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BIODIVERSITY OF THE ENDOPHYTIC FUNGI ISOLATED FROM *CALOTROPIS GIGANTEA* (L.) R.BR.

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Abstract

Calotropis gigantea(L)R.Br., a widely used medicinal plant in India, were exploited for endophytes as a possible source of bioactive secondary metabolites. About 700 segments from 10 plants of *Calotropis gigantea,* collected from different locations of Guindy Campus, University of Madras during the year 2009–2010, were processed for the presence of endophytic fungi. A total of 13 fungal species viz., *Aspergillus niger, Aspergillus flavipes, Alternaria porri, Curvularia lunata, Fusarium oxysporum, Nigrospora sphaerica, Colletotrichum falacatum, Pestalotiopsis sydowiana, Phoma exigua, Phomopsis archeri, Leptosphaerulina chartarum,* and *Mycelia sterilia,* were isolated and identified based on the morphology of the fungal culture and characteristics of the spores.

Introduction

Endophytic fungi often are symptomless symbionts living within the above ground tissues of their angiosperm hosts and are not affected by surface sterilization techniques. De Bary (1866) first defined all organisms that colonize internal plant tissues as endophytes. The study of endophyte distribution, biodiversity and their biochemical characteristics are of immense importance in plant biology to understand and to improve plant fitness. The endophytic fungi are of biotechnological importance as new pharmaceutical compounds, secondary metabolites, agents of biological control and other useful characteristics would be found by further exploration of endophytes. Dryefuss and 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 insects, pests, pathogens and even domestic herbivour (Weber,1981; Malinowski and Belesky, 2006). Almost all plant species harbour one or more endophytic organisms (Tan and Zou, 2001).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 endophytic biodiversity of medicinal plants. *Calotropis gigantea* commonly known as Milk-Weed or Swallow wart is widely used medicinal plant in Indian sub-continent (Kumar and Roy, 2007; Akinloye *et al.,* 2002).It has long ethanobotanical history and extensive uses in traditional medicine. This grows abdundatly in India, Malaysia, Philliphines etc. *Calotropis gigantea* belongs to the family Asclepidaceae. It is a small shrub growing 4 m tall it has clusters of waxy flowers that are either white or lavender in colour , the plant has oval green leaves on milky stem, having cardiotoxic, emetocarthartic and digitalic properties the plant is very effective in treating leprosy, elephantiasis, chronic rheumatism, ulcer and skin diseases. The present study was carried out to determine the endophytic flora in *Calotropis gigantea.*

Fig 1. Pls send the fig as an email attachment with proof correction.

Materials and Methods

Collection of plant samples

Stems, leaves and flowers of *Calotropis gigantea* R.Br. were collected from different locations at University of Madras, Guindy campus. Healthy and mature plants were carefully chosen for sampling.

Isolation of endophytic fungus

The samples were rinsed gently in running tap water to remove dusts and debris. The stem, leaves (lateral and midrib) and flowers were cut into segments (0.5 – 1cm). The samples were surface sterilized by modified method of Dobranic *et al.* (1995). The samples were immersed in 70% ethanol for 5 s, followed by 4% sodium hypochlorite for 90 s and then rinsed in sterile distilled water for 10 s. The excess

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moisture was blotted in a sterile filter paper. The surface sterilized segments were placed in Petridishes containing PDA medium. The Petridishes were sealed using parafilm and incubated at 26 ± 1°C at 12-h light/dark cycle. The Petridishes were monitored every day to check the growth of endophytic fungal colonies from the segments.

Colonization Frequency

Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al.* (2003). Samples were incubated and growth was examined.

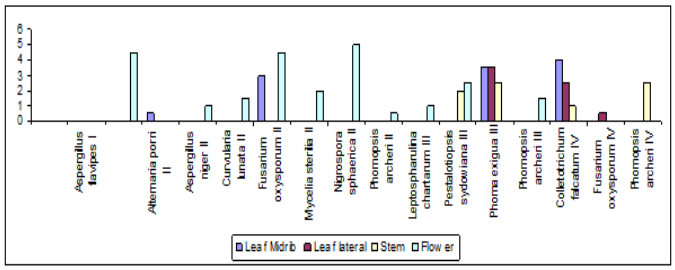
$$CF\% = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total Number of segments analysed}} \times 100.$$

