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Original Research Article

Diversity, antimicrobial and antioxidant activities of fungal endophytes in *Cynodon dactylon* (L.) Pers. and *Dactyloctenium aegyptium* (L.) P. Beauv

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A B S T R A C T

Cynodon dactylon and *Dactyloctenium aegyptium* grass species were studied for endophytic fungi by Potato dextrose agar (PDA) and Malt extract agar (MEA) methods. Species richness, diversity and evenness were determined by Simpson and Shannon indices. Endophyte metabolites were tested for antimicrobial and antioxidant activities. There were 26 and 30 endophytic fungal species of 15 and 16 genera were found in *C. dactylon* and *D. aegyptium*, respectively. More anamorphic ascomycetes were occurred than teleomorphic forms. Species richness, diversity and evenness indices were almost similar in PDA and MEA. *Cochliobolus geniculatus*, *Cochliobolus spicifer* and *Myrothecium roridum* in addition to others were antibacterial to *Enterococcus faecalis* and *Salmonella enterica*, while *Cochliobolus lunatus*, *Chaetomium globosum* and *Myrothecium roridum* were effective to *Staphylococcus aureus* at 1000 µg ml^{-1} concentration. *C. hawaiiensis* and *M. roridum* had shown high antifungal activity against *C. albicans*. In antioxidant assay *C. geniculatus* (71.76) had showed highest percent radical scavenging activity, but less when compare to standard (ascorbic acid, 78.40).

Keywords

Endophytic fungi, grass, anamorphic ascomycetes, antibacterial, antioxidant.

Introduction

Endophytic microorganisms, distinct from mycorrhiza inhabit diverse host plants and become systemic and live asymptomatically in the host system (Li et al., 2007; Rodriguez et al., 2009; Botella et al., 2010; Sakalidis et al., 2011). Endophytic fungi in symbiotic association with plant species confer resistance in plants to climatic (Rodriguez et al., 2004) and soil conditions (Zaurov et al., 2001), and pathogens (Mejía et al., 2008) and herbivores (Cheplick and Faeth, 2009).

Most of the studies were limited to the documentation of the diversity and characterization of endophytes in the above or below-ground plant parts (Liu et al. 2004; Jin et al. 2013; Maheswari and Rajagopal, 2013). A few grass species have been studied for their endophytic association in the above-ground parts (Walsh et al., 2010) and entire plant system (Alfaro et al., 2008).

Previous studies have shown that fungi occur in the rhizosphere of *Cynodon*

dactylon (L.) Pers. (Bermuda grass), *Dactyloctenium aegyptium* (L.) P. Beauv. (Egyptian grass), and *Zoysia tenuifolia* Willd. ex Thiele (Zoysia grass) of sub-family Chloridoideae and become endophytic in roots (Shivanna et al., 1996; Shivanna and Vasanthakumari, 2011). *Cynodon dactylon,* one of the 'Dasapuspam' used in Ayurveda, is ubiquitous in distribution and is considered as a serious weed, invading the cultivated lands. It has been studied in detail for its chemical constituents and importance in traditional medicine (Singh et al., 2007; Amita et al., 2012). It is used in ethnomedicine for the treatment of respiratory disorders, menstrual disorders, skin injuries, dandruff and diarrhoea (Rajakumar and Shivanna, 2009; Shivanna and Rajakumar, 2010; Qureshi et al., 2010). *Dactyloctenium aegyptium,* pan-tropical in distribution, is also a destructive weed in crop fields; it has anti-oxidant, anti-inflamatory, anticancer and antipyretic properties (Khumbongmayum et al., 2005; Hansakul et al., 2009; Jananie et al., 2011) and is used for treating small pox, wounds and ulcers (Heuze et al., 2013). Both the above species find importance as fodder and forage species. Fungal endophytes occurring in certain grass species (Danielsen and Jensen, 1999; Márquez et al., 2010; Ghimire et al., 2011 and Tanaka et al., 2012) produce diverse chemical compounds (Wayne et al., 1999; Koulman et al., 2007), some with interesting biological activities including antimicrobial (Barnabas et al., 2013) and antioxidant properties (Nitya et al., 2011). *Aspergillus fumigatus* associated with *C. dactylon* produced antifungal metabolites (Liu et al., 2004). Endophytic association of fungi with *C. dactylon* might differ depending on the location and such endophytic fungi might produce novel chemical compounds with interesting properties, useful for human welfare. Since the above grass species are the rich source of antioxidants and other chemicals (Liu et al., 2004; Abdullah et al., 2012; Abdallah and El-Ghazali, 2013), produced either individually or in association with endophytes, they are required to be explored for their potentials (Ding et al., 2006; Jananie et al., 2011). *Cynodon dactylon* and *D. aegyptium* growing in the Western Ghats region of peninsular India have not been thoroughly investigated for their association with endophytic fungal species in the above-ground parts. This study assumes importance as the Western Ghats is the heaven for indigenous and endangered medicinal plants (Sringeswara et al., 2009) including grass species.

The main objective of the present study was to characterize and document the endophytic fungal species associated with the above-ground parts of perennial tropical grass species, *C. dactylon* and *D. aegyptium.* The diversity, evenness and richness of the associated fungal endophytes were determined. Certain endophytic fungi occurring in these grass species were also studied for their antimicrobial and antioxidant activities.

