	0.05	24.33
Rainy	69	0.95	3.42	0.23	0.07	42.92
Winter	50	0.91	3.06	0.23	0.06	27.67
Summer	31	0.77	2.12	0.22	0.05	28.39

[a]Data are based on the values of three different locations and arranged in randomized complete block design and carried out in triplicate; 75 segments/location/season

A. fusispora, *G. roseum* and a morphotype in addition to *C. cladosporioides* and *F. oxysporum*. In the present study, 49 and 51 fungal endophytes out of 70 and 71 fungal species colonized all three regions of *D. bicornis* and *P. flavidum,* respectively. However, certain fungal endophytes were tissue-specific; *C. oxysporum, C. geniculatus, Dinemasporium* sp., and *Pyricularia* sp. expressed exclusively in the inflorescence. However, *Alternaria tenuissima, C. spirochaete, Pestalotiopsis guepinii, C. eragrostidis,* and *Cylindrocladium* sp. colonized the culm as well as the leaf of *D. bicornis* and not the inflorescence (Table 3). The rarefaction index depicted that the expected number of endophytic fungal communities from the aerial regions increased with an increase in the number of isolations, depending on the season (Fig. 1). The analysis also pointed at the high incidence of endophytic fungi in the inflorescence during the rainy season in both *D. bicornis* and *P. flavidum* (Fig. 1).

Influence of seasons on fungal communities

The expression of endophytic fungal communities in *D. bicornis* and *P. flavidum* was found to depend on seasons. The rainy season (1657) supported the high expression of endophytic fungi followed by winter (1221) and summer (138) in *D. bicornis* (Table 3). The corresponding seasonal role in the expression of fungal endophytes in *P. flavidum* was rainy (1803) followed by summer (1159) and winter (1130) (Table 3). Examples of fungi with high incidences during the rainy season were *C. cladosporioides, F. oxysporum, Penicillium* sp., and morphotype strains in *D. bicornis* (Fig. 2). Similarly, those of *P. flavidum* were *C. protuberata*, *F. chlamydosporum*, *F. oxysporum*, *T. harzianum*, *Penicillium* sp., and morphotypes (Fig. 2). The colonization frequency of endophytic fungi was less in *D. bicornis* and *P. flavidum* during the summer season. However, certain species of *Helminthosporium* and *Phoma* occurred in

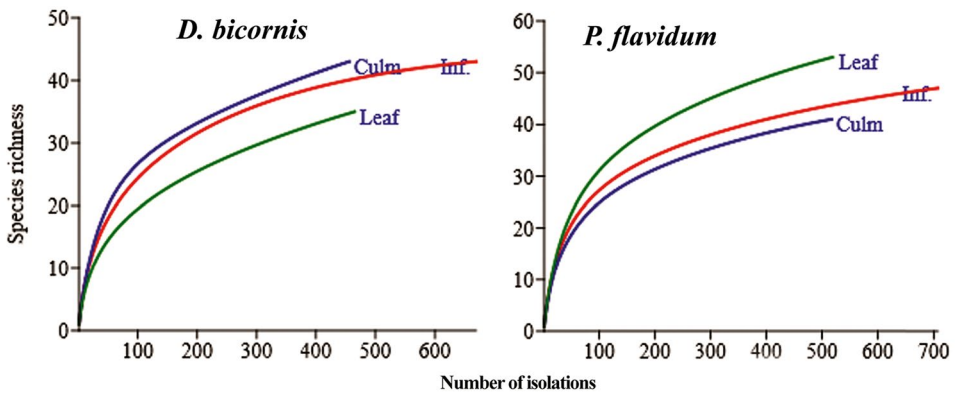

Fig. 1 The endophytic fungal assemblages in the aerial regions of *Digitaria bicornis* and *Paspalidium flavidum* during rainy season as depicted by rarefaction curve

