Research Paper

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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM Acanthus ilicifolius IN MUTHUPET MANGROVES

Botany

KEYWORDS : Muthupet mangroves, Acanthus ilicifolius, Endophytic fungi

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ABSTRACT

Acanthus ilicifolius (Acanthaceae) has received considerable attention due to its wide range of secondary metabolites and its traditional usage in Indian and Chinese system of medicine. Mangrove survives in the most hostile environment with fluctuating tidal and saline regime. Hence, these plants are considered to be rich sources of steroids, triterpenoids, saponins, flavonoids, alkaloids, and tannins. The aim of the research was to isolate and identify the fungal endophytes from the leaves of Acanthus ilicifolius. A total of fourteen isolates of fungal endophytes were isolated from the leaf of Acanthus ilicifolius. The mycoflora such as Absidia,sp Aspergillus fumigatus, A.ochraceous, A.terreus A.candidus, A.janus, A.nidulans, A.ustus, A.granulosis A.versicolor, A. flavus, , A.panamensis , Fusarium sp, Alternaria sp.

INTRODUCTION

Mangrove forests are fascinating and complex ecosystems (Feller *et al*., 2010). Mangrove plants are salt-tolerant plants, which act as primary producers in the estuarine food chain; they produce novel metabolites unique to the environment with various important economic and environmental functions (Bandarnayake 2002). Most mangrove plants studies do not consider the fact that plants in natural ecosystems have symbiotic associations with fungi. *Acanthus ilicifolius* is a gregarious shrub, commonly found with mangroves along the coastal regions of India. The common names of *Acanthus ilicifolius* are Beng. Harkucha kanta, Eng. Holy leaved acanthus, Hind. Harkukanta, Kan. Holechudi, Mal. Payinachulli, Mar. Marandi, Mandli, Tam. Attumulli, Kaludaimulli, Tel. Alasyakampa, Alchi. Leaves are simple, oblong to elliptic, varying in size, smaller in flowering season, measuring 7.5 to 15 cm in length and 5 to 6 cm in width, pinnatified, apex truncate to acute, terminating in a sharp spine, surface glabrous, granular structure. (Xie *et al*., 2005).

For centuries, mangrove plants found in the tropics have been accumulated source of bioactive molecules with different dimensions of activities. They have been used in folklore medicine for treatment of several diseases and extracts from different parts of the plant and their associates being widely used worldwide for medicinal purposes. Endophytic fungi that live inside the tissues of living plants are under-explored group of microorganisms. Recently they have been received considerable attention after they were found to protect their host against insect, pests, pathogens and even domestic herbivores. Almost all the plant species harbour one or more endophytic microorganisms. Endophytes are hidden within healthy host plants are a poorly investigated group of microorganisms, but they represented an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medical, agricultural, and industrial areas (Tan and Zou 2001).

These fungi are important to the structure, function, and health of plant communities and may be responsible for the adaptation of plants to environmental stresses (Clay and Holah 1999). In addition, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Baltruschat *et al.* 2008).

Endophytic fungi have been recognized as possible useful sources of bioactive secondary metabolites (Schulz *et al*., 2002, Strobel *et al*., 2004). There is a need to search new ecological niches for potential sources of natural bioactive agents for different pharmaceutical, agriculture, and industrial applications.

MATERIALS AND METHODS

COLLECTION OF PLANT SAMPLES

The leaves of *Acanthus ilicifolius,* was collected from the Muthupet (Forest). Healthy and mature plants were carefully chosen for sampling. The plants randomly collected and brought to the laboratory in sterile bags for further investigation.

ISOLATION OF ENDOPHYTIC FUNGI

Asymptomatic healthy leaf materials were thoroughly washed in running tap water, then surface sterilized by a modified method of Raviraja (2005). The selected leaf segments were immersed in 95% ethanol for 30sec, 45% sodium hypochlorite solution for 15sec and 95% ethanol for 30sec followed by rinsing with sterile distilled water three times for 10sec and allowed to surface dry under sterile conditions. After drying, each leaf segment was cut into approximately 0.5cm squares and placed on petri plates containing potato dextrose agar medium (PDA). The streptomycin sulphate (100mg/L) was added to prevent the growth of bacteria. Then it was monitored every day for growth of endophytic fungal colonies. Fungi growing out from the samples were subsequently transferred on to fresh PDA plates.

Number of segments colonized by fungi
Colonization frequency of endophytes = **X 100**
Total number of segments analysed

IDENTIFICATION OF ENDOPHYTIC FUNGI

The identification of fungi was done using cultural and microscopic characteristics such as shape, colour, pattern arrangement of mycelium and conidial arrangement, types of spores by using standard manuals such as, A manual of Penicillia by Kenneth (1949). The genus *Aspergillus* by Kenneth Raper (1965), Fungi in Agricultural Soils by Domsch and Gams (1972) and A manual of soil fungi by Joseph C.Gilman (1957). All the isolated fungi were identified up to genus level on the basis of detailed culture and microscopic study and by consulting relevant literature. The pure culture of isolated fungal strains were maintained in PDA slants at 28° C.