The hyphal tips which grew out from the segments were isolated and sub cultured on PDA medium. The pure cultures were maintained on PDA slants. The endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruting structures and spore morphology

Table 1 Endophytic fungus isolated from different parts of *Calotropis gigantea* (Mar 2009 – Feb 2010)

Seasons	Endophytes	CF%			
		Leaf Midrib	Leaf lateral	Stem	Flower
Season I Summer Mar - May 2009	*Aspergillus flavipes*				4.5
	Alternaria porri	0.5			
Season II Pre- monsoon Jun - Aug 2009	*Aspergillus niger*				1
	Curvularia lunata				1.5
	Fusarium oxysporum	3			4.5
	Mycelia sterilia				2
	Nigrospora sphaerica				5
	Phomopsis archeri				0.5
Season III Post monsoon Sep - Nov 2009	*Leptospharulina chartarum*				1
	Pestalotiopsis sydowiana			2	2.5
	Phoma exigua	3.5	3.5	2.5	
	Phomopsis archeri				1.5
Season IV Winter Dec - Feb 2009	*Colletotrichum falcatum*	4	2.5	1	
	Fusarium oxysporum		0.5		
	Phomopsis archeri			2.5	

Fig 2: CF% of Endophytic fungus isolated from *Calotropis gigantea* at different seasons



Results

The plant materials were collected from University of Madras, Guindy campus. About 700 segments (350 segments of leaf, 250 segments of stem, 100 segments of flower) of *Calotropis gigantea* were processed for the isolation of endophytic fungus. A total of 12 fungus of which six form of Hyphomycetes, four form of Coelomycetes, one form of Ascomycete and one form of fungi which do not produce any reproductive structures, as it produce sterile mycelia, was obtained. All the isolated and identified fungus was submitted to Madras University Botany Laboratory (MUBL).

Description of Endophytic Fungi

Alternaria porri (Ellis) Cif

Conidiophores dark, septate, sometimes inconspicuous, simple or branched, bearing conidia at

the apex, porospores solitary or more often produced in acropetal succession to form simple or branched chains, darkly pigmented, ovate to obclavate, tapering abruptly or gradually towards the distal. Overall conidial dimensions are 15-20µm.

Aspergillus flavipes, (Bain & Sart) Thom & Church

Colonies white or silvery white, reverse yellow to orange brown or reddish brown. Conidial heads columnar in size. Vesicles globose to ovate, metulae fertile over entire vesicle, conidial heads splitting over age. Conidia smooth, globose, 2-3 µm in diameter.

Aspergillus niger, Van Tiegh

Colonies spreading rapidly with mycelium white to dark brown to black or purple brown conidial heads, conidial heads globose, radiate, conidiophores arising from the substratum varying from 200µm to several millimeters long and 10-20µm diameter, smooth, vesicle globose, phialides borne directly on the vesicles in some species, but metulae usually present, metulae varying in length from 10-15µm, conidia small, more or less globose, rough, 4-5µm in diameter.

Curvularia lunata(Wakker) Boedjiin

Colonies effuse, brown, cottony, conidiophores are macronematous, mononematous, straight or flexuous, often geniculate, sometimes nodose, brown usually smooth. Conidia solitary, acropleurogenous, simple, often curved, clavate, ellipsoidal, broadly fusiform with 3 – transverse septa, dark brown, usually the end ones paler than the others, sometimes with dark bands at the septa, hilum scarcely or not at all protuberant, smooth – walled, middle septum not median, 20 – 28 X 8 - 14µm.

Fusarium oxysporum Schl

Growth moderate, white, peach, to salmon pink or violet.Conidiogenous cells hyaline, enteroblastic, mono or polyphialidic. Fusarium species produce several types of conidia. Microconidia hyaline, 0-1 or septate, small, macroconidia hyaline, curved, phragmospores, with a foot cell bearing, some kind of heel. Chlamydospores may also be present, borne terminally or intercalary or on the macroconidia. Microconidia are oval to cylindrical or even curved and produced on simple, short phialides. Macroconidia 3-5 septate, 27 - 60 X 3 - 5µm.

Nigrospora sphaerica (Sacc.) Mason

Colonies white later brown to black when sporulation is abundant. Conidiophores micronematous, branched, flexous, colourless to brown, smooth, conidia solitary,

acregenous, simple, spherical or broadly ellipsoidal, compressed dorsiventrally, black , shining, smooth, 0 – septate,10 – 16 µm diameter.