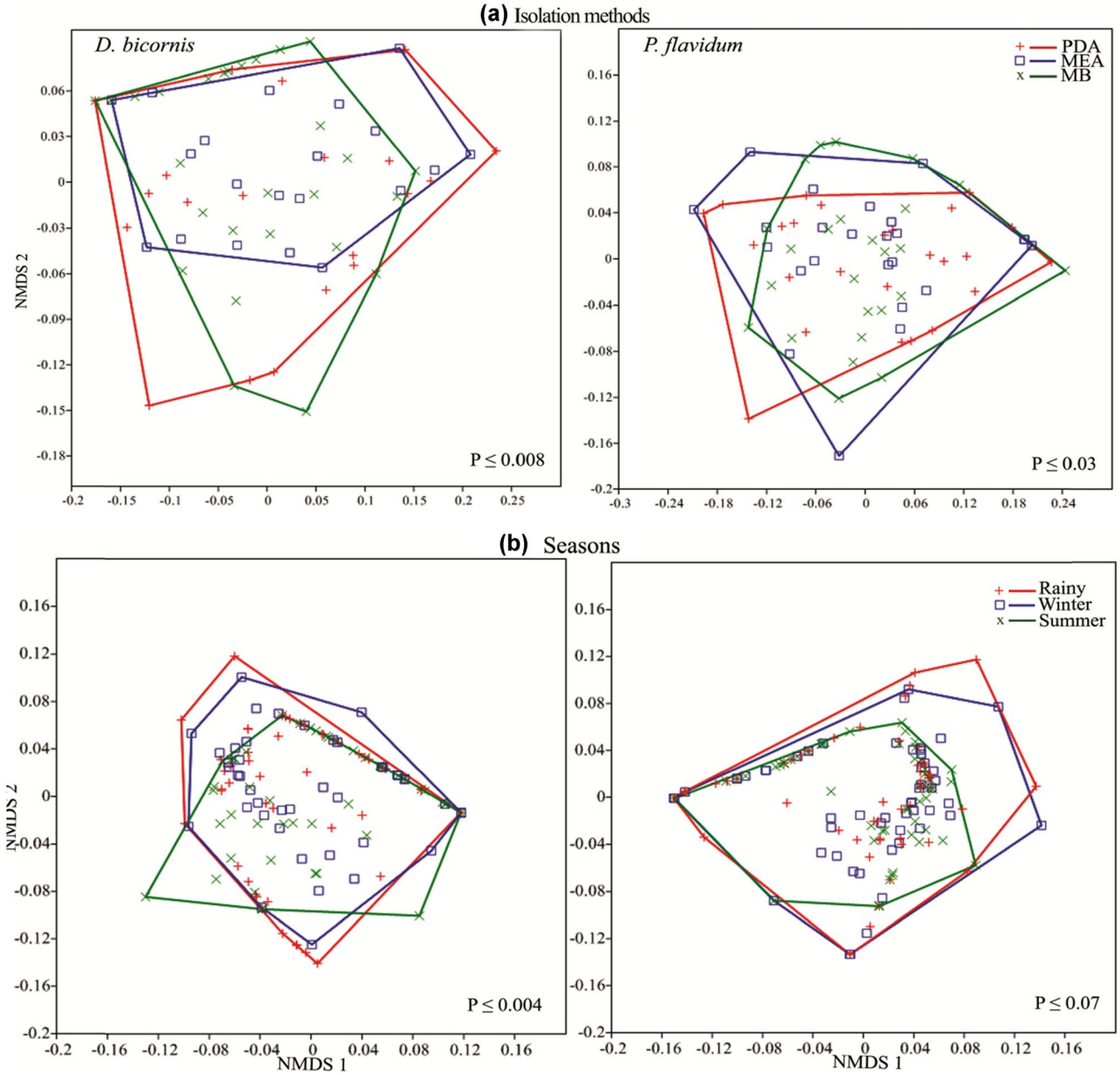

Fig. 2 Nonmetric multidimensional scale (NMDS) of endophytic fungal communities occurring in *Digitaria bicornis* and *Paspalidium flavidum* in relation to isolation methods (PDA—potato dextrose agar; MEA—malt extract agar; MB—moist blotter) and seasons. Polygons indicate clustering of endophytic fungal communities based on the above variables

high frequency in *D. bicornis,* and species of *Alternaria*, *Mycotypha*, *Penicillium*, *Pithomyces,* and *Stachybotrys* expressed high in *P. flavidum,* during the summer season. The frequency of endophytic fungal species was assessed by a nonmetric multidimensional scaling method, and their abundance was used to assess the similarity and correlation of fungal endophytic communities occurring in the aerial regions of *D. bicornis* and *P. flavidum* with different seasons and isolation methods. The NMDS plots were constructed based on Bray–Curtis coefficient analysis that portrayed the significant distribution of fungal endophytes depending on the above parameters (Fig. 2). Shannon (H') and Simpson (D') diversity and evenness indices (J' and E') were high in *P. flavidum* inflorescence during the rainy season on PDA when compared to that in *D. bicornis* (Table 3).

In vitro antagonistic assay of endophytic fungi

Out of 71 endophytic fungal species, six of them (*H. halodes*, *G. roseum*, *C. protuberata*, *P. guepinii, F. oxysporum,*

and a morphotype) from *P. flavidum* were tested for their in vitro antagonistic activity against the test bacterial and fungal strains (Tables 4, 5). Fungal endophytes like *F. oxysporum* and *C. protuberata* showed good activity to *E. coli*, *X. campestris, S. typhi,* and *S. enterica,* and a morphotype isolate and *C. protuberata* were strongly antagonistic to *A. alternata* and *F. oxysporum* (Tables 4, 5).

In vitro antibacterial assay of endophytic fungi

Among the endophytic fungi showing antagonism in vitro, three isolates with high activity from *P. flavidum* (Tables 4, 5) and another three isolates from *D. bicornis* showing moderate activity (data not shown) were selected for determining the antibacterial activity, in their metabolites. Results showed that ethyl acetate extract of *A. flavus, C. spicifer,* and *P. citrinum* of *D. bicornis* exhibited high antibacterial potential against *S. aureus, S. enteric,* and *S. typhi.* Whereas, *C. protuberata, F. oxysporum*, and *G. roseum* of *P. flavidum* showed high inhibitory activity against *E. faecalis*, *S. aureus,* and *K. pneumonia.* followed by methanolic extracts. Among them, *P. citrinum* in *D. bicornis* and *C. protuberata* in *P. flavidum* showed very high antibacterial activity against the test bacterial strains (Table 6).