Lacto phenol Cotton Blue Mounting:

A loopful culture was picked up with the help of a sterile inocu-

lation loop and semi permanent slides were prepared using lacto phenol cotton blue. The slides were gently heated in a spirit lamp so as to release the air bubbles, if any present inside the cover glass. The excess stain was removed by using tissue paper and the cover glass was sealed with white nail polish.

RESULTS

This resulted in association with *Acanthus ilicifolius,* was selected and its leaves only taken for isolation of endophytic fungi. Each isolates were sub-cultured into a PDA agar plates to remove the adherent plant metabolite from the mycelia and stored at 4°C for further studies.

A total of fourteen species have been isolated from leaves of *Acanthus ilicifolius,* The isolated organisms were *Absidia, Aspergillus fumigatus, A.ochraceous, A.terreus A.candidus A.janus, A.nidulans, A.ustus, A.granulosis A.versicolor, A. flavus, A.panamensis, Fusarium* sp and *Alternaria* sp.

Absidia sp

Mycelium formed as in the genus Rhizopus by frequently branched stolons; more or less in curved into arches and producing at the point of contact with the substratum more or less richly branched rhizoids. Sporangiophores straight, rarely single, more often in groups of two to five, occurring at the curve of the stolon (internodal) and not at the point of origin of the rhizoid. A cross-wall is placed at a definite distance below the sporangium. Spores small, 5-6µ, round l (not angular) with a smooth wall, rarely echinulate, colorless or bluish-black. Zygospores formed on the stolons. They are surrounded by circinate filaments, cutinized, which are borne in a whorl from one or both of the suspensors.

Aspergillus fumigatus

Colonies green to dark green conidiophores short, usually densely crowded submerged hyphae or as branches from aerial hyphae septate or non septate, gradually enlarged conidia dark green in mass, globose.

A.ochraceous

Colonies on czapeks agar ochraceous shades, consisting of conidiophores and conidial heads with little aerial mycelium. Conidiophores variable in length, commonly several millimeters, pitted, yellow, bearing large, radiate conidial heads. Vesicles 60-75µ in diameter; phialides in two series, primary commonly 15-30µ long although some times longer, secondary 7-10x1.5-2µ. Conidia globose to elliptical, smooth or delicately spinulose, yellow, 3.5-5µ or 3.5-4 purple sclerotia commoly present.separated into radially arranged.

A.terreus

Colonies on czapeks agar from tints of pinkish cinnamon through shades in age, spreading, velvety, anastomosing ropes of aerial hyphae. Reverse and agar from pale or bright yellow to fairly deep brown conidiophores to 150µ long × 5-8µ, more or less flexuous with walls smooth, septate with apex enlarged to form a vesicle commonly 12-18µ in diameter.

A. candidus

Surface growth usually stalks and heads with scanty sterile mycelium or anastomosing ropes of hypae bearing short fertile stalks. Conidiophores very with the strain. Walls thick, smooth heads white globose radiate. Conidia colourless, globose, smooth sclerotic occasionally produced.

A.janus

Colonies varying in colour and texture, depending on substratum and temperature incubation; on czapeks agar at 24°C . spreading irregulary, consisting of a central floccose mass, 1-2cm deep, pale yellow-buff, bearing few to abundant fruiting structures, surrounded by an irregular zone of crowed dark green heads adjacent to the substratum with numerous long- stalked white heads projecting above this later. Hulle cells commonly present, light yellow in color: reverse dull yellow to light brown. White conidial heads loose in texture, with radiating and divergent chains of conidia, commonly 150-200µ in diameter. Conidiophores long, thin, 2-3.5 mm in length by 8-10.5µ in diameter, erect, walls smooth colourless.

A.nidulans

Colonies on czapeks agar plane, spreading broadly, dark cress-green from abundant conidial heads during the first two weeks; cleisothecia developing from the center of the colony outward, separately produced, often abundant ; sectoring occasionally. Reverse of colony in shades of purplish-dark in age. Conidial heads short columnar, 40-80x 25-40µ. stalks sinuous, with smooth walls, in shades of cinnamon- brown, ranging from 60-130u, about 2.5-3u,in diameter near foot, to 3.5-5µ below the terminal visicle; visicle 8-10u in diameter.

A.ustus

Colonies floccose, with fine hyphae, color, in old cultures purplish, vinaceous at times. Reverse through shades of yellow, orange and brown stalks when rising from submerged hyphae up to 1,000u, most branches of aerial hyphae up to 500x5-10µ, five septate conidia globose about 3.6u (3.5-4u) spinulose some strains show sterile.

A.granulosis

Colonies on czapeks agar growing well, floccose, uneven in texture , buff to dull brown from felted sterile mycelium. Conidial heads few, arising from substrate, in clusters, pale blue- green. Colonies semi- granular in appearance from abundant, small colorless clusters of globose, ovoid, or elliptical thick-walled hulle cells. Reverse dull yellow and brown; with slight mushroom odor. Conidial heads few clustered in small groups mostly at margin, hemispherical to radiate, 75-125µ in diameter, very loose, pale Niagara green. Conidiophores erect, straight, non septate, 350-500×5.5-8µ, uniform in width, thin –walled, smooth, tan to light brown often slightly constricted beneath vesicle. Vesicles ovate to elliptic 12-18µ in diameter, thin walled, largely covered by phialides.

