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Mycodiversity and biotechnological potential of endophytic fungi isolated from hydrophytes

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Abstract

Foliar endophytic fungi were isolated from regular hydrophytic plants such as *Eichhornia crassipes (*Pontederiaceae*), Nymphaea nouchali* (Nymphaeaceae), *Vallisneria spiralis* (Hydrocharitaceae) growing in a pond in the Anna Zoological Park, Chennai, Tamil Nadu. Five hundred leaf segments from each plant species were inoculated in Potato Dextrose Agar (PDA) medium. A total of 18 different endophytic fungi could be isolated from three host surveyed. Hyphomycetes group was the most prevalent endophyte than ascomycetes, coelomycetes, and sterile form. *Nymphaea nouchali* had more endophytic fungi (18 species) followed by *Eichhornia crassipes* (12 species), and *Vallisneria spiralis* (11 species). To our knowledge *Vallisneria spiralis* is studied for endophytic fungi diversity for the first time. Among the 18 endophytic fungi isolated, nine were present in all three hosts investigated for endophytic fungi. *Curvularia lunata, Chaetomium indicum, Nigrospora oryzae, Pestalotiopsis* sp. showed <5% of colonization frequency in all three host species studied. The culture filtrate of *Curvularia lunata, Nigrospora oryzae, Chaetomium indicum and Pestalotiopsis microspora* investigated contained alkaloids, flavonoids, terpenoids, tannins and steroids. The dominant endophytic fungi were tested for production of extracellular enzymes like amylase, cellulase, L- asparaginase, laccase and protease. *Curvularia lunata, Chaetomium indicum* and *Pestalotiopsis microspora* produced all the enzymes tested, whereas *Nigrospora oryzae* did not produce L- asparaginase. The culture filtrate of *Chaetomium indicum* and *Pestalotiopsis microspora* significantly increased the cell division in *Allium cepa* root meristem and the radical plumule length in AD8 rice variety.

Key words – *Eichhornia crassipes* – *Nymphaea nouchali* – *Vallisneria spiralis* – Hydrophyte – Leaves – Bioactive compounds

Introduction

The interior parts of plants like the tissues and organs are inhabited by microorganisms like fungi and bacteria that do not cause any damage to the host tissue. They are known as endophytes or endophytic microorganisms (Almeida et al. 2015). Endophytic fungi represent an interesting

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group of microorganisms associated with the herbs, shrubs, trees, ethnopharmaceutically important medicinal trees, coastal mangroves, temperate, alpine areas, tundra, dry desserts and tropical rain forests from the Artic to Antarctica. (Tejesvi et al. 2006, Espinosa & Langenheim 1990, Luiz et al. 2012). The host plant receives some benefits from the plant-endophyte interactions such as modification in plant physiology and protection against insect pests and phytopathogenic microorganisms. Moreover, compounds like enzymes, antibiotics, alkaloids, with biotechnological importance have been reported to be produced by endophytes (Azevedo et al. 2002, Peixoto et al. 2002). Endophytic studies on true hydrophytic hosts are very limited, however few studies have been carried out on the distribution of endophytic fungi in aquatic plants (Li et al. 2010, Tejesvi et al. 2006, Young-Hyun et al. 2015, Das et al. 2013). Endophytes are horizontally transmitted and they form an endosymbiotic relationship with host plants of all kind. The universal occurance of endophytic fungi has been reported from plants from various ecological niches. Besides these fungi are known to produce various secondary metabolites with distinct bioactivities. This has led to their use in various biotechnological applications (Suryanarayanan 2017). Recently, Raman & Suryanarayanan (2017) reported that the plant-feeding insects' relation is influenced by fungus plant interaction. Endophytic fungi are also involved in multiple balanced antagonisms (Schulz et al. 2015). The evidence for broad trophic status of leaf endophytic fungi of *Quercus gambelii* was first showed by Szink et al. (2016). Wang et al. (2016) unraveled the chemical interactions of endophytic fungi for exploitation as microbial factories and its sustainable applications in biotechnology. In the present study, three true hydrophytic plants were screened for endophytic fungi. For the first time *Vallisneria spiralis* leaf tissue was screened for endophytic fungi diversity.