Characterization of secondary metabolites in endophytic fungal extracts

The OHR LC–MS is highly sensitive and yielded the separation of a large number of compounds. As many as 2352 and 2500 annotatable compounds of *C. protuberata* and *P. citrinum*, respectively, were separated by positive- and negative-ion modes. Compounds included those with prominent peaks and high retention values as well as those with insignificant peaks represented by certain other compounds. There were several compounds with high peaks with no known activities, as per the available database searches. Such of them could be identified as the novel chemicals that might be relevant to the antibacterial and antagonistic activities, observed in the present study. Furthermore, such compounds need to be properly distinguished based on their chemical profiling and associated activities. On the other hand, certain well-established peaked compounds have been identified with known activities as per the literature search. The ion chromatograms (Figs. 3, 4) containing prominent peaks of 24 and 23 compounds in extracts of *C. protuberata* and *P. citrinum,* respectively, were selected. Out of 24 in *C. protuberata,* 23 compounds were detected in the positive mode and one was detected in the negative mode; and in the case

Table 4 Antagonistic activity of fungal species endophytic in *Paspalidium flavidum* against test bacterial strains

Endophytic fungi	Inhibition (%)/test bacterial strains							
	Sa	E f	Ec		Pa	Xc	Se	Kp	St
C. protuberata	43.6 ± 1.5	35.6 ± 1.5	45.0 ± 2.6	46.6 ± 1.5*		41.0 ± 1.0	43.6 ± 1.5	35.0 ± 2.6	51.0 ± 1.0*
F. oxysporum	43.3 ± 1.5	47.0 ± 2.0	54.3 ± 1.5*	45.6 ± 1.5		51.0 ± 1.0*	54.0 ± 1.0*	45.3 ± 0.5	43.6 ± 2.0
G. roseum	36.0 ± 2.6	34.0 ± 2.0	26.3 ± 2.5	55.0 ± 1.0*		42.6 ± 2.0	34.3 ± 2.0	40.6 ± 1.1	34.3 ± 2.0
P. guepinii	20.0 ± 1.0	23.3 ± 1.5	23.3 ± 1.5	21.6 ± 0.5		14.6 ± 1.5	24.3 ± 2.0	40.0 ± 1.0*	22.0 ± 1.0

Values are mean ± SE of three replicates

C. protuberate Curvularia protuberata, *P. Guepinii pestalotiopsis guepinii*, *F. oxysporum Fusarium oxysporum*, *G. roseum Gliocladium roseum*. *Sa Staphylococcus aureus* and *Ef Enterococcus faecalis, Ec Escherichia coli, Pa Pseudomonas aeruginosa*, *Xc Xanthomonas campestris*, *Se Salmonella enterica*, *Kp Klebsiella pneumonia, and St Salmonella typhi* obtained from IMTECH, Chandigarh, India; *The means of endophytic fungi in a row are significantly different at $p \leq 0.05$ according to DMRT

Table 5 Antagonistic activity of fungal species endophytic in *Paspalidium flavidum* against test fungal strains

Endophytic fungi	Inhibition (%)/test fungal strains		
	Alternaria alternata	*Fusarium oxysporum*	*Sclerotium rolfsii*
Curvularia protuberata	62.3 ± 0.5*	50.0 ± 1.0*	31.0 ± 1.0
Fusarium oxysporum	45.6 ± 0.5*	45.0 ± 3.2	33.3 ± 1.5
Gliocladium roseum	44.1 ± 1.7	44.6 ± 2.5*	32.0 ± 1.0
Helminthosporium halodes	49.6 ± 0.5	40.0 ± 1.0	22.3 ± 2.5
Pestalotiopsis guepinii	54.3 ± 1.1*	46.0 ± 1.0	34.0 ± 2.0
Morphotype	64.3 ± 1.1*	53.0 ± 2.6*	41.6 ± 1.5*

Values are mean ± SE of three replicates; The test fungal strains were obtained from infected samples in the field of crop plants; *The means of endophytic fungi in a row are significantly different at $p \leq 0.05$ according to DMRT

Table 6 Antibacterial activity of ethyl acetate and methanol extracts of fungal species endophytic in *Digitaria bicornis* and *Paspalidium flavidum*