A.versicolor

Colonies on czapeks agar with vegetative mycelium largely submerged sparse, spreading slowly, producing green heads freely in center, less abundantly in outer areas, large gray cleistothecia in clusters at center, and scattered toward the margins; reverse in shades of purple- red. Conidial heads green ; columnar, relatively long, 100-200× 30-40µ. Conidiophores from submerged hyphae, straight with smooth walls, cinnamon brown, 140-200×3-5µ, broadening gradually to hemispherical vesicles 8-10µ in diameter. Primary phialides 7-8×3-4µ; secondary phialides, 8-9×2.5-3µ. Conidia globose, rugulose, 3-3.5µ.

A. flavus

Very rapid rate of growth, maturing in about three days. Surface is greenish-yellow to olive and may have a white border. Septate hyphae with rather long conidiophores (~400-800 X 8-17 µm) which have a rather rough texture or even spiny, especially just below the vesicle. Vesicles are spherical to elongate and about 20 – 45 µm wide. can be variable in seriation with most strains being about 20% biseriate however some strains can be almost entirely uniseriate. Metulae (8-10 X 5-7 µm) cover three quarters to the entire surface of the vesicle from which the phialides (7-12 X 3-4 µm) form. Conidia are globose to ellipsoidal (3-6 µm) with smooth to finely roughened walls.

A.panamensis

Colonies on czapeks agar very thin almost wholly submerged,

with widely scattered conidial structures with radiate heads, light brown; in maltose agar fruiting luxuriantly, reddish-brown, often in concentric zones. Fertile conidiophores bearing globose to radiating heads light brown with red sterile structure; in age developing a loose floccose overgrowth. Reverse dull brown odor none. Conidial heads globose, with loosely radiating chains of conidia, variable, 250-450µ in diameter.

***Fusarium* sp**
Colonies on PDA medium white conidial layer cushion shaped or somewhat extended without a spindle or sickle shaped, many celled with indistinct cross walls stroma brownish, white to violet sclerotial hard bodies. Medium, high aerial mycelium but are lacking in the typical fruiting layers of the macroconidia. Chlamydospores terminal and intercalary globose.

***Alternaria* sp**
Sterile hyphae creeping, septate, conidiophores single or in groups erect, septate, mostly unbranced, short, conidia inverted club shaped mostly elongate at the tip muriform in the lower portion dark colored lighter at the points – usually simple chains.

DISCUSSION
The 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 substance for host (Weber, 1981: Shiomi *et al*., 2006). Endophytic organisms have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivorous (Weber, 1981). Fungi have been widely investigated as a source of bioactive compounds, an excellent example is anticancer drug taxol, which had been previously to occur only in the plants (Strobel and Daisy, 2003).

Endophytic fungi were isolated from the healthy and mature stem pieces of *piper nigrum* collected from a local farm.surface sterilization of the plant material was carried out following the protocol described by Aravind *et al* (2009).

The endophytic fungi *P.citrinum* was isolated from the *B.gymnorrhiza*. The fungus was identified by morphological characteristics and analysis of the rDNA. The sequence data derived from the fungal strain have been deposited at Gen Bank with the accession number HM008916 discribed by Wang and He (2006).

The endophytic fungal community associated with the native *cactus opuntia* in the united states was investigated and its potential for providing antifungal compo bunds. A hundred-eight endophytic fungal isolates were obtained and identified by molecular methods into 17 different taxa of the genera *Alternaria, Aureobasidium, Biscogniauxia, Cladosporium, Cryptococcus, Curvularia Diaporthe, Epicoccum, Paraconiothyrium, Pestalotiopsis and Phoma*. The most frequent species associated with *O. humifusa* were *Alternaria* sp. 3, *Aureobasidium*, *Pullulans* and *Diaporthe* sp. The fungal community of *O. humifusa* had a high richness and diversity; additionally, the species richness obtained indicates that the sample effort was enough to recover the diversity pattern obtained.

In the present investigation I have been isolated fourteen species were screened from the leaves of *Acanthus ilicifolius* such as *Absidia, Aspergillus fumigatus, A.ochraceous, A.terreus A.candidus, A.janus, A.nidulans, A.ustus, A.granulosis A.versicolor, A. flavus, A.panamensis*, *Fusarium* sp, *Alternaria* sp.

CONCLUSION
Resistance to drugs used in the treatment of bacteria, but also for fungal or parasitic origin is necessary and new resistance problems are emerging, further complicating and rendering many infectious diseases, not only treatment of critical infectious illness more difficult. Increasing prevalence of multi resistant bacteria has turned the search for new antimicrobial agent, an important strategy for alternative therapies useful in the handling of difficult infections. The present study leads to the need of these isolated bioactive endophytic fungal isolates. Further, the best proved active isolates should be identified using available methods to place these fungi in the fungal kingdom.

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