Materials and Methods

Selection of study site and identification of grass species

Lakkavalli forest (13° 34' - 13° 39' N latitude , 75° 30' - 75° 39' E longitude) located in the boundary line of the Bhadra Wildlife Sanctuary, that lies in the Central Western Ghats region of Karnataka, was selected as the study area. *Cynodon dactylon* (L.) Pers. and *Dactyloctenium aegyptium* (L.) P. Beauv., growing abundantly in two study sites were selected for studying the association of endophytic fungal flora during 2009-2011.

Three quadrats (1×1 m each) were identified in each study site, located 2 km apart from each other. The study area with red loam soil (pH 5.32) received an annual rainfall of 150-250 cm and experienced the temperature of 14.5-31.5°C. The plant species flowered profusely during August-September period. The two plant species were collected and identified based on the habit and flower characteristics (Bhat and Nagendran, 2001; Vasanthakumari et al., 2010). The morphological and floral characteristics of grass species were confirmed with those described in standard manuals and flora (Bor, 1960; Yoganarasimhan et al., 1982; Sreekumar and Nair, 1991).

Isolation and characterization of endophytic fungal species

The selected grass species, growing in study sites at flowering stage were identified for the collection of samples like leaf, inflorescence and pseudo-stem. Samples were collected in moistened polypropylene covers and processed in the laboratory within 12 h. The plant samples were washed in running tap water, surface disinfected (sodium hypochlorite 0.5%, 2 min followed by 70% ethanol, 2 min), and rinsed with sterile distilled water. Samples were cut into 1-cm-long segments in aseptic condition and incubated by two methods- potato dextrose agar and malt extract agar media (PDA/MEA, Himedia Laboratories, Mumbai) The surface disinfected samples were placed on PDA and MEA supplemented with chloramphenicol (100 mgl^{-1}) contained in Petri dishes (9-cm-dia) and incubated under 12/12h light/nUV light regime at 21±2° C for 7 to 12 days (Achar and Shivanna, 2013). The fungal species expressing on incubated segments were identified based on features of fruiting bodies and spores using standard mycological reference manuals (Barnett, 1972; Ellis, 1976; Arx, 1981). The identification of the species was confirmed by visiting Index Fungorum (Anon, 2012). The fungal isolates failing to sporulate in any one of the above incubation methods and lacking in croziers or clamps were considered as non-sporulating fungi (NSF).

The genomic DNA from the mycelia mat of non-sporulating isolates was extracted by CTAB method to amplify the ITS1 and ITS2 regions (5.8S and D1/D2 domains of 28S region) of nuclear rDNA using ITS1 and ITS4 primers (White et al. 1990). The reaction mixture (25 µl) consisted of 150 ng of DNA, 1 mM of each dNTP, 1X PCR buffer (1.5 mM $MgCl_2$)**,** 1 unit *Taq* polymerase (Chromous Biotech, Bangalore, India) and 5 pM ITS1and ITS4 primers. The reaction was performed in an Eppendorf thermal cycler set to an initial denaturation at 94^{o} C for five min, followed by 40 cycles of denaturation at 95^{o} C for one min, annealing at 53^{o} C for one min, extension at 72^{o} C for five min and final extension at 72^{o} for 10 mins. The amplicons were sequenced at Chromous Biotech, Bangalore, India (Vasanthakumari and Shivanna, 2013).

Preparation of crude extracts for phytochemical and biological assays

The endophytic fungal isolates (*Aspergillus brasiliensis, Aspergillus terreus, Chaetomium globosum, Cochliobolus hawaiiensis, Cochliobolus lunatus, Cochliobolus spicifer, Cochliobolus geniculatus, Fusarium oxysporum, Myrothecium roridum, Penicillium citrinum,* and *Khuskia oryzae* from *C. dactylon* and *D. aegyptium*) were randomly selected for determining their biological activities. The isolates are initially cultured on PDA and incubated, as described previously. Culture discs (5-mm-dia) were obtained from the actively growing margin of colony culture 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 a period of 8-10 days under stationary conditions, with intermittent shaking. The culture broth was filtered through three-layered muslin cloth to separate out the mycelial mat (MM) from the culture filtrate (CF). The filtrate was filtered through three layered Whatman no.1 filter paper discs. To the filtrate, equal volume of ethyl acetate (Himedia, Mumbai) was added, mixed well for 10 min and kept for 5 min until two clear immiscible layers were formed. The upper layer of ethyl acetate containing the extracted compounds was extracted twice and the pooled ethyl acetate fractions of CF were evaporated to dryness at room temperature using the rotary flash evaporator (Heidolph, Germany). The MM was dried in an oven (34°C) for 24 h and the dried mat was ground into a fine powder in a pestle and mortar using the liquid nitrogen. The powder was then transferred into a vial containing methanol (Himedia, Mumbai) and shaken in a water bath at 60°C for 3-4 h and filtered with cheese cloth. The filtrate was collected and evaporated to dryness using the rotary flash evaporator. The extract residue was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored at 4°C to be used as stock solution for determining the antioxidant and antimicrobial activities.

Samples of the above grass species were collected, dried in shade, ground and extracted separately in methanol. The ethyl acetate and methanol extracts were evaporated to dryness and subjected to phytochemical, antimicrobial and antioxidant assays *in vitro*.