Fungal isolates	Crude extracts (100%)	Zone of inhibition (mm)[a]/test bacterial isolates							
		Sa	Ef	Ec	Pa	Xc	Se	Kp	St
Digitaria bicornis									
A. flavus	Ethyl acetate	17.6 ± 0.57	12.3 ± 0.57	16.6 ± 0.57*	14.6 ± 0.57	14.0 ± 1.0	0.0 ± 0.0	10.3 ± 0.57	11.0 ± 1.0
	Methanol	17.6 ± 0.57	12.3 ± 0.57	16.6 ± 0.57*	14.6 ± 0.57	14.0 ± 1.0	0.0 ± 0.0	10.3 ± 0.57	11.0 ± 1.0
C. spicifer	Ethyl acetate	19.3 ± 1.15*	17.6 ± 0.57*	14.0 ± 1.73	15.3 ± 1.15	16.3 ± 1.15*	17.3 ± 1.10*	5.30 ± 1.15	16.3 ± 1.15
	Methanol	17.6 ± 0.57	17.3 ± 1.15	15.0 ± 1.73	13.3 ± 1.15	15.6 ± 0.57	17.0 ± 1.73*	4.00 ± 1.73	16.3 ± 1.15
P. citrinum	Ethyl acetate	20.3 ± 1.15*	16.3 ± 1.15	9.33 ± 1.15	15.0 ± 1.73	15.3 ± 1.15	10.3 ± 1.15	9.30 ± 1.15	18.0 ± 1.73*
	Methanol	20.6 ± 1.15*	15.3 ± 1.15	7.66 ± 1.15	15.3 ± 1.15	15.3 ± 2.88	15.6 ± 0.57*	10.3 ± 1.15	17.3 ± 1.15*
Paspalidium flavidum									
C. protuberata	Ethyl acetate	16.3 ± 1.15	20.3 ± 1.1*	16.6 ± 0.57	16.3 ± 1.15	15.3 ± 1.15	10.3 ± 1.15	18.6 ± 1.15*	16.3 ± 1.15
	Methanol	16.3 ± 1.15	19.3 ± 1.1*	16.3 ± 1.10*	15.3 ± 1.15	16.3 ± 1.1*	10.6 ± 0.57	18.3 ± 0.50*	15.3 ± 1.15
F. oxysporum	Ethyl acetate	15.3 ± 1.15	13.3 ± 1.15	16.3 ± 1.15	13.3 ± 1.15	0.0 ± 0.0	16.3 ± 1.15	0.0 ± 0.0	10.3 ± 1.15
	Methanol	16.3 ± 1.15	13.3 ± 1.1	15.3 ± 1.15	12.6 ± 1.15	0.0 ± 0.0	16.6 ± 0.57*	0.0 ± 0.0	10.6 ± 0.57
G. roseum	Ethyl acetate	18.0 ± 1.73*	0.0 ± 0.0	12.3 ± 1.15	17.0 ± 1.73*	15.0 ± 1.73	15.6 ± 0.57	18.0 ± 1.73	17.0 ± 1.73
	Methanol	17.0 ± 1.73*	0.0 ± 0.0	12.6 ± 0.57	16.3 ± 1.15	15.6 ± 0.57	16.3 ± 1.15	18.0 ± 1.73	18.0 ± 1.70*
Amoxicillin		15.3 ± 1.15	15.3 ± 1.15	17.0 ± 1.73	18.6 ± 1.15	17.6 ± 2.30	19.3 ± 1.15	17.0 ± 1.73	18.0 ± 1.73
Ciprofloxacin		18.3 ± 2.88	20.3 ± 1.15	21.6 ± 1.15	25.3 ± 0.57	19.6 ± 0.57	21.3 ± 1.15	22.0 ± 1.73*	22.3 ± 1.15
Chloramphenicol		22.6 ± 0.57*	25.3 ± 0.57*	22.6 ± 1.15*	28.6 ± 1.15*	25.6 ± 1.15*	24.0 ± 1.73*	23.0 ± 1.73	23.6 ± 2.30*

Crude extracts were dissolved in DMSO@ 10 mg ml^{-1}; Each well received 6.66 µl/well (20 µg ml^{-1})

Sa Staphylococcus aureus, *Ef Enterococcus faecalis, Ec Escherichia coli, Pa Pseudomonas aeruginosa, Xc Xanthomonas campestris, Se Salmonella enterica, Kp Klebsiella pneumonia, St Salmonella typhi* obtained from IMTECH, Chandigarh, India

*Means in a column under each grass species as compared to the standard control are significantly different at $p \leq 0.05$ according to DMRT

