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BIODIVERSITY OF THE ENDOPHYTIC FUNGI ISOLATED FROM *CALOTROPIS PROCERA* (AIT.) R. BR.

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Abstract

Calotropis procera (Ait.) R. Br., a widely used medicinal plant in Indian sub-continent, was investigated for endophytic mycoflora as a possible source of bioactive secondary metabolites. Four hundred seventy three segments from 9 plants of *Calotropis procera*, collected from different locations of Karachi University campus during 2003-2005, were processed for the presence of endophytic fungi. A total of 8 fungal species viz., *Aspergillus flavus, A. niger, Aspergillus* sp., *Penicillium sublateritium, Phoma chrysanthemicola, P. hedericola, Phoma* sp., and *Candida albicans* were isolated. Among the endophytic flora, *Phoma* was the most prominent genus. Interestingly, no endophyte was isolated from 118 leaves samples and overall colonization frequency from surface sterilized stem was 8.86%.

Introduction

Endophytic fungi that live inside the tissues of living plants are under-explored group of microorganisms. Dreyfuss & Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone. Recently they have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivores (Weber, 1981; Shiomi *et al*., 2006; Malinowski & Belesky, 2006). Almost all the plant species (~400,000) harbour one or more endophytic organisms (Tan & Zou, 2001). To date, only a few plants have been extensively investigated for their endophytic biodiversity and their potential to produce bioactive secondary metabolites. Endophytic fungi generally live peacefully with their host, while these fungi under different conditions may act as facultative pathogen. One of the important roles of endophytic fungi is to initiate the biological degradation of dead or dying host-plant, which is necessary for nutrient recycling (Strobel, 2002).

Medicinal plants are reported to harbour endophytes (Strobel, 2002), which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. It is therefore important to determine the endophyte diversity of medicinal plants.

Calotropis procera (Ait.) R. Br., commonly known as calotrope, rubber tree and akando, is a widely used medicinal plant in the Indian Sub-continent (Kumar & Roy, 2007; Akinloye *et al*., 2002). It has long ethnobotanical history and extensive uses in traditional medicine. This grows abundantly in this geographical region. *C. procera* belongs to the family Asclepidaceae. It is a shrub or small tree, drought-resistant, salt-tolerant to a relatively high degree (Fig. 1). The plant contains cardiotoxic, emeto-cathartic and digitalic properties. It also possesses bactericidal and vermicidal activities. An infusion of bark powder is used to treat leprosy and elephantiasis (Sing *et al*., 2002). The present study was carried out to determine endophytic mycoflora in *C. procera* (Ait.) R. Br., a widely used medicinal plant.

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Fig.1. *Calotropis procera*

Materials and Methods

Isolation of endophytic fungi from plants: Stems and leaves of *C. procera* (Ait.) R. Br. were sampled for the investigation of endophytic fungal communities. Healthy and mature plants were carefully chosen for sampling. Samples from different sites of each plant were randomly collected and brought to the laboratory in sterile bags and processed within a few hours after sampling to reduce the chances of contamination.

Isolation of endophytic fungi was carried out according to the method described by Petrini *et al*., (1986). The samples were rinsed gently in running water to remove dust and debris. After proper washing, stem samples were cut into long 0.5-1 cm pieces, whereas leaves were cut into 3-4 mm x 0.5-1 cm pieces with and without midrib under aseptic conditions. Surface sterilization was done by 1-13% Sodium hypochlorite (NaOCl) according to the type of tissues (for example higher concentration was used for root samples). Each set of plant material was treated with 75% ethanol for 1 min followed by immersion in Sodium hypochlorite and again in 75% ethanol for 30 sec. Later the segments were rinsed three times with sterile distilled water. The plant pieces were blotted on sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint method of Schulz *et al*., (1993). In each Petri dish, 5-6 segments were placed on Potato Dextrose Agar (PDA) supplemented with penicillin-G @ 100 units/mL and streptomycin @ 100 µg/mL concentrations. The dishes were sealed with parafilm and incubated at 27 ^{o}C ± 2 ^{o}C for 4-6 weeks in dark. When liquid medium was used, single segment was inoculated in each test tube.