Phytochemical assay

The crude extracts of selected fungal endophytes and two grass species were assayed for the presence of secondary metabolites like alkaloids, phenols, flavonoids, glycosides, lignans, saponins, tannins and triterpenoids by following the standard methodologies described by Harborne (1984).

Antimicrobial assay in vitro

One hundred milligrams of crude extract of CF or MM from the previously listed endophytic fungal species were dissolved in one millilitre of DMSO to obtain the stock solution. Subsequently, 100 µl of stock solution was added to 900 µl of DMSO to obtain a concentration of 1000 µg ml^{-1}. From the latter, 500 µl was added to 500 µl of DMSO to obtain the concentration of 500 µg ml^{-1}, and from this concentration 500 µl was taken and added with 500 µl of DMSO to obtain the concentration of 250 µg ml^{-1}. From each of these concentrations, 20 µl (20, 10 or 5 µg ml^{-1}) was added to each well to determine the antimicrobial activity according to the modified method of Nath (2012). All the test organisms were obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh, India. Two Gram-positive bacteria - *Staphylococcus aureus* (MTCC 3160) and *Enterococcus faecalis* (MTCC 439), three Gram-negative bacteria - *Escherichia coli* (MTCC 723), *Pseudomonas fluorescens* (MTCC 9768) and *Salmonella enterica* (MTCC 734), one clinical fungal pathogen, *Candida albicans* (MTCC 3017) and three other fungal species - *Aspergillus flavus* (MTCC 2813), *Fusarium oxysporum* (MTCC 2485) and *Trichoderma harzianum* (MTCC 936) were selected for the study.

Antioxidant assay

The ethyl acetate extract of CF and methanol extracts of MM and grasses were tested for 2, 2'-Diphenyl-1-picrylhydrazyl

(DPPH, *Sigma Aldrich*, USA) radical scavenging activity. The fungal and grass extracts were prepared by dissolving the crude in methanol and concentrations were derived as described previously. The assay was performed according to Sharma and Bhat (2009) with slight modification. The reaction mixture was incubated at 37°C for 30 min and the absorbance was measured (517 nm) using the enzyme-linked immunosorbent assay (ELISA) plate reader (TECAN, Switzerland). Ascorbic acid (*Sigma-Aldrich*, USA) was used as the standard. The antioxidant activity is given as DPPH scavenging activity (%) using the formula: [(control absorbance – extract absorbance) ÷ (control absorbance) × 100].

Characterization of partially purified crude

The ethyl acetate and methanol extracts of CF and MM, respectively of the endophytic fungus *C. lunatus* isolate 57 from *C. dactylon*, which showed prominent biological activities, were characterized by employing Fourier transform infrared (FT-IR) spectroscopy and Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) techniques. The crude samples were purified and subjected to FT-IR and NMR analysis by Radiant Research Services Pvt. Ltd., Bangalore.

Each of the extract was mixed well with potassium bromide and this mixture was made into circular pellets and placed in the IR holder and transmission was recorded in the range of 4000 - 400 nm (JASCO, Model FT/IR-5300).

The analysis of the sample was also done using the NMR (400 MHz Supercon multi nuclei probe BRUKER, West Germany). The samples were studied for H^1 and ^{13}C nuclei and determined for the CH, CH_2 and CH_3 carbons. The samples were first dissolved in $CDCl_3$ (deuterated chloroform) and DMSO solvents and then subjected to NMR study and H^1 and ^{13}C detections were recorded.

Statistical methods

The colonization frequency (%) of fungal isolates was calculated as described by Suryanarayanan et al (2000). The data of endophytic fungal occurrence were analyzed by Simpson and Shannon diversity and evenness indices and species richness by using PAST version, 1.00 (Hammer et al., 2001). The mean values of antibacterial activity were subjected to arcsine transformation before analysing by Duncan's multiple range test (DMRT, P=0.05). The mean values of antifungal and antioxidant activities were used for determining the standard error.

Results and Discussion

Lakkavalli forest region of the Western Ghats supported abundant individuals of *C. dactylon* and *D. aegyptium*, throughout the study period and area. Incubation of the above-ground parts of these grass species resulted in the expression of fungal communities of anamorphic and teleomorphic ascomycetes and non-sporulating fungi (Table 1). There were 26 species of 15 genera of 9 families and with uncertain placement - Incertae sedis (*Myrothecium roridum*) in *C. dactylon* and 30 species of 16 genera of 11 families in *D. aegyptium*. Reports are available on the occurrence of anamorphic and teleomorphic ascomycetous endophytic fungi from *Dactylis glomerata* and other coastal grass species, respectively, (Marquez et al., 2007, 2008) and certain medicinal plants (Budhiraja et al., 2012; Sadrati et al., 2013). The occurrence of endophytic fungal species in plant segments varied; more anamorphic than teleomorphic ascomycetous isolates

expressed on PDA followed by MEA. Attempts have been made to identify endophytic fungal species by the above methods (Amirita et al., 2012) and MEA has been suggested to be better than PDA (Sun et al., 2013). The abundant expression of endophytic fungal species in *C. dactylon* and *D. aegyptium* on PDA (Table 1) could be attributed to nutrients in the medium (Shivanna and Vasanthakumari, 2011). Previous work on these grass species showed that PDA rather than Czapek dox agar medium supported better expression of rhizosphere and rhizoplane fungal species (Shivanna and Vasanthakumari, 2011).

