academicJournals

Vol. 12(27), pp. 4317-4323, 3 July, 2013
DOI: 10.5897/AJB2013.12154
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Diversity of fungal endophytes and their bioactive metabolites from endemic plants of Tirumala hills-Seshachalam biosphere reserve

D. Pragathi[1], T. Vijaya[2]*, K. C. Mouli[1] and D. Anitha[1]

[1]Department of Biotechnology, Sri Venkateswara University, Tirupati-517 502, A.P, India.
[2]Department of Botany, Sri Venkateswara University, Tirupati-517 502, A.P, India.

Accepted 31 May, 2013

This study investigates the endophytic fungal diversity of three endemic plants of Tirumala hills also their capabilities for the production of hydrolysing enzymes and secondary metabolites. Our study provides the first evidence on the diversity, enzyme and metabolite charecterisation of fungal endophytes from the untapped endemic plants of Tirumala Hills of Seshachalam Biosphere Reserve, Easternghats, Andhra Pradesh, India. A total of 13 endophytic fungi isolates were obtained and grouped into seven genera based on the morphological traits, indicating endophytic fungi in *Shorea thumbuggaia, Boswellia ovalifoliolata*, *Pterocarpus santalinus* were diverse and abundant. *Fusarium, Penicillium, Aspergillus* and *Colletotrichum* were the dominant genera, whereas the remaining genera were less frequent. The 13 representative species of the distinct genera were capable of producing hydrolysing enzymes. Phytochemical analysis showed the production of various secondary metabolites that included saponins, carbohydrates, phenolics, glycosides and flavonoids. This investigation also reveals that the metabolites produced by a variety of endophytic fungi can be a potential source of novel natural therapeutic agents.

Key words: Endemic plants, fungal endophytes, hydrolyzing enzymes, secondary metabolites, Tirumala Hills.

INTRODUCTION

Endophytic fungi are microorganisms that reside in living plant tissues, apparently without imposing negative effects. They are ubiquitous and have been found in all plant species examined to date (Stone et al., 2000). There is growing interest in endophytes and their origins, their biodiversity, endophyte-host interactions, their role in ecology and the characterisation of their secondary metabolites (Arnold, 2007; Saikkonen et al., 2004). However, only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology (Strobel et al., 2004). Fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Tan and Zou, 2001; Strobel and Daisy, 2003; Kumar and Hyde, 2004; Kumar et al., 2005). Hence, there are major efforts to isolate and characterize endophytes from plants. At present, much research has focused on isolation of endophytic fungi from pharmaceutical plants, such as *Camptotheca acuminata* (Lin et al., 2007), *Taxus* plants (Zhang et al.,

*Corresponding author. E-mail: tarttevijaya@yahoo.co.in

Abbreviations: PDA, Potato dextrose agar; **PVLG,** polyvinyl lactic acid glycerol; **CF,** colonization frequency; **DMSO,** dimethylsulphoxide.

2007), discovering a vast number of undescribed endophytic fungi species, some of which have potential to be used in the production of medicine. Ongoing global efforts to discover novel compounds from endophytic fungi isolated from medicinal plants are yielding good results (Liu et al., 2004; Li et al., 2005; Phongpaichit et al., 2006; Zhou et al., 2007). Therefore, investigations of endophytic fungi are crucial for conservation and utilization of fungal resources in plants. Tirumala hills, Seshachalam biosphere reserve of Easternghats possesses rich floristic wealth and diversified genetic resources of medicinal plants. However, the community of endophytic fungi of endemic plants at Tirumala Hills in India has not been described. The present study was undertaken to investigate the diversity and characterization of endophytic fungi and in endemic medicinal tree taxa in the Tirumala Hill region, Eastern Ghats of Andhra Pradesh, Southern India.