Fungi growing out of the plant segments were purified and identified after reference to Domsch *et al*., 1980; Kenneth *et al*., 1965; Thom & Raper, 1951; Sutton, 1980. Species of *Aspergillus* and *Penicillium* were grown on Czapek's Dox agar for identification.

Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al*., (2003). Samples were incubated and growth was examined daily during 6 weeks and colonizing frequency was calculated:

$$\text{Colonization frequency (\%)} = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total number of segments analyzed}} \text{ X } 100$$

Table 1. Endophytic fungi isolated from different parts of *Calotropis procera*.

Site of isolation	(Number of samples)	Number of fungi isolated
Leaves	(118)	0
Stems	(355)	9
Total number of isolates		9

Table 2. Name and colonizing frequency of Endophytic fungi isolated from *Calotropis procera.*

S. No.	Fungi	KUCC Number	% Frequency of colonization		Number of isolates
			Leaf	Stem	
	Ascomycota				
1.	*Candida albicans*	209	-	2.5	1
	Deuteromycota				
1.	*Aspergillus flavus*	379	-	30	1
2.	*Aspergillus niger*	346	-	50	1
3.	*Aspergillus* sp.	286	-	4	1
4.	*Penicillium sublateritium*	211	-	16	1
5.	*Phoma chrysanthemicola*	197	-	7.14	1
6.	*Phoma hedericola*	177	-	31.5	2
7.	*Phoma* sp.	136	-	7.14	1

Results

The plant materials were collected from Karachi and sample specimen was deposited at the Herbarium University of Karachi (C.H. NO: 69310) and identified by Prof. Dr. Surayya Khatoon (Taxonomist), Department of Botany, University of Karachi.

Four-hundred-seventy-three (118 leaf samples and 355 stem samples) segments from 9 plants of *C. procera* were processed for the isolation of endophytic fungi. A total of 9 fungi, belonging to 8 species, were isolated (Table 1). Except *Candida albicans,* which belongs to the class Ascomycetes, all the other isolates belong to the class Deuteromycetes. Most fungi isolated from this plant produced only sterile mycelium, while a few fungi produced pycnidia. Most prominent endophyte in *C. procera* was found to be genus *Phoma*. Different species of *Phoma* including *Phoma chrysanthemicola* and *Phoma hedericola* were isolated and identified. Interestingly all the fungi were isolated only from stems of plant while 118 leaf segments were also examined. All the isolated and identified fungi were submitted to the Karachi University Culture Collection (KUCC) (Table 2). Many fungi did not produce any reproductive structure, as they produced sterile mycelia and in some cases sterile pycnidium.

Description of Endophytic fungi

i. *Aspergillus flavus* Link: *Aspergillus flavus* grew rapidly with floccosity when cultured on Czapek's Dox agar. It produced light greenish-yellow colour colony. Reverse side of the colonies were yellowish at primary stage of growth and brownish in mature age.

Conidiophores arose from submerged hyphae, were 400-1000 x 5-15 µ in size. Walls of conidiophores were pitted, rough and uncoloured. Conidial heads were hemispherical to subglobose. Vesicles were dome-like and 10-30 µ in diameter. Sterigmata were mostly in two series. Single series sterigmata were also produced. Primary sterigmata were 7-10 x 3-4 µ and secondary sterigmata were 10-15 x 3-5 µ in size. Conidia were pyriform to almost globose, nearly colourless and varied in size between 3-4 µ.

ii. *Aspergillus niger* van Tieghem: *Aspergillus niger* grew rapidly on Czapek's Dox agar. Colonies were carbon black in colour. It produced abundant submerged mycelia in the medium. Conidiophores were smooth with thick walls, unseptate, 200-1000 µ long and 7-10 µ thick. They were uncoloured near the vesicle. Conidial heads were fuscous black, globose, up to 300-500 µ in diameter. Vesicles were colourless and globose, thick-walled up to 35 µ in diameter. Conidial chains were present over the entire surface of vesicles. Conidia were rough, globose and 3-4 µ in diameter.