Seventeen and 18 species each of 11 genera of anamorphic ascomycetes occurred in *C. dactylon* and *D. aegyptium,* respectively. Among them, *Cladosporium* sp. that occurred in high percentage in *C. dactylon* have been commonly identified as endophytic fungi in other plant species (Wang et al., 2007; Purmale et al., 2012). In case of *D. aegyptium, T. harzianum* and species of *Aspergillus* and *Cladosporium* occurred in high percentage. Some of the above have also been documented in tissues of *Calotropis procera* and *Acalypha indica*. (Rezwana et al., 2007; Banerjee et al., 2010). The PDA and MEA supported the good expression of several anamorphic ascomycetes. Anamorphic ascomycetes generally occur in large number upon incubation of living and dead plant tissues (D'Souza and Bhat, 2013). Some of the other fungal species that occurred in at least one of the methods and grass species are listed in Table 1.

In respect of teleomorphic ascomycetes, there were 11 and 9 species each of five and four genera in *C. dactylon* and *D. aegyptium,* respectively (Table 1). High expression of anamorphic rather than the teleomorphic ascomycetes (Pragathi et al., 2013) could be due to the common occurrence of asexual rather than the sexual stage. *Phoma* sp. occurred in high percentage on PDA in *C. dactylon* than in *D. aegyptium*; *Phoma* spp. expressed on PDA, when infected plant materials were incubated (Rebecca et al., 2011). *Glomerella cingulata* occurred exclusively in *D. aegyptium* on both PDA and MEA.

On the other hand, the NSF occurred more frequently in MEA than PDA. Three NSF isolates (NSF CB 28, NSF 21 and NSF 62) failed to sporulate on culture for a long period or under nUV; they failed to produce clamps or crosiers. Based on the ITS regions of rDNA and BLAST search, one of the isolates (NSF CB 28) was characterized as *Cochliobolus geniculatus* (Acc. no. KF590040, length 539 bp, identity 99%, Homologue – HE861840.1). *Cochliobolus* species generally sporulate well upon incubation; however the inability of *C. geniculatus* isolate to sporulate in PDA/MEA is not well understood.

The latter two NSF isolates with buff or pink mycelia were identified as *Phoma herbarum* (Acc. no. KF590039, length 475 bp, identity 100%, Homologue – JX867225.1 and Acc. no. – KC821515, length 503 bp, identity 99%, Homologue – JX867225.1). The above non-sporulating isolates of *P. herbarum* failed to produce pycnidia and microsclerotia on PDA. The genus *Phoma* is a complex group of several species that are difficult to identify. The molecular characterization of all the NSF helped in their inclusion under teleomorphic ascomycetes (Table 1). Another NSF from *C. dactylon* with pale brown mycelium on PDA, was comparable to the previously identified *Fusarium oxysporum* (isolate 71) from the root region of both *C. dactylon and D. aegyptium* (Shivanna and Vasanthakumari, 2011).

The species richness in *D. aegyptium* (28) and *C. dactylon* (22) was high on PDA than on MEA (21 and 18) (Table 2). Shivanna and Vasanthakumari (2011) showed high rhizosphere and rhizoplane fungal species richness on PDA in *D. aegyptium*. The Shannon and Simpson diversity indices values on MEA and PDA in the above grass species were almost similar except simpson diversity index on MEA. Most of the endophytic fungal species from *C. dactylon* and *D. aegyptium* have also been reported from the rhizosphere and rhizoplane of the above grass species, in the same region of the Western Ghats (Shivanna and Vasanthakumari, 2011). This suggested the possibility of establishment and localization of endophytic fungi in aerial plant tissues following colonization of rhizosphere and rhizoplane regions. Further experimental studies are required for confirmation of root to shoot transmission of endophytic fungi.

All fungal isolates used were positive for triterpenoids and negative for alkaloids and lignans. However, alkaloid production by grass endophytes have been reported by Clay (1990) and Tan and Zou (2001). Most fungal isolates except *K. oryzae* produced saponins. Flavonoids and tannins were produced by species of *Aspergillus* and *K. oryzae*, while flavonoids and glycosides were produced by *P. citrinum*, *C. spicifer* and *K. oryzae*; glycosides and phenols were produced by *C. globosum* and *C. lunatus*, respectively. The flavonoids produced by many endophytes and grasses might have an active role in antimicrobial activity. Chandrappa et al (2013) reported that flavonoids as well as phenols contributed to the antimicrobial property. In this study, both grass species produced only phenols and flavonoids. In other studies, the above grass species were shown to produce alkaloids (Asthana et al., 2012; Abdallah and El-Ghazali, 2013) that possessed certain biological activities (Sofowora, 1984). The reason for the lack of alkaloids in these grass species is not understood, however their induction due to edaphic and other factors and association with certain endophytic communities cannot be ruled out.