MATERIALS AND METHODS

Sample collection

Twenty segments of each leaf and stem samples from *Shorea thumbuggaia* (*Dipterocarpaceae*), *Boswellia ovalifoliolata* (*Burseraceae*) and *Pterocarpus santalinus* (*Fabaceae*) were randomly collected from Tirumala Hills of Seshachalam range, Easternghats, Andhra Pradesh, India. Immediately after the collection, plant parts were washed with tap water and processed for isolation of endophytic fungi.

Isolation of fungal endophytes

Surface sterilization of samples was done by cleaning leaves and stems under running tap water and cutting them into 1 cm segments followed by stepwise washing with 70% ethanol for 2 min, 3% calcium hypochlorite solution for 5 min and 70% ethanol for 30 s followed by two rinses in sterile distilled water, then allowed to surface dry under sterile conditions (Arnold et al., 2000).

The Leaf segments were placed on 9 cm Petri plates containing potato dextrose agar (PDA, Hi Media Laboratories, Mumbai, India) medium amended with streptomycin (250 mg L^{-1}) to suppress bacterial growth. The efficacy of surface sterilization was confirmed by pressing the sterilized segments on to the surface of PDA medium. The absence of growth of any fungi on the medium confirmed that the surface sterilization procedure was effective (Schulz et al., 1993). Petri plates were incubated at 28 ±1°C with a 12 h photoperiod, and sporulation was induced by incubation in a light chamber under near ultraviolet (UV) light for 1 to 12 days. Fungi growing out from the cut ends of the segments were subsequently transferred onto fresh PDA plates. Pure cultures were spread on fresh PDA slants.

Identification of endophytic fungi

Fungal mycelium was stained in cotton blue and mounted in polyvinyl lactic acid glycerol (PVLG) by heating at 65°C for two to three days and observed under microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, colour of colony on the medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spore (Barnett and Hunter, 1956). An Olympus CX13 with interference contrast was employed for examination by light microscopy. Cultures that failed to sporulate were recorded as sterile form. The identification of the isolates was confirmed by expert taxonomists at the Agarkhar Research Institute, Pune, Maharashtra, India.

Colonization frequency of fungal endophytes

The colonization frequency (CF%) of each endophyte was calculated according to the method of Hata and Futai (1995):

$$CF = (N_{COL}/N_t)\times 100$$

Where, N_{COL} is the number of segments colonized by each fungus and N_t is the total number of segments.

Tests for enzymatic activities

Thirteen (13) endophytic fungal isolates from three endemic plants were tested for hydrolytic capabilities. Enzymes hydrolyzing complex carbon molecules, amylase, cellulase, polyphenol oxidases were tested (Keerthi et al., 2010). Confirmed endophytes also were tested for gelatinase. Each test medium was inoculated with a 6 mm fungal plug cored from PDA, and each isolate was incubated in triplicates for two weeks at room temperature. Polyphenol oxidase test were incubated for three weeks. A basal medium (Caldwell et al., 1991) composed of mineral salts was used for amylase and cellulase evaluation.

Fermentation and extraction

All the endophytic fungal isolates were grown on PDA plates at 30°C for seven to 14 days depending on growth rate. Six pieces (8×8 mm^2) of the grown culture cut from the plate were inoculated into a 1000 ml Erlenmeyer flask containing 200 ml of PDA broth (Paterson and Bridge, 1994). After incubation at 25°C for 21 days under stationary condition, the fungal culture was filtered to remove mycelium. The filtered broth was then extracted with 200 ml of dichloromethane three times. The organic phase was evaporated to dryness under reduced pressure using a rotary evaporator and weighed to constitute the crude broth extract. The fungal mycelia were freeze dried and then disrupted using a spatula and extracted twice by soaking in a mixture of dichloromethane/methanol (1:1, v/v) for 1 h. The two mycelial extracts from each fungus were pooled and air-dried and weighed to constitute the crude mycelial extract. Crude extracts from the culture broth and mycelium were dissolved separately in dimethylsulphoxide (DMSO, Merck) depending on solubility. Equal amounts of the crude extracts obtained from culture broth and mycelium were combined.