Colletotrichum falcatum Went, Arch

Colonies grayish white, with sparse aerial mycelium and small dense felty patches, elsewhere reverse white to grey, conidial masses salmon pink. Some cultures have abundant greyish white aerial mycelium with poor sporulation and no distinct acervuli. Sclerotia absent from both races. Setae sparse. Conidia falcate, fusiform apices obtuse, 15.5 – 26.5 X 4 - 5µ. Appresoria sparse, medium brown, clavate or circular, edge entire, 12.5 – 14.5 X 9.5 – 12.5µ.

Pestalotiopsis sydowiana Bresadola

Conidia clavate to fusiform, straight, rarely curved, equilateral, 5 – celled, smooth walled, 23 – 29 X 80 – 95 (-11)µm mean 25 X 90 µm. Apical and basal cell hyaline, apical hyaline cells long and broad cylindric, the basal hyaline cells broad – conic. Median 3 cells coloured, guttulate, together 16 - 20 µm long, slightly or hardly constricted at the septa, the lowest coloured cell is light brown, apical appendage (2-3-4) divergent or recurved, hyaline, cylindrical with abtuse apices, 18 - 40 µm long. Basal appendage hyaline, straight or slightly curved, 3-6 µm long.

Phoma exigua Desm

Colonies very variable with a scalloped or lobed margin, usually with dense felty white black or dark olivaceous aerial mycelium, not concentrically zoned. Conidia 5.5 – 10 X 2.5 – 3.5 µ straight or slightly curved, ellipsoid or cylindrical, often biguttulate and becoming 1 septate.

Phomopsis archeri Nom.nov

Conidiomata up to 1mm diameter, globose to sub globose. Conidiophores sparingly septate and branched, filiform up to 15µ long. Conidia α- conidia, ellipsoid, less often fusiform, each end obtuse, 0.2 guttulate, 5.5 – 9 X 2 – 2.25µ; β - conidia straight, curved or lanate, 15 - 19µ long. These dimensions are somewhat lower than those reported in the original account where α – conidia were described as 7 – 10 X 2.5µ and β – conidia as 20 – 30 µ long.

Leptosphaerulina chartarum Cec. Roux

A filamentous ascomycetous fungus that produce dark coloured psuedothecia. The asci of *Leptosphaerulina* are shortly clavate to saccate and have bitunicate. Bitunicate asci are characterized by an inner extensable wall. Ascospores are hyaline to brown in colour and ellipsoid, cylindrical or oblong.

Mycelia sterilia

Many fungi do not produce any recognizable sexual/ asexual conidia state in culture. Such forms are frequently classified for convenience in the Mycelia sterilia. This group is catchcall which may include a few well defined and easily recognizable genera, but more

often is a repository for a large number of non descript mycelial isolates.