[a]Values are mean ± SE of three replicates

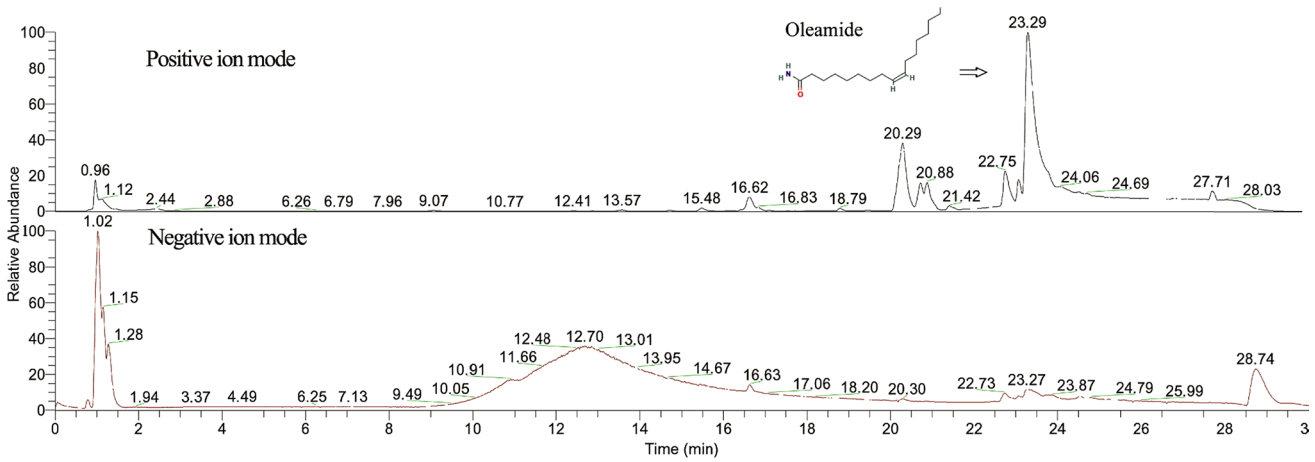

Fig. 3 OHR LC–MS chromatogram of *Curvularia protuberata* endophytic in *Paspalidium flavidum.* Major peak compound is indicated with molecular structure

of *P. citrinum,* 22 compounds were detected in the positive mode and one was detected in the negative mode (Figs. 3, 4). In the case of extracts of *C. protuberata* and *P. citrinum,* compounds numbered 1, 7, 10, 13, and 19, and 6, 7, 10, 11, and 15, respectively, were related to the antibacterial, antifungal, and antiviral activities; and compounds with number 8 and 17, and 3, 5, 17, 19, and 21 (Tables 7, 8), respectively, were identified with insecticidal, nematicidal and pesticidal activities. However, compounds numbering 4, 12, 14 and 20, and 4, 10, 12, 14 and 23, respectively, were

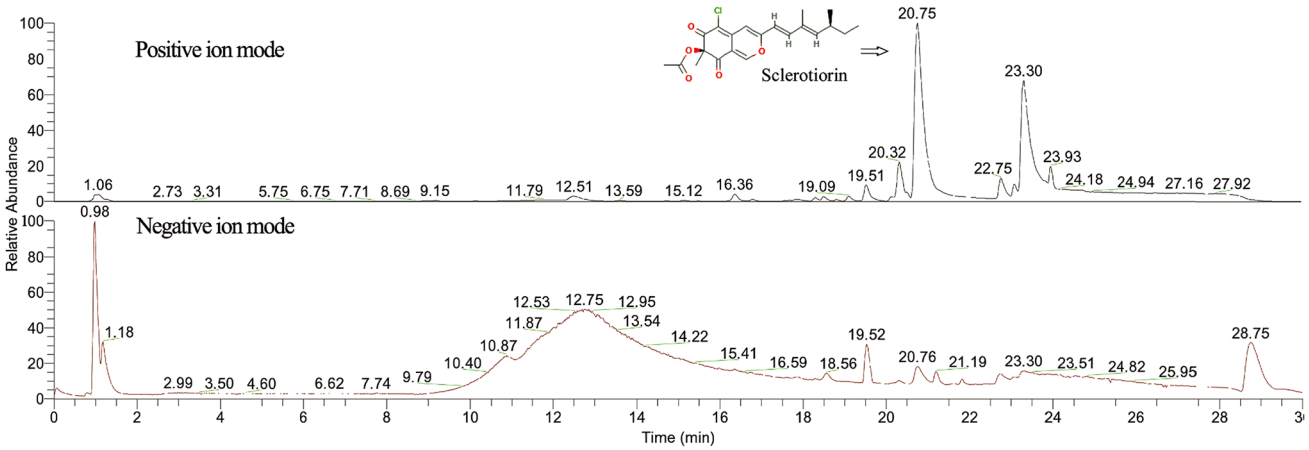

Fig. 4 OHR LC–MS chromatogram of *Penicillium citrinum* endophytic in *Digitaria bicornis.* Major peak compound is indicated with molecular structure

related to the anticoagulant, anti-inflammatory, antioxidant, antipsychotic, antiseptic, and antitumor activities (Tables 7, 8). Among the above compounds, oleamide ($t_R = 23.29$) (5) and α-linolenic acid ($t_R = 20.29$) (9) in *C. protuberata* and sclerotiorin ($t_R = 20.75$) (7) and 1-decyl-2-pyrrolidinone ($t_R = 23.30$) (5) in *P. citrinum* associated with high peaks (Figs. 3, 4; Tables 7, 8) were shown with algaecidal, antimicrobial, anticancer, and pesticidal properties.