iii. *Penicillium sublateritium* Biourge: Colonies of *P. sublateritium* were restricted when grown on Czapek's Dox agar. It reached 1-2 cm in diameter in 12 days at 27 °C. Colonies were velvety, orange-green in colour with thin white margin. Reverse side of the colony were pale orange. Mature colonies were deeply radiantly wrinkled. Spores were abundant with grey-green shades. Colonies did not produce odour and exudates. Conidiophores were mostly 70-80 x 2 µ in size and smooth walled. Phyloides were strictly monoverticillate, consisting of small verticels. Five to eight or ten parallel sterigmata were present on verticels. Sterigmata were mostly 10-12 x 0.2-2.5 µ, occasionally 15 µ in length. Spores arranged in chain. Conidial chains were up to 100µ long. The mature conidia were elliptical, smooth and 4.0-5.0 x 3.0µ in size.

iv. *Phoma chrysanthemicola* Hollos: *Phoma chrysanthemicola* (Fig. 2) produced irregular, olivaceous grey colonies with darker patches on PDA medium. Reverse side of the colonies were blackish brown. Mycelia were immersed, branched, septate and brown in colour. Conidiomata were pycnidial, dark brown in colour, semi-immersed and thin-walled. Pycnidia of *P. chrysanthemicola* developed separately or aggregately on medium. Pycnidia sometimes became erumpent, unilocular and globose. Conidia were straight cylindrical with 1-4 guttules, 5-6.5 x 1-1.5µ in size. Clamydospores were multicellular. Pseudosclerotia were formed by aggregated clamydospores which were pale to dark brown in colour.

v. *Phoma hedericola* (Dur. & Mont.) Boerema: *Phoma hedericola* (Fig. 3) produced brown coloured colonies with regular margin on PDA medium. Reverse side of the colonies were brown. Mycelia were immersed, branched, septate and dark brown in colour.

The fungus produced semi-immersed pycnidia in medium which were globose, thin-walled and brown in colour. Sometimes the pycnidia were erumpent and unilocular. Pycnidia were separate or aggregated and occasionally confluent on medium. Conidia 4-6.5 x 2.5-3.5µ in size.

vi. *Candida albicans* (C.P. Robin) Berkhout: Colonies of *C. albicans* were smooth and off-white in colour when it grew on PDA medium. Reverse side of the colonies were also off-white. The diameter of colonies was 3-4 cm at 10 days at 27 °C. Older colonies developed radial furrows. Pseudomycelia developed by elongation of cell. Cells were small, oval, budding, 2.5 x 4 x 6 µ in size. *C. albicans* produced clamydospores in medium.

Discussion

Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host (Weber, 1981; Shiomi *et al*., 2006). Fungi have been widely investigated as a source of bioactive compounds. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plants (Strobel & Daisy, 2003).