Phytochemical screening of endophytic fungal extracts

Preliminary phytochemical screening of the crude extracts of the endophytic fungal isolates was carried out with the methods outlined by Trease and Evans (1983).

RESULTS AND DISCUSSION

A total of 13 fungal isolates were recovered from 120 segments incubated from 3 endemic medicinal shrubs. These isolates belonged to Ascomycetes (7.69%), Coelomycetes (15.3%), Hyphomycetes (53.8%), and ste-

Table 1. Colonisation frequency (%) of fungal endophytes isolated from endemic plants of Tirumala Hills.

Endemic tree taxa	Fungal isolate	Fungal class	Macro and microscopic identification	Colonisation frequency (%)
Shorea thumbuggaia	EF1	*Hyphomycetes*	*Pencillium corylophilum*	15
	EF2	*Coelomycetes*	*Phyllosticta* sp.	4
	EF3	*Coelomycetes*	*Pestalotiopsis maculans*	6
Boswellia ovalifoliolata	EF4	*Hyphomycetes*	*Fusarium* sp.	12
	EF5	*Hyphomycetes*	*Fusarium oxysporum*	4
	EF6	Not identified	Sterile	8
	EF7	*Hyphomycetes*	*Pencillium* sp.	18
	EF8	*Hyphomycetes*	*Aspergillus aculeatus*	8
	EF9	*Hyphomycetes*	*Aspergillus* sp.	12
Pterocarpus santalinus	EF10	*Ascomycetes*	*Cochliobolus lunatus*	20
	EF11	Not identified	Sterile	10
	EF12	*Hyphomycetes*	*Colletotrichum gloeosporioides*	16
	EF13	Not identified	Sterile	4

EF, Endophytic fungal isolates.

rile forms (23.0%) (Table 1). Colletotrichum, *Fusarium*, *Penicillium*, *Aspergillus* were the most frequently isolated genera. Colonization did not differ significantly among endemic medicinal species but ranged from 30.7% in *P. santalinus* and *B. ovalifoliolata* to a maximum of 38.4% in *S. thumbuggaia*. Sterile forms were isolated frequently from *B. ovalifoliolata* (7.69 %) and *P. santalinus* (15.3 %). Figures 1 and 2 depict the details of each morphologically identified taxon of the endophytic fungal isolates.

Results of enzyme hydrolysis are provided (Table 2). Most isolates tested positive for all extracellular enzyme activities. The intensity of enzyme hydrolysis varied among taxa. Gelatinase was also produced by all isolates. Polyphenol oxidases are produced by all isolates except *Phyllosticta* sp*., Pestalotiopsis maculans*, *Cochliobolus lunatus* and *Colletotrichum gloeosporioides*. The phytochemical analysis of the fungal extracts indicated the presence of various secondary metabolites including flavonoids, carbohydrates, phenolics and glycosides, saponins (Table 3).

In our study, calcium hypochlorite (Arnold et al., 2001) was used as a surface disinfectant because it damages less the tissue than sodium hypochlorite (Boccon-Gibod, 1982). The sterility check test showed that our surface sterilization procedure was sufficiently effective. Therefore, we conclude that all isolated fungi are endophytes. The colonization frequency of endophytes in this study was within the range of many host plants studied in the tropics (Frohlich et al., 2000; Photita et al., 2001; Suryanarayanan et al., 2003). *Colletotrichum*, *Phyllosticta*, *Pestalotiopsis*, *Fusarium* sp., which were isolated in this study, have been reported as endophyte genera in a wide host range in the tropics (Azevedo et al., 2000; Suryanarayanan et al., 2002; Photita et al., 2005). In the present study, mycelia sterilia (23.0 %) were isolated as endophytes from three endemic plants and the results are in agreement with studies (Arnold et al., 2000; Frohlich et al., 2000). In a study on the medicinal plant *Tripterygium wilfordii*, mycelia sterilia were isolated as the second most dominant taxa (23.6%) next to coelomycetes (35.0%) (Kumar and Hyde, 2004). Since these non-sporulating mycelia sterilia cannot be provided with taxonomic names without reproductive structures in conventional classification, they are now generally categorized as ‘‘morphotypes’’ based on similar cultural characteristics (Guo et al., 2003; Promputtha et al., 2005).