The ethyl acetate and methanol extracts at the concentration of 20 µg ml^{-1} of most fungal isolates showed prominent antibacterial activity to *E. faecalis, S. enterica* and *P. fluorescens* (Table 3). Moderately high antibacterial activity (>30 mm ZI) was expressed by the methanol extract of *C. hawaiiensis* to *S. enterica.* However, moderate antibacterial activity (15-20 mm ZI) was shown by CF of *P. citrinum* to *E. faecalis* and *P. fluorescens, C. lunatus* to *S. enterica* and *C. hawaiiensis* and *C. geniculatus* to *E. faecalis.* As compared to standard control, endophytes like *C. geniculatus, P. citrinum, C. globosum* and *C. lunatus* produced moderately high activity to *P. fluorescens.* On the other hand, moderate activity of mycelial metabolite was recorded in *C. lunatus* to all bacteria; *M. roridum* to all except *P. fluorescens* and *C. globosum* to all except *E. coli*. Garcia et al (2012) reported that one of the metabolites extracted from the *Cochliobolus intermedius* showed activity to *E. coli*, *S. aureus*, S. *typhi*, and other bacteria.

In the present study, the methanol extract from *C. globosum* mycelia had moderately high antibacterial activity to most bacterial species. Pervez et al (2012) showed that species of *Myrothecium* have strong antibacterial effect. Moderate activity was also shown by *P. citrinum, C. spicifer, C. geniculatus* and *K. oryzae* only to *E. faecalis* (Table 3). The literature review indicated that methanol and ethyl acetate were commonly used in the extraction of antimicrobial compounds (Tayung et al.,

2012; Wang et al., 2013). However, ethyl acetate extract of endophytic fungal species showed high antibacterial rather than the antifungal activities (Garcia et al., 2012; Rhoden et al., 2012).

In comparison to the high antibacterial activity expressed by 10 endophytic fungi, seven isolates expressed low (< 15 mm ZI) to moderately high (< 20 mm ZI) antifungal activity *in vitro* in the ethyl acetate and methanol extracts (Table 4). *Penicillium citrinum* produced low activity (< 15 mm ZI) to *T. harzianum.* The CF and mycelial extracts of all seven fungal species (except CF of *P. citrinum*) produced low to moderately high activity to *C. albicans*. The CF of *M. roridum* was effective to *C. albicans* even at 5 µg ml^{-1} (15 mm) and 10 µg ml^{-1} (21 mm). Similarly, the MM extract of *C. hawaiiensis* produced moderate activity (17 mm) to the above pathogen. Several reports are available on the antifungal activity of endophytic extracts on *C. albicans* (Powthong et al., 2012; Jirayu et al., 2011) as compared to a few on phytopathogenic fungi (Tejesvi et al., 2007; Camila et al., 2011). The CF of *C. globosum* caused less inhibition to *F. oxysporum*. However, *C. globosum* has been reported to produce metabolites with broad antifungal activity (Zhang et al., 2013).

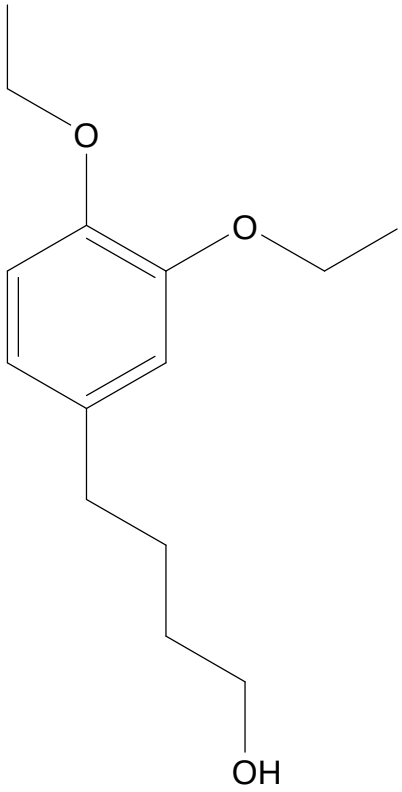
The methanol extracts of *C. dactylon* and *D. aegyptium,* that contain phenolic and flavonoid compounds, did not exhibit either antibacterial or antifungal activity even at 20 µg ml^{-1} of crude extract suggesting that antimicrobial compounds could be produced by certain endophytic fungal species in association and not by grass species alone. However, the reported antimicrobial activities of the above grasses (Asthana et al., 2012; Abdallah and El-Ghazali, 2013) might also depend on the chemical constituents following specific endophytic association.

The DPPH radical scavenging activity of endophytic fungal extracts has also been documented in other studies (Jananie et al., 2011, El-Khawaga et al., 2013).

The crude extracts of endophytic fungal isolates and grass species expressed antioxidant activity very much similar to that of the standard-ascorbic acid. The CF of *C. lunatus* and *C. geniculatus* showed high radical scavenging activity even at the low concentration of 2.5 and 5 µg ml^{-1} in comparison to other endophytic fungal extracts. On the other hand, the CF extract of *C. spicifer* and the MM extract of *C. lunatus* showed high antioxidant activity at 10 µg ml^{-1} of the crude. In comparison, the concentration of 20 µg ml^{-1} of methanol extract of mycelial mat of *K. oryzae* was required to exhibit good antioxidant activity. Govindappa et al (2013) reported that former two endophytes as well as *Trichoderma* sp. have potential *in vitro* antioxidant activities. Nitya et al (2011) reported that *Aspergillus* extract followed by that of *Penicillium* and *Mucor* showed high total antioxidant activity.