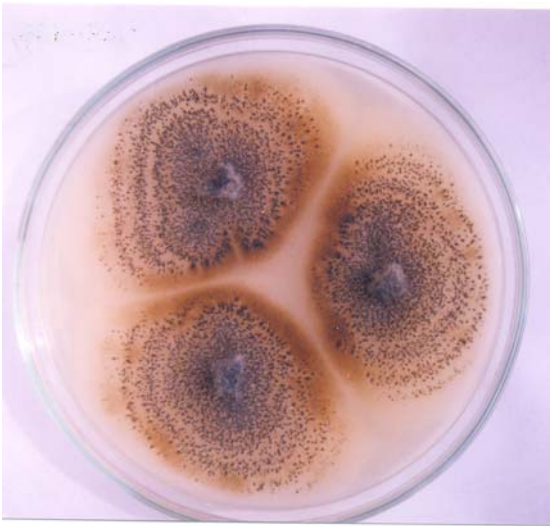

Fig. 2. *Phoma chrysanthemicola*

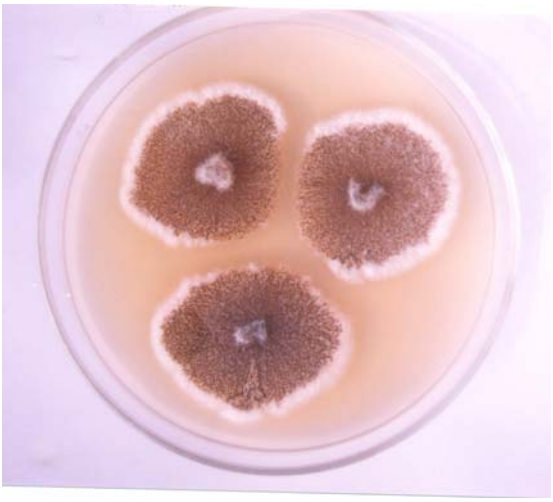

Fig. 3. *Phoma hedericola*

Endophytic organisms have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivorous (Weber, 1981). However only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive compounds. Recently studies have been carried out about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effect on host (Petrini, 1986; Arnold *et al*., 2001; Clay & Schardl, 2002; Selosse & Schardl, 2007). Endophytes, are now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel & Daisy, 2003; Strobel *et al*., 2004).

Endophytic fungi from medicinal plants can therefore be used for the development of drugs. The endophytic flora, both numbers and types, differ in their host and depends on host geographical position (Gange *et al*., 2007; Arnold & Herre, 2003).

Calotropis procera is a well known medicinal plant and its different parts are reported to have anti-inflammatory, antioxidant and anticancer activities (Kumar & Arya, 2006; Choedon *et al*., 2006). The medicinal properties of the plant could be attributed to their endophytic fungi. Therefore, the present work was initiated to find out endophytic flora associated with in this widely used medicinal plant.

A study of endophyte biodiversity of the two dry tropical forest of the Nilgiri Biosphere Reserve in India was conducted by the Suryanarayanan *et al*., (2003). They have reported diversity of fungal species varying from 10 to 26 in the host. Amongst the 24 plant species, the lowest number of fungal diversity was 10 in *Gmelina arborea* Roxb. and the highest number was 26 in *Shorea roxbufghii* G. Don. In the present study 8 different species with 8.86% colonization frequency were isolated from *C. procera*, which is slightly less than the above cited study (Suryanarayanan *et al*., 2003).

During the present study, mainly *Aspergillus, Penicillium* and *Phoma* sp. were isolated as endophytic fungi. Deuteromycota fungi were largely prevalent. Majority of endophytic fungi belongs to Ascomycota and Deuteromycota (Frohlich & Hyde, 1999). Many species of *Phoma* also causes disease in plants. *Phoma macdonaldii* causes black stem disease in Sunflower (Siddique *et al*., 1988).

In this study profuse pycnidia were found on the dead plants of *C. procera* which is the characteristic of the fungus *Phoma*, the most common endophyte of this plant. The *Phoma* species are reported to produce cellulytic enzymes, necessary to degrade plant material (Urbanek *et al*., 1998).

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References

Akinloye, A.K., M.O. Abatan, O. Alaka and B.O. Oke. 2002. Histomorphometric and histopathological studies on the effect of *Calotropis procera* (Giant Milkweed) on the male reproductive organs of wistar rats. *African Journal of Biomedical Research*, 5: 57-61.

Arnold, A.E. and E.A. Herre. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia*, 95(3): 388-398.

Arnold, A.E., Z. Maynard and G.S. Gilbert. 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research*, 105: 1502-1507.

Choedon, T., G. Mathan, S. Arya, V.L. Kumar and V. Kumar. 2006. Anticancer and cytotoxic properties of latex of *Calotropis procera* in a transgenic mouse model of hepatocellular carcinoma. *World Journal of Gastroenterology*, 12(16): 2517-2522.

Clay, K. and C. Schardl. 2002. Evolution origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist*, 160: 99-127.

Domsch, K.H., W. Gamas and T-H. Anderson. 1980. *Compendium of Soil Fungi*, Vol. I. Academic Press, New York. p. 205-205.