Our study reports the amylase, cellulase, gelatinase polyphenol oxidase producing capabilities of the isolated endophytes. By contrast, polyphenol oxidases are not produced by some of the isolates, namely *Phyllosticta* sp*., Pestalotiopsis maculans*, *Cochliobolus lunatus* and *Colletotrichum gloeosporioides*. Mandyam and Jumpponen (2005) reviewed the observations of enzymatic activities of endophytic fungi and these included amylase, cellulase, lipase, pectinase, polyphenol oxidase (laccase and tyrosinase), protease and xylanase. Another study with *Periconia* and *Microdochium* isolates produced enzymes capable of metabolizing complex substances (Keerthi et al., 2010). The enzymatic capabilities of the isolated endophytes suggest their potential to access detrital C, N and P with a potential to aid host nutrient uptake.

Our study also investigates the secondary metabolites (saponins, carbohydrates, phenolics, glycosides, flavornoids) isolated from fungal endophytes to understand their ecology and to determine their potential against therapeutic targets. Nitya et al. (2011) reported that the antioxidant capacities and total phenolic contents of endophytic fungi present in the leaves of *Lobelia nicotianifolia*

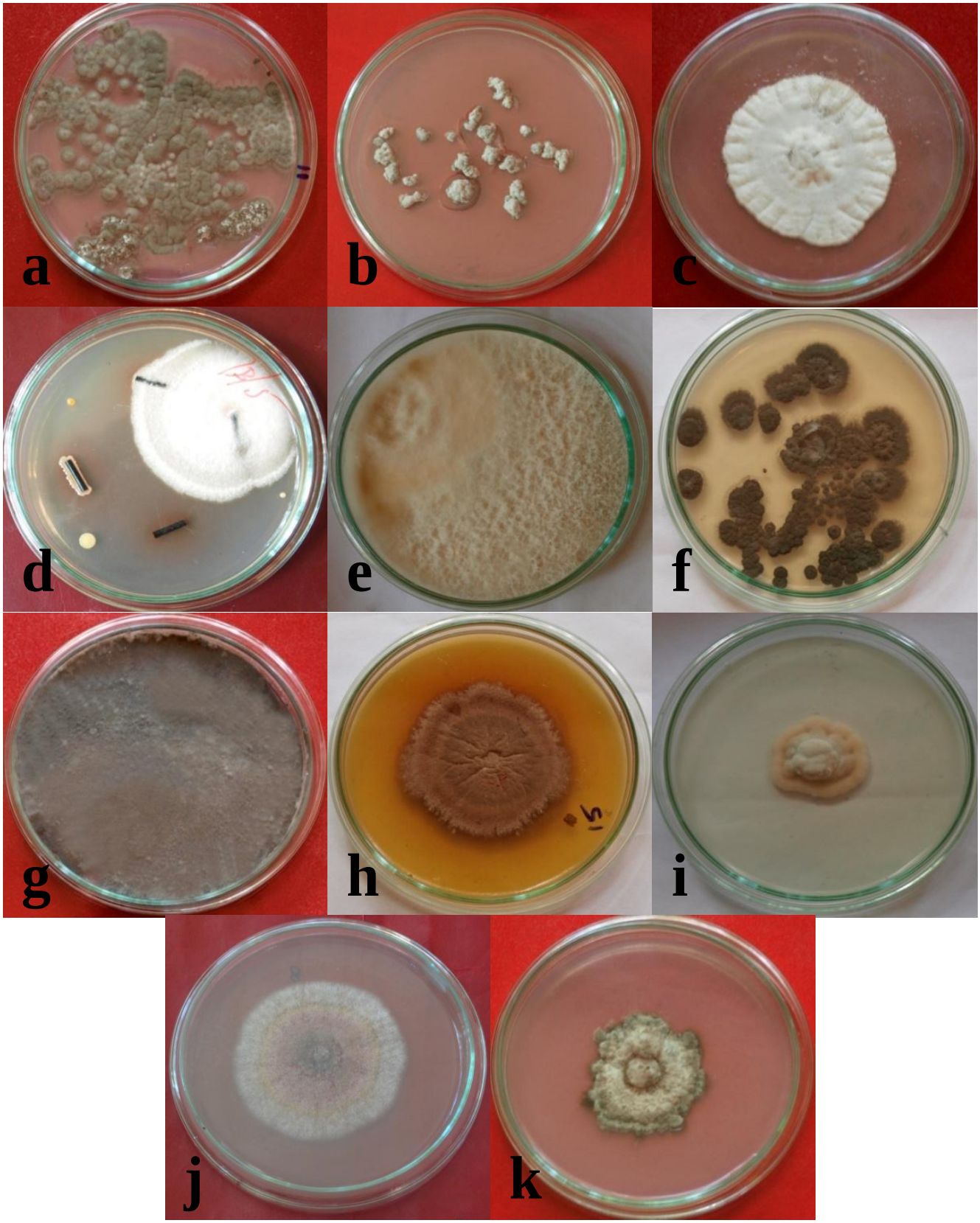

Figure 1. Colony morphology of fungal endophytes isolated for endemic plants of Tirumala Hills. A, *Pencillium corylophilum*; b, *Phyllosticta* sp.; c, *Pestalotiopsis maculans*; d, *Fusarium* sp.; e, sterile, f, *Pencillium* sp.; g, *Aspergillus aculeatus*; h, *Cochliobolus lunatus*; i, sterile; j, *Colletotrichum gloeosporioides;* k-sterile.