The antioxidant activity of *C. dactylon* and *D. aegyptium* was expressed at 20 and 10 µg ml^{-1}, respectively. This property of the grass species could be attributed to their association with endophytic fungi like *Cochliobolus* species and *K. oryzae* and others harboured in tissues. The flavonoids and phenols in *C. dactylon* and *D. aegyptium* could also be accounted for antioxidant activities as opined by Lee et al (2003). The antioxidant activity of *C. dactylon* is well documented in the literature (Jananie et al., 2011; Saroja et al., 2012). *Cynodon dactylon* has been an important component in certain Ayurvedic formulations (Kumar et al., 2011) for treating diseases relating to oxidative stress (Saroja et al., 2012).

Structural formula of 4- [3, 4, diethoxy phenyl-]-1-butanol



Structural formula of Tetradeca-6-enyl acetate

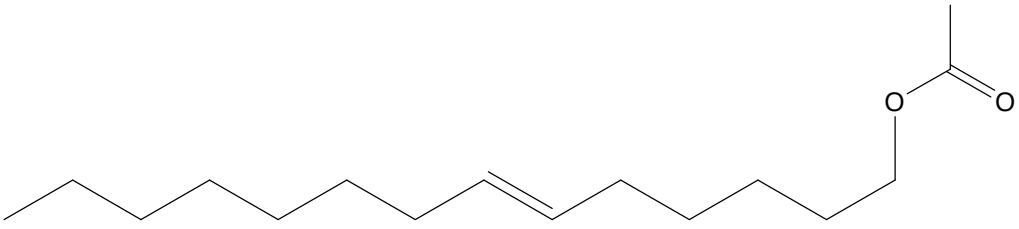


Table.1 Colonization frequency of endophytic fungal species occurring in the aerial parts of *Cynodon dactylon* and *Dactyloctenium aegyptium* by potato dextrose agar (PDA) and malt extract agar (MEA) methods

Endophytic fungal isolates	Frequency of occurrence (%)[1] / Incubation methods			
	Cynodon dactylon		***Dactyloctenium aegyptium***	
	PDA	MEA	PDA	MEA
Anamorphic ascomycetes				
Alternaria alternata (Fr.) Keissl.	0.37	0.0	0.18	0.0
Aspergillus spp.[3]	0.36 (2)[4]	1.83(5)	7.94(6)	16.66(3)
Cephalosporium acremonium Corda	1.48	0.0	0.0	0.0
Cladosporium spp.[5]	22.21(2)	5.37(1)	13.88(2)	17.22(1)
Fusarium spp.[6]	1.85(2)	3.51(2)	2.03(1)	4.62(2)
Macrophomina phaseolina (Tassi) Goid	0.18	0.37	3.7	2.40
Monodictys fluctuata (Tandon & Bilgrami) M.B. Ellis	0.0	0.0	0.37	0.0
Penicillium spp.[7]	0.18(1)	2.96(1)	0.0	0.55(2)
Pestalotiopsis mangiferae (Henn.) Steyaert	0.0	0.92	0.0	0.37
Phomopsis vexans (Sacc. & P. Syd.) Harter	0.0	0.37	0.37	0.0
Trichoderma harzianum Rifai	1.11	0.0	19.07	0.18
Myrothecium roridum Tode	0.92	0.0	1.48	0.0
Total frequency	**28.66**	**15.33**	**49.02**	**42.0**
Teleomorphic ascomycetes				
Chaetomium globosum Kunze	0.37	0.18	1.66	0.0
Cochliobolus spp.[8]	12.39(4)	5.17(5)	4.24(5)	6.65(4)
Khuskia oryzae H.J. Huds	10.73	4.62	0.74	7.03
Phoma spp.[9]	14.62(2)	0	7.58(3)	1.11(1)
Glomerella cingulata (Stoneman) Spauld. & H. Schrenk	0.0	0.0	3.7	2.22
Total frequency	**38.11**	**9.97**	**17.92**	**17.01**

Note: [1]Frequency of fungal endophyte occurrence was calculated based on the number of segments colonized by each fungus over the total number of segments studied and represented as percentage ; [2]Data is an average of three replicates, each with 90 samples; [3]*Aspergillus* species = *A brasiliensis* sensu auct. pro parte, pre (0.18-15.18), *A. flavus* Link (0.18-0.74), *A. fumigatus* Fresen (0.37-0.92), *A. ochraceus* G. Wilh. (0.18-1.11), *A. terreus* Thom (0.18-0.92), *A. stellatus* Curzi (0.0-0.18); [4]Figures in parenthesis indicate total number of species of genera which may vary in different media; [5]*Cladosporium* species = *C. cladosporioides* (Fresen.) G.A. de Vries (5.37-17.22), *C. herbarum* (Pers.) Link (5.74-7.03); [6]*Fusarium* species = *F. oxysporum* E.F. Sm. & Swingle (0.18-2.03), *F. oxysporum* NSF isolate 71 (0.37-3.88) ; [7]*Penicillium* species = *P. citrinum* Thom (0-0.18), *P. commune* Thom (0.37-2.96); [8]*Cochliobolus* species = C. *geniculatus* R.R. Nelson (0.18-0.92), *Cochliobolus hawaiiensis* Alcorn (0.37-2.22), *Cochliobolus lunatus* R.R. Nelson & F.A. Haasis (0.37- 3.14), *Cochliobolus spicifer* R.R. Nelson (0.55-6.29), and *C. geniculatus* R.R. Nelson NSF isolate CB 28 (0.55-3.51) ; [9]*Phoma* species = *Phoma* sp. (0.00-5.92), *P. herbarum* Westend NSF isolate 21 (0.92-10.55), *P. herbarum* NSF isolate 62 (0.74-4.07).