Fig 3 Colony morphology of *Alternaria porri*

Fig 4 Colony morphology of *Aspergillus flavipes*

Fig 5 Colony morphology of *Curvularia lunata*

Fig 6 Colony morphology of Fusarium oxysporum

Fig 7 Colony morphology of *Nigrospora sphaerica*

Fig 8 Colony morphology of *Colletotrichum falcatum*

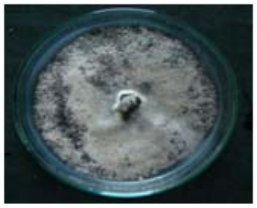


Fig 9 Colony morphology of *Pestalotiopsis sydowiana*

Fig 10 Colony morphology of *Phoma exigua*

Fig 11 Colony morphology of *Phomopsis archeri*

Fig 12 Colony morphology of *Leptosphaerulina chartarum*

Fig 13 Spore of *Alternaria porri*

Fig 14 Spores of *Aspergillus flavipes*

Fig 15 Spores of *Curvularia lunata*

Fig 16 Spores of *Fusarium oxysporum*

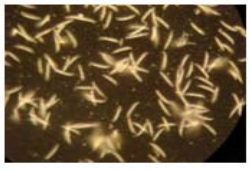


Fig 17 Spores of *Nigrospora sphaerica*

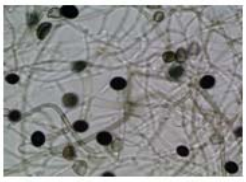


Fig 18 Conidia of *Colletotrichum falcatum*

Fig 19 Conidia of *Pestalotiopsis sydowiana*

Fig 20 Conidia of *Phoma exigua*

Fig 21 Conidia of *Phomopsis archeri*

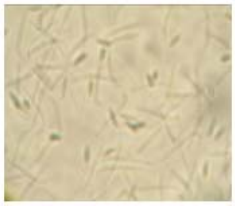
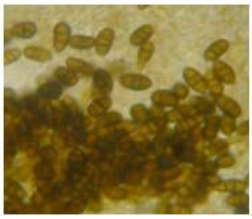


Fig 22 Ascospore of *Leptosphaerulina chartarum*



The spores photos are taken at a magnification of 40x

Discussion

Herbal medicine is one of the oldest forms of health care known, every plant on earth is known to harbor at least one endophytic microbe. Plants have a long history of use in treatment of cancer (Hartwell, 1982). Endophytic fungi are one of the most unexplored and diverse group of organisms having symbiotic associations with higher life forms and may produce beneficial substances for host (Weber, 1981;). *Calotropis gigantea* is a common medicinal plant its latex is used in treating leprosy, eczema, inflamation, cutaneous infections, syphilis, malarial and low hectic fevers, and as abortifacient

(Kumar and Basu, 1994), rheumatism, as an anti-inflmmatory and antimicrobial hepatoprotective agents, against colds and coughs, syphlis and elephantiasis, as an anti-inflammatory, analgesic, antimalarial and antimicrobial. cytostatic, arbortifacient and antimalarial, in asthama and piles and villagers in Bikaner district ingest almost all plant parts in various dietary combinations for malarial fevers and pyrexias (Sharma and Sharma, 2000). The seasonal variation which plays a main role in endophyte harvesting where environmental conditions paves way for the symbiotic microbes to survive or to explore, some literature points the precipitation is one of the major factors that influence the infection of endophytes. A strong correlation has been observed between endophytes primary growth, cumulative and precipitation (Wilson, 2000). In many instances leaves sampled during wet season harbour more Endophytes than those screened during dry season (Rodrigues,1994; Wilson & Carroll,1994; Suryanarayanan *et al.,* 1998). In the present study , altogether 12 fungi were isolated as endophytes from the flower, leaves, and stem parts of *C.gigantea* collected from Guindy Campus, University of Madras for various seasons.

Some hyphomycetous forms viz., *Alternaria porri, Aspergillus niger, Aspergillus flavipes, Fusarium oxysporum, Nigrospora sphaerica, Curvularia lunata* (Blodgett *et al*.,2000; Suryanarayanan *et al* 1998, 2002) were isolated as endophytes in the present study which have been previously reported as endophytes.

Among coelomycetous fungus *Colletotrichum falcatum, Phoma exigua,, Phomopsis archeri, Pestalotiopsis sydowiana* have been previously reported as endophytes (Bussan ban, 2001, Suryanarayanan *et al* 2002). Majority isolates belonged to ubiquitous genera (egg. *Alternaria , Fusarium , Phoma, Leesport*) concurring with previous results reviewed by Petrini(1986) who found that many endophytes belonged to ubiquitous taxi. Ascomycetes and their anamorphic states invariably constitute the endophytic populations of leaves (Petrini,1986; Wilson,2000). In the present study a single Ascomycetous form *Leptospharulina chartarum* was obtained. The occurrence of sterile mycelia as endophytes demand the use of molecular techniques, for classification and induction of sporulation is suggested by means of incubation under near U.V or low temperature (Bills,1996). Previous studies reported distinct endophyte community compositions in different host plants suggesting host preference (Cannon and Simmons, 2002; Cohen, 2006). This study shows such a trend was apparent with the leaves, stem and flower

parts of *Calotropis gigantea*. However, high colonization frequency were observed during the month of June – August where the leaves are mature and there was very little precipitation, endophytic species can be affected by season (Petrini, 1991). In the present investigation, a significant variation was detected in the colonization frequency of endophytic species at different seasons of the year, indicating the environmental factors such as rainfall and atmospheric humidity and their effect on host plant. Therefore, surveys of endophytic fungal communities at different seasons of the year might favour a higher recovery of particular species.

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