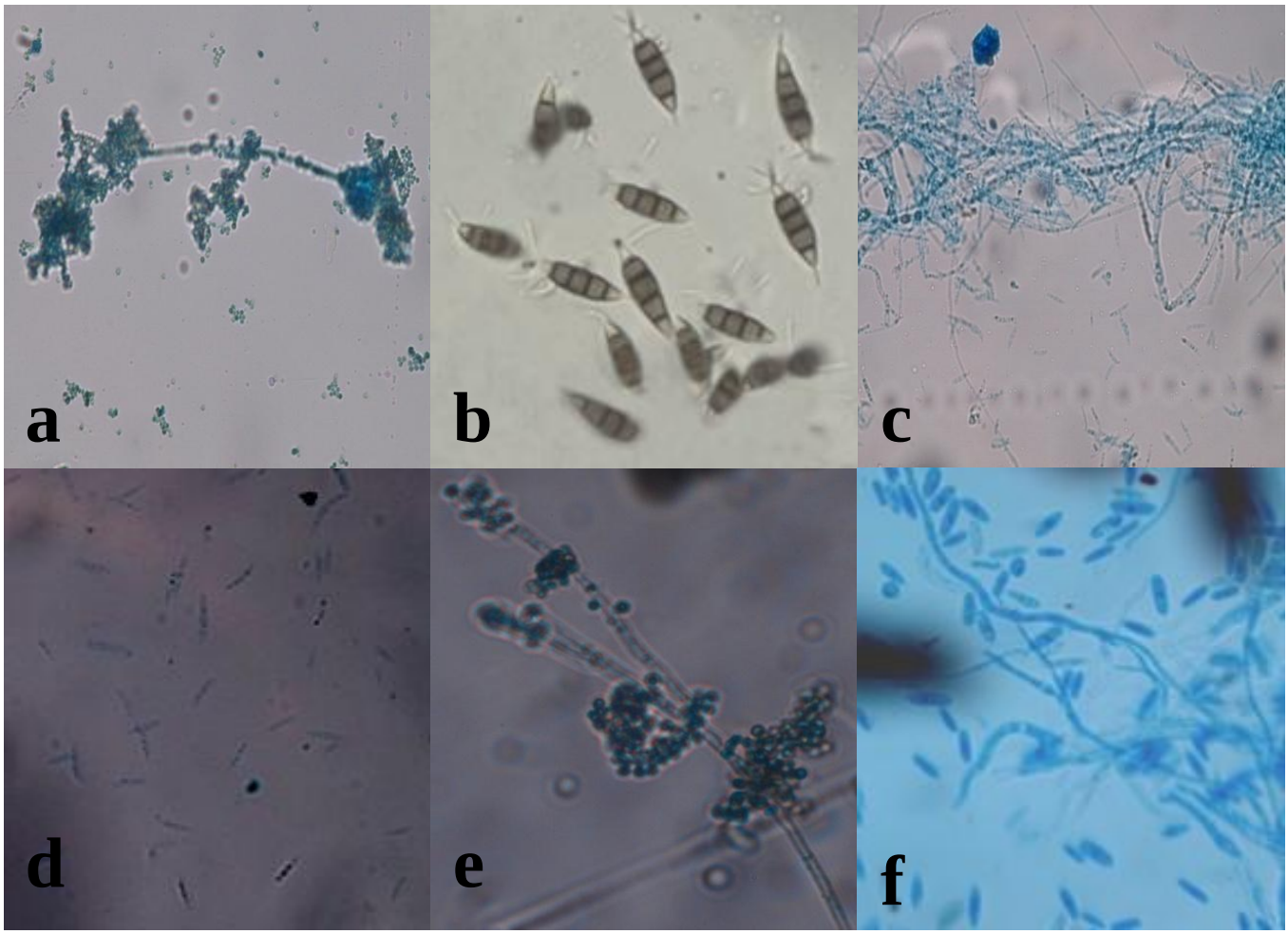

Figure 2. Spore morphology of fungal endophytes isolated for endemic plants of Tirumala Hills. A, *Pencillium corylophilum*; b, *Pestalotiopsis maculans*; c, *Fusarium* sp; d, *Fusarium oxysporum*; e, *Pencillium* sp.; f, *Colletotrichum gloeosporioides*.

Table 2. Extracellular enzymatic activities of the fungal endophytes isolated from three endemic plants collected at Tirumala Hills, India.

Endophyte	Enzyme hydrolysis			
	Amylase	Cellulase	Polyphenol oxidase	Gelatinase
Pencillium corylophilum	+	+	+	+
Phyllosticta sp.	+	-	-	+
Pestalotiopsis maculans	+	+	-	+
Fusarium sp.	+	-	+	+
Fusarium oxysporum	+	-	+	+
Sterile	-	+	+	+
Pencillium sp.	+	+	+	+
Aspergillus aculeatus	+	+	+	+
Cochliobolus lunatus	-	+	-	+
Sterile	-	-	-	+
Colletotrichum gloeosporioides	+	-	-	+
Sterile	-	-	-	+

-, Absence of clearing around fungal mat, negative for test; +, presence of clearing around fungal mat, positive for test.

Table 3. Phytochemical screening of extracts of fungal endophytes isolated form endemic plants of Tirumala Hills.

Endophyte	Metabolite				
	Saponin	Carbohydrate	Phenolic	Glycoside	Flavonoid
Pencillium corylophilum	-	-	+	+	-
Phyllosticta sp.	+	+	-	+	+
Pestalotiopsis maculans	+	+	-	-	+
Fusarium sp.	-	-	-	+	+
Fusarium oxysporum	-	-	-	+	+
Sterile	+	+	+	-	+
Pencillium sp.	-	-	+	+	-
Aspergillus aculeatus	-	+	-	+	-
Cochliobolus lunatus	+	+	-	-	+
Sterile	+	+	-	+	+
Colletotrichum gloeosporioides	-	-	+	+	+
Sterile	-	+	-	-	+