Discussion

The present study established a plethora of anamorphic ascomycete occurrence followed by teleomorphic ascomycetes and morphotypes in the aerial regions of both grass species. The high expression of anamorphic ascomycetous rather than the teleomorphic forms and morphotypes (Rekha and Shivanna 2014) is not understood. However, the nutrient content of the isolation media might play an important role in the enhanced expression. The study showed a systematic difference in the occurrence of certain fungal endophytes depending on the isolation methods. The enhanced expression of endophytic fungi on PDA is attributed to high nutrient availability facilitating the growth of the fast colonizing fungal endophytes and suppression of slow-growth forms (Vasanthakumari and Shivanna 2011). In this context, PDA is shown to support the high expression of fungi (Singh et al. 2016). High fungal abundance in *P. flavidum* could be attributed to the inherent ability of endophytic fungi to associate with certain grass species. Certain dominant species of *Penicillium, Aspergillus*, *Cladosporium*, *Colletotrichum,* and *Cochliobolus* in the study were also documented from the rhizosphere and rhizoplane/root regions of *D. bicornis* and *P. flavidum* (Vasanthakumari and Shivanna 2009), and some of the above fungal endophytes were also documented in *Halophia ovalis* (Devarajan et al. 2002). Much similar to ascomycetes, morphotypes in grasses expressed very well on PDA followed by MEA and MB methods. Such an increase in the occurrence of morphotypes on PDA is also documented in the literature (Desmukh et al. 2010; Rekha and Shivanna 2014).

Intriguingly, the high expression of endophytic fungi in the inflorescence could be due to the elevated nutrient level during flowering and seed setting, and possibly the inflorescence acting as the region for fungal transmission from the mother plant to seeds. Such an observation has not been documented in the literature. In contrast, several reports documented the association of a large number of endophytic fungi in the foliage of plants (Abdelfattah et al. 2016; Rajamani et al. 2018). Attempts were also made to document endophytic fungi from the root regions of grasses and other plants. The increased incidence of endophytic fungi in the root as compared to those of the aerial regions was attributed to the exposure of roots to the rhizosphere mycoflora and root colonization (Sarma et al. 2018). High incidences of mycoflora in the rhizoplane and rhizosphere were also documented in *D. bicornis* and *P. flavidum* (Vasanthakumari and Shivanna 2009). The present study showed that the endophytic fungal assemblages could be host part-specific. For example, *C. trifolii, C. boninense,* and *F. moniliforme* were localized to the inflorescence, while *C. australe, P. commune,* and *S. lobulata* and a morphotype to leaves of *P. flavidum*. Such instances of host tissue specificity were also documented in *Zea mays* associated with *F. moniliforme* and *P. commune* (Pamphile and Azevedo 2002; Hosseini et al. 2013). The restricted occurrence of endophytic fungal communities to the aerial parts was also shown to be influenced by seasonal fluctuations (Gomes et al. 2018).

There was a striking similarity in the occurrence and frequency of endophytic assemblages in 2 years of study. A

Table 7 Physico-chemical and pharmacological characteristics of compounds produced in culture by *Penicillium citrinum* endophytic in *Digitaria bicornis*

Sl. No	Compound	Molecular formula	Molecular weight and RT (min)	Properties	References
1	Stearamide	$C_{18}H_{37}NO$	283.28627; 27.92	Antifoaming and anticoagulating	PubChem CID: 31292
2	Oleamide	$C_{18}H_{35}NO$	281.27066; 24.94	Algaecidal	Shao et al. (2016)
3	1-Stearoylglycerol	$C_{21}H_{42}O_4$	358.30692; 24.18	Pesticidal	Murata et al. (2011)
4	Caffeic acid	$C_9H_8O_4$	180.0416; 23.93	Antioxidant and anti-inflammatory	Oršolić et al. (2016)
5	1-Decyl-2-pyrrolidinone	$C_{14}H_{27}NO$	225.20844; 23.30	Pesticidal	PubChem CID: 171,427
6	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.13322; 22.75	Antibacterial	Sidoryk et al. (2014)
7	Sclerotiorin	$C_{21}H_{23}ClO_5$	390.12167; 20.75	Fungicidal and anticancer	Giridharan et al. (2012)
8	Benzophenone	$C_{13}H_{10}O$	182.07316; 20.32	Photosensitizing	Wang et al. (2013)
9	5-Chloro-3-[(1E,3E)-3,5-dimethyl-1,3-heptadien-1-yl]-7-methyl-6,8-dioxo-2,6,7,8-tetrahydro-7-isoquinolinyl acetate	$C_{21}H_{24}ClNO_4$	389.1375; 19.513	Unknown	Unknown
10	Ergosterol peroxide	$C_{28}H_{44}O_3$	428.32905; 19.09	Anti-Inflammatory and antiviral	Yasukawa et al. (1996)
11	Mycophenolic acid	$C_{17}H_{20}O_6$	320.12467; 16.36	Antimicrobial and antiviral	Oh et al. (2015)
12	Cannabidiol	$C_{21}H_{30}O_2$	314.22328; 15.12	Analgesic and antioxidant	PubChem CID: 644019
13	Hexadecanamide	$C_{16}H_{33}NO$	255.25524; 13.59	Paranoid schizophrenia	Hua and Jenke (2012)
14	α-Eleostearic acid	$C_{18}H_{30}O_2$	278.22359; 12.51	Antitumor	Tsuzuki et al. (2004)
15	Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	244.12032; 11.79	Antibacterial	Kannabiran (2016)
16	Noroxymorphone	$C_{16}H_{17}NO_4$	287.11467; 9.15	Analgesic	PubChem CID: 5497189
17	Isophorone	$C_9H_{14}O$	138.10398; 8.69	Pesticidal	Daoubi et al. (2005)
18	4-Acetamidoantipyrine	$C_{13}H_{15}N_3O_2$	245.11554; 7.71	Antipyrine	PubChem CID: 65743
19	Solanine	$C_{45}H_{73}NO_{15}$	867.49468; 6.75	Insecticidal	PubChem CID: 262500
20	L-Phenylalanine	$C_9H_{11}NO_2$	165.07904; 3.31	Antidepressant	Aydaş et al. (2013)
21	Carbofuran	$C_{12}H_{15}NO_3$	221.10454; 2.73	Pesticidal	Wille et al. (2013)
22	DL-carnitine	$C_7H_{15}NO_3$	161.10467; 1.06	Treatment of heart and blood	Bazotte and Bertolini (2012)
23	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.19237; 28.75	Antiseptic	Tigges et al. (2010)