Dreyfuss, M.M. and I.H. Chapela. 1994. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In: *The discovery of natural products with therapeutic potential.* (Ed.): V. P. Gullo. Butter- worth-Heinemann, London, United Kingdom. p. 49-80.

Fröhlich, J. and K.J.D. Hyde. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimated realistic? *Biodiversity and Conservation*, 8: 977-1004.

Gange, A.C., S. Dey, A.F. Currie and B.V.C. Sutton. 2007. Site- and species-species differences in endophyte occurrence in two herbaceous plants. *Journal of Ecology*, 95(4): 614-622.

Kenneth, B.R., I.F. Dorothy and K.C.A. Peter. 1965. The genus *Aspergillus*. The Williams & Wilkins Company, Baltimore. p. 214-219.

Kumar, V.L. and S. Roy. 2007. *Calotropis procera* latex extract affords protection against inflammation and oxidative stress in freund's complete adjuvant-induced monoarthritis in Rats. *Mediators of Inflammation*. Doi: 10, 1155/ 2007/ 47523. pp. 1-7.

Kumar, V.L. and S. Arya. 2006. Medicinal uses and pharmacological properties of Calotropis procera. In: *Recent Progress in Medicinal Plants*. (Ed.): J.N. Govil, vol. 11.Studium Press, Houston, Tex., USA. p. 373-388.

Malinowski, D.P. and D.P. Belesky. 2006. Ecological importance of *Neotyphodium* sp. Grass endophytes in agroecosystems. *Grassland Science*, 52(1): 23-28.

Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the phylosphere.* (Ed.): N.J. Fokkema and J. Van-den Heuvel. Cambridge University Press, Cambridge. p. 175-187.

Schulz, B., S. Wanke, S. Draeger and H.J. Aust. 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol*. *Res*., 97: 1447-1450.

Selosse, M.A. and C.L. Schardl. 2007. Fungal endophytes of grass: hybrids rescued by vertical transmission? An evolutionary perspective. *New Phytologist*, 173(3): 452-458.

Shiomi, H.F., H.S.A. Silva, I.S. De Melo, F.V. Nunes and W. Bettiol. 2006. Bioprospecting endophytic bacteria for biological control of coffee leaf rust. *Sci. Agric.*, 63(1): 32-39.

Siddique, M.M., A.R. Masood and M. Ayub. 1988. Sunflower diseases in Pakistan in the period 1980 to 1987. In: *Proc. 12th Inter. Sunflower Conf.* Organizing Committee of the International. Sunflower Conference, Novi-Sad, Yugoslavia. p. 2-25.

Sing, A.K., A.S. Raghubanshi and J.S. Sing. 2002. Medical ethnobotany of the tribals of Sonaghati of Sonbhadra district, Uttar Pradesh, India. *Journal of Ethnopharmacology*, 81(1): 31-41.

Strobel, G.A. 2002. Microbial gifts from rain forests. *Can. J. Plant Pathol*., 24: 14-20.

Strobel, G. and B. Daisy. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Review,* 67: 491-502.

Strobel, G., B. Daisy, U. Castillo and J. Harper. 2004. Natural Product from endophytic microorganisms. *Journal of Natural Products*, 67: 257-268.

Suryanarayanan, T.S., G. Venkatesan and T. S. Murali. 2003. Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. *Current Science*, 85(4): 489-492.

Sutton, B.C. 1980. *The Coelomycetes, Fungi Imperfecti with pycnidia Acervuli and Stromata*. Robert Mac Lechose and Co. Ltd. University of Glasgow. England. p. 378-385.

Tan, R.X. and W.X. Zou. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep*, 18: 448-459.

Thom, C. and K.B. Raper. 1951. *A Manual of the Aspergilli*. The Williams & Wilkins Company, pp. 259-261.

Urbanek, H., J. Zalewska-Sobczak and A. Borowińsk. 1998. Isolation and properties of extracellular cellulose hemicellulase complex of *Phoma hibernica*. *Archives of Microbiology*, 118(3): 265-269.

Weber, J. 1981. A natural control of Dutch elm disease. *Nature*, London, 292: 449-451.

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