Table.2 Species richness, diversity and evenness indices of endophytic fungal communities occurring in the aerial parts of Cynodon dactylon and Dactyloctenium aegyptium by PDA and MEA methods*

Grass species/ Incubation method	Species richness	Diversity index		Evenness index	
		Shannon (H')	Simpson (D')	Shannon (J')	Simpson (E')
Cynodon dactylon					
PDA	22	2.42	8.32	0.78	0.37
MEA	18	2.36	8.41	0.80	0.44
Dactyloctenium aegyptium					
PDA	28	2.67	9.02	0.79	0.31
MEA	21	2.24	6.38	0.73	0.30

Note: * Data is an average of three replicates, each with 90 samples

Table.3 Antibacterial activity of solvent extracts of endophytic fungi isolated from *Cynodon dactylon* and *Dactyloctenium aegyptium*

Crude extract of fungal isolates	Zone of inhibition (mm)[1]/ test bacterial isolates				
	Sa [2]	**E f** [2]	**Se** [3]	**Ec** [3]	**Pf** [3]
Ethyl acetate extract of culture filtrate					
A. terreus isolate 72 [4]	0	13 b	15 a	11 c	11 c
C. geniculatus NSF isolate 28 [4]	12 b	15 a	11 c	10 d	14 a
C. hawaiiensis isolate 56 [4]	11 b	17 a	0	11 b	10 b
C. lunatus isolate 57 [4]	13 b	12 b	19 a	11 b	13 b
C. spicifer isolate 58 [4]	0	13 a	15 a	0	11 b
K. oryzae isolate 60 [4]	12 b	15 a	11 c	10 d	14 a
M. roridum isolate 87 [5]	0	11 a	11 a	10 a	0
P. citrinum isolate 81 [5]	0	20 a	0	12 c	15 b
Methanol extract of mycelial mat					
A. brasiliensis isolate 67 [5]	0	10 b	12 a	10 b	13 a
A. terreus isolate 72 [5]	0	10 b	13 a	0	10 b
C. geniculatus NSF isolate 28 [4]	0	16 a	13 a	11 b	11 b
C. globosum isolate 54 [4]	16 b	20 a	16 b	13 c	15 b
C. hawaiiensis isolate 56 [4]	0	17 b	31 a	17 b	0
C. lunatus isolate 57 [4]	18 a	18 a	15 b	15 b	15 b
C. spicifer isolate 58 [4]	0	17 a	13 b	11 c	10 c
K. oryzae isolate 60 [4]	14 b	18 a	13 b	12 c	0
M. roridum isolate 87 [5]	16 b	16 a	18 a	16 a	13 c
P. citrinum isolate 81 [5]	14 b	16 a	10 c	0	14 b
Chloramphenicol [6]	40 a	40 a	40 a	40 a	25 b

Sa= *Staphylococcus aureus*, **Ef**= *Enterococcus faecalis*, **Se**= *Salmonella enterica*, **Ec**= *Escherichia coli*, **Pf**=*Pseudomonas fluorescens*

Note: [1]Values were subjected to arcsine transformation before statistical analysis; Values are mean inhibition zone of three replicates (n=3).

[2] Gram positive bacteria; [3] Gram negative bacteria;

[4] Isolates of *Cynodon dactylon*

[5] Isolates of *Dactyloctenium aegyptium*

[6] Chloramphenicol and crude extracts were used at 20 µg ml^{-1} (per well).

Table.4 Antifungal activity of solvent extracts of endophytic fungi isolated from *Cynodon dactylon* and *Dactyloctenium aegyptium*

Crude extract of fungal isolates	Zone of inhibition (mm)[1] ± standard error/ test fungal isolates		
	Candida albicans[2]	*Fusarium oxysporum*[3]	*Aspergillus flavus*[3]
Ethyl acetate extract of culture filtrate			
A. terreus isolate 72 [5]	13±0.67	0	0
C. geniculatus NSF isolate 28 [4]	13±0.33	0	0
C. globosum isolate 54 [4]	10±0.33	10±0.33	0
C. lunatus isolate 57 [4]	13±1.67	13±0.33	0
K. oryzae isolate 60 [4]	13±0.88	0	0
M. roridum isolate 87 [5]	26±0.33	0	16±1.73
P. citrinum isolate 81 [5]	0	0	12±0.88
Methanol extract of mycelial mat			
C. geniculatus NSF isolate 28 [4]	10±0.0	0	0
C. globosum isolate 54 [4]	13±0.88	11±0.58	0
C. hawaiiensis isolate 56 [4]	21±0.58	0	0
C. lunatus isolate 57 [4]	12±0.0	0	0
K. oryzae isolate 60 [4]	14±0.88	0	0
M. roridum isolate 87 [5]	16±0.33	0	0
P. citrinum isolate 81 [5]	14±0.33	12±0.33	18±0.58
Control	30±0.0 (flucanozole)	30±0.0 (bavistin)	30±0.0 (bavistin)

Note: [1]Values are mean inhibition zone of three replicates (n=3); the crude extracts did not show antifungal activity to *T. harzianum.*
[2]Clinical pathogen; [3]Plant pathogen, saprophyte and antagonistic (*T. harzianum*) fungi, respectively.
[4] Isolates of *Cynodon dactylon*
[5] Isolates of *Dactyloctenium aegyptium*
An antifungal compound- Mancozeb for *T. harzianum* (28 mm ZI); crude extracts were used at 20 µg ml^{-1} (per well).