Material & Methods

Collection of plant material and surface sterilization

Fresh and healthy leaves were collected from hydrophytic plants growing in the pond of Anna Zoological Park, Chennai, South India. Ten hosts of each species were chosen for this investigation and about 10-12 leaves were collected from each plant species at the water level. Plant samples were transported to the laboratory in a closed sterile zip bags and processed within a day of collection (Fisher & Petrini 1987). From the sample collected, 2 to 3 segments with approximately 5 mm length were cut from the healthy leaves (Cabral et al. 1993). The surface sterilization of the leaf segments was done by dipping in 70% alcohol for 15 secs then in 4% NaOCl for 30 seconds followed by a rise in autoclaved distilled water (Modified method of Dobranic et al. 1995). Five hundred leaf segments from each plant species were placed on Potato Dextrose Agar (PDA) medium. The growth medium is amended with streptopenicillin (150 mg/L) for arresting the growth of bacteria. The petri dishes were incubated at 27°C ±1°C under a 12 hours fluorescent light: 12 hours of dark cycle (Bills & Polishook 1992, Suryanarayanan & Rajagopal 1998). Petri dishes were observed for 3-4 weeks for endophytic fungal growth. The petri dishes were observed regularly and endophytic fungi which grew out from the leaf segments were subcultured to fresh PDA slants. Those fungi which failed to sporulate were given identification numbers (Dobranic et al. 1995) based on the morphological characters. The colonization frequency was calculated as the percentage of leaf segments that were colonized by one or more isolate(s) from the total number of segments incubated × 100 (Maheshwari & Rajagopal 2011). The Simpson dominance index and Shannon-wiener's diversity index were calculated for fungal diversity (Poole 1974, Groth & Roelfs 1987).

Qualitative test for chemical compounds

Different qualitative tests were conducted to deduct chemical groups like alkaloids, flavonoids, terpenoids, steroids and tannins (Prashant et al. 2011).

Test for alkaloids

Endophytic fungal extracts were dissolved individually in dilute hydrochloric acid at a concentration of about 2 mg/ml and then filtered. Dragendroff's Test- This filtered solution is treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Presence of alkaloids is confirmed by the formation of red precipitate. Wagner's Test- Presence of alkaloids is also is tested using Wagner's reagent (Iodine in Potassium Iodide) with the formation of brown/reddish precipitate.

Test for flavonoids

Each endophytic fungal extracts were tested for flavonoids individually.

Alkaline Reagent Test

Few drops of sodium hydroxide solution was added to individual endophytic fungal extracts. Intense yellow color formation and immediate decolorization on adding dilute acid shows the presence of flavonoids.

Lead Acetate Test

Few drops of lead acetate solution added to endophytic fungal extracts and the presence of flavonoids is indicated by yellow colour precipitation.

Test for diterpenoids

Copper Acetate Test

Copper acetate solution was added to each endophytic fungal extracts dissolved in water and formation of emerald green indicates the presence of diterpenes.

Test for phenol

Few drops of ferric chloride solution was added to endophytic fungal extracts and formation of bluish black colour indicates the presence of phenol.

Effect of endophytic fungal extract on rice seed germination

Four dominant endophytic fungi *Chaetomium indicum*, *Curvularia lunata*, *Nigrospora oryzae* and *Pestalotiopsis microspora* were grown in PD broth as shake culture for 14 days. The resultant mycelium was filtered using 3 layers of cheese cloth. 15% H_2O_2 was used to soak rice grains (Aduthurai -ADT-36 variety) for 10 min for surface sterilization (Helton & Dilbeck 1982). Surface sterilized grains were later soaked in the culture filtrate for 48 h and then placed on a sterile, wet cotton bed set in enamel coated trays and then incubated for 4 days at 30°C in darkness at an elevated angle of 45° from the horizontal plane. This causes the geotropic straightening of radical thereby enabling accurate growth measurements (Helton & Dilbeck 1982). Uninoculated liquid medium was used as a control.