similar observation was also documented in several Indian medicinal plants (Jalgaonwala and Mahajan 2015). The challenge of the present study is to understand the season-induced variability in the occurrence of endophytic fungal assemblages. The predominant expression of fungal endophytes during the rainy season could be explained by the enhanced availability of soil nutrient contents which supported the good plant health and colonization ability of endophytic fungi (Singh et al. 2016). While endophytic fungal isolates were encountered in high incidences during the rainy season (Deshmukh et al. 2010), they were associated with the low expression during the summer season (Vasanthakumari and Shivanna 2009). The high richness and diversity of certain endophytic fungal species with lower evenness during summer than in other seasons were also observed by Konig et al. (2018). The PDA is a suitable isolation medium, since it supported a variety of endophytic fungal species through different seasons. This observation is supported by the finding that seasons influenced profoundly the increased occurrence of fungal endophytes and this is concurrent with the growth-supporting role played by the isolation/incubation method (Giauque 2016). The fact that certain dominant endophytic fungi are also season-specific is well demonstrated by the NMDS plots generated based on

Table 8 Physico-chemical and pharmacological characteristics of compounds produced in culture by *Curvularia protuberata* endophytic in *Paspalidium flavidum*

Sl. no	Compound	Molecular formula	Molecular weight and RT (min)	Properties	References
1	Diphenyl sulfone	$C_{12}H_{10}O_2S$	218.03945; 28.03	Antifungal	Murafuji et al. (2011)
2	Dodecylbenzene	$C_{18}H_{30}$	246.23378; 27.71	Detergent	Kurt (2005)
3	Myristamide	$C_{14}H_{29}NO$	227.22405; 24.69	Antistatic	PubChem CID: 69492
4	4-Hydroxycoumarin	$C_9H_6O_3$	162.03102; 24.06	Anticoagulant	Au and Rettie (2008)
5	Oleamide	$C_{18}H_{35}NO$	281.27066; 23.29	Algaecidal	Shao et al., (2016)
6	2-Pyrazolin-5-ol, 5-tridecafluorohexyl-3-methyl-1-(3-pyridylcarbonyl)-	$C_{16}H_{10}F_{13}N_3O_2$	523.05726; 22.75	Unknown	Unknown
7	7-Hydroxycoumarine	$C_9H_6O_3$	162.03102; 21.42	Antifungal and phytoalexin	Li and Ellis (2012)
8	Triphenylphosphine oxide	$C_{18}H_{15}OP$	278.08516; 20.88	Organophosphorus compound	Corbridge, (1996)
9	α-Linolenic acid	$C_{18}H_{30}O_2$	278.22368; 20.29	Cardiovascular disease	Pan et al. (2015)
10	Griseofulvin	$C_{17}H_{17}C_1O_6$	352.07014; 18.79	Antifungal	Mauro et al. (2013)
11	4-Methoxycinnamaldehyde	$C_{10}H_{10}O_2$	162.06743; 16.83	Nematicidal	Wang et al. (2009)
12	Dexamethasone	$C_{22}H_{29}FO_5$	392.19826; 16.62	Anti-inflammatory	Goswami et al. (2018)
13	β-Asarone	$C_{12}H_{16}O_3$	208.10929; 15.48	Antifungal	Venkatesan et al. (2019)
14	2-Chloro-9-[3-(dimethylamino) propylidene]thioxanthene	$C_{18}H_{18}ClNS$	315.08608; 13.57	Antipsychotic	PubChem CID: 667467
15	Vanillyl alcohol	$C_8H_{10}O_3$	154.0625; 12.41	Flavoring ingredient	PubChem CID: 62348
16	5,6-Dichloro-3-methoxy-1H-isoindole	$C_9H_7Cl_2NO$	214.99028; 10.77	Unknown	Unknown
17	Isophorone	$C_9H_{14}O$	138.10405; 9.07	Pesticidal	Daoubi et al. (2005)
18	2-phenylbenzimidazole-5-sulfonic acid	$C_{13}H_{10}N_2O_3S$	274.04015; 7.93	Cyclobutane pyrimidine dimmers	Bastien et al. (2010)
19	N-Butylbenzenesulfonamide	$C_{10}H_{15}NO_2S$	213.08167; 6.79	Neurotoxic and myotoxics	Rider et al. (2012)
20	Meprednisone	$C_{22}H_{28}O_5$	372.19228; 6.26	Anti-inflammatory	PubChem CID: 5284587
21	1-Benzoyl-4-(3,5-dibromobenzoyl)piperazine	$C_{18}H_{16}Br2N_2O_2$	449.95604; 2.88	Unknown	Unknown
22	S-Benzyl-N-[(benzyloxy)carbonyl]cysteinyltyrosine	$C_{27}H_{28}N_2O_6S$	508.16469; 1.12	Unknown	Unknown
23	DL-carnitine	$C_7H_{15}NO_3$	161.10462; 0.96	Heart complication in diphtheria	Bazotte et al. (2012)
24	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.19234; 28.74	Surfactant	Tigges et al. (2010)