Table.5 Antioxidant activity expressed by certain endophytic fungal isolates and grasses *Cynodon dactylon* and *Dactyloctenium aegyptium*

Crude extract of fungal isolates	Radical scavenging activity (%) ± standard error
Ethyl acetate extract of CF	
C. lunatus isolate 57 [1]	69.63±0.02
C. spicifer isolate 58 [1]	67.98±0.01
C. geniculatus NSF isolate 28 [1]	71.76±0.01
Methanol extract of MM	
C. lunatus isolate 57 [1]	67.21±0.01
K. oryzae isolate 60 [1]	68.97±0.05
Grass species	
C. dactylon	69.27±0.04
D. aegyptium	66.59±0.02
Ascorbic acid (Standard)	78.40±0.01

Note: The radical scavenging activity of the crude extract was determined by DPPH method; The reaction mixture received 30 µl of crude extract (30 µg ml^{1}).
[1] Isolates of *Cynodon dactylon*
[2] Isolates of *Dactyloctenium aegyptium*

The antioxidant activity of certain endophytic fungal isolates in the study assumes greater importance in human welfare as their association in these forage grass species could be used in alternative traditional system of medicine".

Spectral characteristics of fungal extracts

Among the fungal isolates used for determining the biological activities, *C. lunatus* isolate 57 from *C. dactylon* yielded high quantities of the metabolite in the ethyl acetate and methanol fractions. These fractions showed moderate to high antibacterial, antifungal and antioxidant activities. The mycelia mat (RR 1384) extract of *C. lunatus* was brown and paste like (162 mg g^{-1}) and the ethyl acetate (RR 1385) fraction was a dark brown paste (0.077 mg l^{-1}). Both the extracts were partially purified using the respective solvent systems. The phytochemical investigation by qualitative assay indicated the presence of triterpenoids and phenolic compounds in both the extracts. The compound obtained from the ethyl acetate fraction (RR1385) could be an alcohol having the molecular formula $C_4H_{22}O_3$. The IR spectrum of this compound, exhibited OH group (3413 cm^{-1}), C-H group (2920 cm^{-1}) and a carbonyl group (1704 cm^{-1}). The ^1H NMR spectrums exhibited two signals for free phenolic group at δ_H 9.62 and 9.55. The aromatic protons showed the presence of trisubstituted benzene ring system at δ_H 7.19, 6.53 and 6.45. There was a methyl group at δ_H 0.96 and long chain methylene

groups at δ_H 1.26. The ^{13}C NMR spectrum exhibited the benzene ring system at δ_C 147.73, 146.00, 126.63 and 128.35. Minor signals between δ_C 55.55 to 61 and δ_C 24 to 32 were due to carbon atoms under oxygen fraction and methylene carbons, respectively. Based on this information the compound was identified as 4- [3, 4, diethoxy phenyl-]-1-butanol.

This natural compound has not been isolated from any biological materials and characterized in any of the previous reports. The present study indicated that the above two novel compounds extracted from *C. lunatus* could be exploited for antimicrobial and antioxidant properties. The functionality of these compounds produced by this endophytic fungus in *C. dactylon* grass species is not known. However these compounds might have implications in the protection of plants from microbial infection. The antioxidant activities of these compounds could be contributing to the health of the herbivore vertebrates that consume grasses as fodder.

The NMR and FT-IR data analysis of the methanol extract indicated that the compound might be an unsaturated ester with the molecular formula $C_{16}H_{30}O_2$ **.** The IR spectrum exhibited the presence of hydroxyl group (3419 cm^{-1}), C-H group (2849 cm^{-1}) and ester carbonyl group (1742 cm^{-1}). The ^1H NMR spectrum exhibited signal for unsaturated protons δ_H at 5.36 and two signals for CH_2OH protons at δ_H 4.32 and 4.14. The signals at δ_H 2.32 and 2.64 were due to methylene group adjacent to C=C bond. On the other hand ^{13}C NMR spectrum exhibited signals at δ_C 14.08 for a methyl carbon, δ_C 130.02 for C=C carbon atoms. The signals at δ_C 22.67, 27.12, 29.12, 29.51, 29.68, 31.90 and 40.99 were due to methylene groups in the long chain hydrocarbon. Further the ^{13}C NMR did not show the presence of carbonyl group and the double bonds were positioned randomly. Based on the above the structure has been established, which might belong to compound Tetradeca-6-enyl acetate.

This natural compound obtained from *C. lunatus* could be related to a compound extracted from *Muscodor yucatanensis,* a tropical endophytic fungus occurring in *Bursera simaruba*, where in the compound with the molecular formula of $C_{16}H_{32}O_3$ was identified as Tridecan-3-yl 2-methoxyacetate. **(**Martha *et al.,* 2010). Other than this there was no information available in the literature on the compound extracted from the mycelia of *C. lunatus*.

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