Effect of endophytic fungal extract on onion root mitosis

The dominant endophytic fungi from each group *Chaetomium indicum*, *Curvularia lunata*, *Nigrospora oryzae* and *Pestalotiopsis microspora* were grown in liquid Potato Dextrose Broth (PDB) (pH 6.2) for 14 days. An equal amount of ethyl acetate was used to extract 50 ml of the culture filtrate. Evaporation of the organic phase was done and the residue left behind was dissolved in 25 ml of distilled water (Modawi et al. 1992). For control, ethyl acetate extract from inoculated medium was used. Distilled water was used to root fresh onion bulbs. 1-2 cm long roots were treated with culture filtrate extracts for 48h. Acetic acid:ethanol (1:3) was used to treat root-tips for 24 hrs. Then washed and hydrolyzed in 1N HCl at 30 °C for 30 min. Once again washed and treated with acetocarmine stain (2%) for 15 min. The processed roots were squashed on a microscopic slide for observing cell division. About 1000 cells for each treatment were observed. The mitotic index was calculated using the following formula:

$$\text{Mitotic Index} = \frac{\text{Total number of dividing cells}}{\text{Total number of cells observed}} \times 100$$

Detection of extracellular enzyme production by endophytic fungi (Hankin & Anagnostakis 1975, Rohrmann et al. 1992, Rajagopal 1999)

CDA was used as the basic medium for this study. Petri dishes (90 mm dia) inoculated with mycelia discs (5 mm diam) obtained from the growing edge of the colony on CDA medium was used for conducting the enzyme tests at 26°C. Semiquantitative method was used to determine the production of extracellular enzyme. Here, respective substrates or reagents were incorporated or added into the medium or over a period of growth. The appearance of colored or clear zones were recorded in arbitrary units.

Laccase (EC 1.10.3.2)

CDA medium of pH 6.0 containing 0.05 g 1- naphthol/L was used. 1-naphthol is oxidized by laccase thereby turning the colorless medium blue (Higuchi & Kitamura 1953).

Amylase

CDA medium with 0.2% soluble starch was used. When Iodine solution was used to flood the growth, a yellow zone was developed around the colony in an otherwise blue medium which shows the presence of amylase.

Protease

CDA medium with 0.4% gelatin (8% solution of gelatin in water was sterilized separately and added to the CDA medium at the rate of 5 ml/100ml medium) was taken and inoculated. After incubation, gelatin was degraded and it was evident with a clear zone around the colonies. Aqueous saturated solution of ammonium sulphate was used to flood the petri dish so that a white precipitate was formed making the agar more opaque and enhancing the clear zone around the colony.

Cellulase C3 (endoglucanase EC 3.2.1.91)

0.5% Na-carboxy methyl cellulose was incorporated in the CDA medium. After incubation, growth was flooded with 0.2% aqueous congo red and destained with 1 M NaCl (15 min each). A yellow zone around the fungal colony in the red substrate showed the presence of cellulase activity (Teather & Wood 1982).

L- asparaginase (L- asparaginase aminohydrolase EC 3.5.1.1)

CDA medium (PH 6.2) containing 1% L- asparagine and 0.009% phenol red was used. The activity of L- asparaginase was confirmed with the formation of a clear zone around the colony margin (Gulati et al. 1997).

Results and Discussion

Various groups of plants growing in different ecological systems have been studied for endophytic fungal distribution and for their bioactive compound production. However, very scant attention has been accorded to the distribution of endophytic fungi in hydrophytic plants. This paucity of research prompted us to investigate few hydrophytic plants of tropics for endophytic fungi distribution and bioactive production. In this study, all the three species of host plant were found to be colonized by endophytes. Hyphomycetes were the dominant group followed by ascomycetes, coelomycetes and sterile form (Table 1). Basidiomycetes were absent and are usually isolated in very low numbers as endophytic fungi (Petrini 1986, Suryanarayanan et al. 1998, Rajagopal 1999). Among the three host species studied *Nymphaea nouchali* leaves yielded a total of 18 endophytic fungi while *Eichhornia crassipes* yielded 12 endophytes and *Vallisneria spiralis* yielded 11 endophytic species respectively (Table 1). Almeida et al. (2015) isolated 8 different fungal endophytes from the leaves of *Eichhornia crassipes.* They were identified as *Bipolaris* sp.,