Bray–Curtis coefficient. This kind of association of endophyte assemblage with seasons was also studied in the olive and perennial ryegrass plant systems (Martins et al. 2016; Konig et al. 2018).

Among the endophytic fungal species, one morphotype strain and *C. protuberata* in *P. flavidium* expressed high antagonistic activity in vitro to fungal and clinical bacterial pathogens. The inhibitory effect of endophytic fungi against test fungi and bacteria could be attributed to the competition for nutrients between the two or the production of secondary metabolites (Talapatra et al. 2017) with antimicrobial properties. This prompted authors to screen metabolites of selected endophytic fungi for their antibacterial activity, in CF and MM extracts. The metabolites of *C. protuberata* and *P. citrinum* from *P. flavidum* and *D. bicornis*, respectively, showed very high activity against Gram-positive and negative bacterial test strains and activity was higher than the standard antibiotics amoxicillin or chloramphenicol. This suggested that the endophytic fungal metabolites contain active principles that inhibited bacterial growth by targeting the biosynthesis of cell walls and proteins. Metabolites of *Cochliobolus* species in perennial grasses and medicinal plants were also shown with wide antimicrobial activity (Raviraja et al. 2006; Rekha and Shivanna 2014). The compounds produced in the endophytic fungi could be possibly contributing to broad-spectrum activity against several test bacterial isolates.

Based on the high antimicrobial activity in their extracts, *C. protuberata* of *P. flavidum* and *P. citrinum* of *D. bicornis* were subjected to an orbitrap high-resolution LC–MS. The extracts were found to contain a wide range of biomolecules. Searches in the literature on biomolecules of *C. protuberata* such as diphenyl sulfone, 7-hydroxycoumarine, griseofulvin, or *β*-asarone revealed

their antibacterial and antifungal principles. This suggested that the above compounds could be involved in the antimicrobial activity in the present study. On the other hand, compounds such as cyclo (phenylalanyl-prolyl) or mycophenolic acid in *P. citrinum* were identified with antiviral activities (Adam et al. 2009; Du et al 2012). The compounds with a high peak in both positive- and negative-ion modes might be working synergistically with certain novel compounds with no known activity resulting in antifungal and antibacterial activities higher than the standard controls. In addition to the above compounds, 4-hydroxycoumarin, dexamethasone, 2-chloro-9-[3 (dimethylamino) propylidene] thioxanthene, and meprednisone in *C. protuberata* and ergosterol peroxide, cannabidiol, α-eleostearic acid, and 4-dodecylbenzenesulfonic acid in *P. citrinum* were also documented for anticoagulant, anti-inflammatory, antipsychotic, antioxidant, antiseptic, and antitumor activities (Yasukawa et al. 1996; Tsuzuki et al. 2004; Goswami et al. 2018). Certain compounds in the metabolites of the endophytic fungi require a detailed study as they might play an important role in the antibacterial activity. It is very interesting to note that compounds such as oleamide (5) and sclerotiorin (7) with high peaks, in case of *C. protuberata* and *P. citrinum,* respectively, have algaecide and fungicide activities in addition to their role in anticancer activity. The compound sclerotiorin was also documented in *Cephalotheca faveolata* endophytic in *Eugenia jambolana* (Giridharan et al. 2012; Shao et al. 2016).

In conclusion, the endophytic fungal assemblages were high in both *P. flavidum* and *D. bicornis*. The PDA and MEA media supported the expression of a large number of endophytic fungal species. Certain endophytic fungi were specific to the inflorescence or foliage and were influenced by the rainy season. Some species showed antimicrobial activities in vitro and produced metabolites with the ability to inhibit test bacterial strains. The LC–MS spectral studies of some endophytic fungal extracts revealed the association of compounds with several pharmacological activities having antimicrobial and antibiotic properties. The compounds with unknown biological activity require detailed study as they are compounds of promise. The results of the study suggested that endophytic fungi of grasses are a good source of bioactive compounds that might find application in medicine and agriculture, as well.

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Author contributions MBS conceived the initial work idea and NR performed the laboratory experiments and data analysis. MBS and NR worked on the data interpretation of the work. NR prepared the first draft copy of the manuscript, and the corrections were made and finalized by MBS and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflicts of interest.

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