-, Absence of metabolite; +, presence metabolite.

nicotianifolia were evaluated for the first time and the study showed positive correlation between the phenol content of the extracts with their antioxidant activity. The presence of different phytochemicals viz. saponins (Khanna and Kannabiran, 2008), phenolic compounds (Lai et al., 2010), glycosides (Ahmad et al., 2005), and napthoquinone (Vinothkumar et al., 2010) in endophytic fungal extracts has also been reported and they are known to possess strong antimicrobial, antioxidant activity and immune modulating potentials. Our results suggest that these endophytes have a potential use as sources of bioactive secondary metabolites that could be valuable candidates for novel natural therapeutic agents.

ACKNOWLEDGEMENT

The authors thank the Department of Science and Technology (DST, New Delhi) for providing financial support in the form of Research Project (No.SR/SO/PS-85/09) sanctioned to Dr.T.Vijaya (Corresponding Author).

REFERENCES

Ahmad R, Ali AM, Israf DA, Ismail NH, Shaari K, Lajis NH (2005). Antioxidant, radical-scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some *Hedyotis* species. Life Sci. 76 (17):1953-1964.

Arnold AE (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol. Rev. 21: 51-66.

Arnold AE, Maynard Z, Gilbert GS (2001). Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycol. Res. 105:1502-1507.

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000). Are tropical fungal endophytes hyperdiverse?. Ecol. Lett. 3: 267-274.

Azevedo JL, Macchroni W, Pereira JO, Araujo WL (2000). Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Biotechnology 3:40-65.

Barnett HL, Hunter BB (1956). "Illustrated Genera of Imperfect Fungi", 2nd Edn., Burgess Publishing, Minneapolis, MN. p. 218.

Boccon-Gibod J (1982). La technologie de la culture in vitro. In: Auge´ R, Beauchesne G, Boccon-Gibod J, Decourtye L, Digat B, Minier R, Morand JC, Oudin Y, Vidalie H (eds) La culture *in vitro* et ses applications horticoles. Techniques et Documentation, Lavoisier, Paris. pp. 47-73

Caldwell BA, Castellano MA, Griffiths RP (1991). Fatty acid esterase production by ectomycorrhizal fungi. Mycologia 83:233-236.

Frohlich J, Hyde KD, Petrini O (2000). Endophytic fungi associated with palms. Mycol. Res. 104:1202-1212.

Guo LD, Huang GR, Wang Y, He WH, Zheng WH, Hyde KD (2003). Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. Mycol. Res. 107:680-688.

Keerthi M, Loughin T, Jumpponen A (2010). Isolation and morphological and metabolic characterization of common endophytes in annually burned tallgrass prairie. Mycologia 102 (4):813-821.

Khanna VG, Kannabiran K (2008). Antimicrobial activity of saponin fractions of the leaves of *Gymnema sylvestre* and *Eclipta prostrata*. World J. Microbiol. Biotechnol. 24 (11):2737-2740.

Kumar DSS, Hyde KD (2004). Biodiversity and tissue-recurrence of endophytic fungi from Tripterygium wilfordii. Fungal Diversity 17:69-90.

Kumar DSS, Lau CS, Wan JMF, Yang D, Hyde KD (2005). Immunomodulatory compounds from *Pestalotiopsis leucothes* (HKUCC 10197), an endophytic fungus of *Tripterygium wilfordii*. Life Sci. 78:147-156.

Lai HY, Yau YY, Kim KH (2010). *Blechnum orientale* Linn- a fern with potential as antioxidant, anticancer and antibacterial agent. BMC Complement. Altern. Med. 10:15.

Li H, Qqing C, Zhang Y, Zhao Z (2005). Screening for endophytic fungi with antitumour and antifungal activities from Chinese medicinal plants. World J. Microbiol. Biotechnol. 21: 1515-1519.

Lin X, Lu CH, Huang YJ, Zheng ZH, Su WJ, Shen YM (2007). Endophytic fungi from a pharmaceutical plant, *Camptotheca acuminata*: isolation, identification and bioactivity. World J. Microbiol. Biotechnol. 23:1037-1040.

Liu JY, Song YC, Zhang Z, Wang L, Guo ZJ, Zou WX (2004). *Aspergillus fumigatus* CY018, an endophytic fungus in *Cynodon dactylon*, as a versatile producer of new and bio active metabolites. J Biotechnol. 114:279-289.

Mandyam K, Jumpponen A (2005). Abundance and possible functions of the root-colonising dark septate endophytic fungi. In: The Missing Lineages: Phylogeny and ecology of endophytic and other enigmatic root-associated fungi, ed. Summerbell, R. Currah, R.S. and Sigler, L.

Stud Mycol 53:173-189.
Nitya KM, Pushpalatha KC, Chandrashekhar G (2011). Antioxidant activity and phytochemical analysis of endophytic fungi isolated from *Lobelia nicotianifolia*. J. Chem. Pharm. Res. 3 (5):218-225.
Paterson RRM, Bridge PD (1994). Biochemical techniques for filamentous fungi. Oxon: CAB International. pp. 5-7.
Phongpaichit S, Rungjindamal N, Rukachaisirikul V, Sakayaroj J (2006). Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol. Med. Microbiol. 48:367-372.
Photita W, Lumyong S, Lumyong P, Hyde KD (2001). Endophytic fungi of wild banana (Musa acuminata) at Doi Suthop Pui National Park, Thailand. Mycol. Res. 105:1500-1513.
Photita W, Taylor PWJ, Ford R, Hyde KD, Lumyong S (2005). Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Diversity 18:117-133.
Promputtha I, Jeewon R, Lumyong S, McKenzie EHC, Hyde KD (2005). Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). Fungal Diversity 20:167-186.
Saikkonen K, Wäli P, Helander M, Faeth SH (2004). Evolution of endophyte-plant symbioses. Trends Plant Sci. 9:275-280.
Schulz B, Wanke U, Draeger S (1993). Endophytes from herbaceous and shrubs: effectiveness of surface sterilization methods. Mycol. Res. 97:1447-1450.
Stone JK, Bacon CW, White Jr. JE (2000). An overview of endophytic microbes: Endophytism defined. In: Microbial Endophytes, (eds) Bacon, C.W. and White, Jr., J.F. Marcel Dekker, Inc., New York, New York, USA. pp. 3-29.
Strobel G, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67:491-502.
Strobel G, Daisy B, Castillo U, Harper J (2004). Natural products from endophytic microorganisms. J. Nat. Prod. 67:257-268.
Suryanarayanan TS, Murali TS, Venkatesan G (2002). Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. Can. J. Bot. 80:818-826.
Suryanarayanan TS, Murali TS, Venkatesan G (2003). Endophytic fungal communities in leaves of tropical forest trees: diversity and distribution patterns. Curr. Sci. 85:489-493.
Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18: 448-459.
Trease GE, Evans WC (1983). Textbook of Pharmacognosy. 12th ed. Bailliere Tindal, London. pp. 343-383.
Vinothkumar SP, Murali K, Gupta JK (2010). Antioxidant effect of synthetic hydroxy naphthoquinone derivatives. J. Pharm. Res. 3 (11):2784-2787.
Zhang CL, Liu SP, Lin FC, Kubicek CP, Druzhinina IS (2007). *Trichoderma taxi* sp. nov., an endophytic fungus from Chinese yew *Taxus mairei*. FEMS Microbiol. Lett. 270:90-96.
Zhou X, Wang Z, Jiang K, Wei Y, Lin J, Sun X, Tang K (2007). Screening of taxol producing endophytic fungi from *Taxus chinensis var. mairei*. Appl. Biochem. Microbiol. 43:439- 443.