Microsphaeropsis arundinis, Curvularia trifolii, Bipolaris papendorfii, Cercospora kikuchii, Plectosphaerella cucumerina, Phoma sp. and *Bipolaris sorokiniana.* In the present investigation, except for *Bipolaris* sp., none of the above mentioned endophytic species have been reported in *Eichhornia crassipes* from Almeida et al. (2015) but were reported in our study. This indicates that endophyte distribution differ due to local environmental conditions and climate. Similarly, Ranga et al. (2016) reported 8 different endophytic isolates from leaves of *Nymphaea nouchali*. Among the eight endophytic fungi isolated, *Chaetomium* sp., *Colletotrichum* sp. and *Fusarium* sp. were found to be dominant in leaves. In the current study also similar endophytic fungi were isolated. To our knowledge, no reports have been made on the isolation of endophytic fungi from *Vallisneria Spiralis*, but all the endophytic fungi isolated were common endophytic fungi already been reported in other studies. Even though 18 different endophytic fungal species were reported in three hosts (Table 1), only 9 endophytic fungi showed appreciable colonization frequency, above 5% (Table 1). *Alternaria alternata, Aspergillus niger, Bipolaris* sp, *Curvularia lunata, Drechslera hawaiiensis, Nigrospora oryzae, Penicillium* sp, *Chaetomium indicum and Pestalotiopsis microcarpa* could be isolated from all the three host species studied. Some endophytic fungi were host specific viz., *Aspergillus stellatum, Drechslera halodes, Colletotrichum* sp. and sterile mycelia were present only in *Nymphaea nouchali* (Table 1). The endophytic fungi assemblage all the three species dominated by *Pestalotiopsis microspora, Chaetomium indicum, Curvularia lunata* and *Nigrospora oryzae* (Table 1). Suryanarayanan et al. (1998), Petrini (1986) stated that only one or a few endophytic fungi dominate single host species. Thus, the variation in endophytic fungi composition and colonization frequency indicated that host specificity was shown by certain endophytic fungi. Ellis et al. (1988), Mungai et al. (2012) reported that *Sporormiella* and *Chaetomium* as coprophilous fungi. The presence of coprophilous fungi as endophytes even though is not common Petrini (1986) but reported in the present study. Suryanarayanan et al. (1998) isolated *S. minima* and *C. globosum* as endophytes from two *Rhizophora* sp. Among the various groups, mitosporic fungi (asexual states of mostly ascomycetes and basidiomycetes) represented 61.1 %, ascomycetes 16.6%, coelomycetes (16.6%) and sterile mycelia (5.5%). Basidiomycetes and Zygomycetes were absent and are usually isolated in low numbers in endophytic research (Suryanarayanan et al. 1998, Maheshwari & Rajagopal 2011). In the current study also basidiomycetes and zygomycetes were not isolated. In comparison to other tropical trees screened for endophytes, our host plants harboured more endophytic fungal taxa. The restricted number of endophytes in these three hosts could be attributed to several anti- microbial particularly anti-fungal compounds present in the leaf tissue (Thamaraiselvi et al. 2012, Duraipandiyan & Ignacimuthu 2011, Qiming et al. 2006).

Table 1 Distribution and Mean Colonization frequency of endophytic fungi isolated from the leaf tissues of hydrophytic host**s**

Endophyte	**Host Plants**		
	Eichhornia crassipes	***Nymphaea nouchali***	***Vallisneria spiralis***
Hyphomycetes			
Alternaria alternata	4.2	5.1	2.2
Aspergillus stellatum	-	3.8	-
Aspergillus niger	1.0	2.1	2.1
Bipolaris sp.	2.1	5.0	2.0
Cladosporium cladosporioides	-	5.2	3.0
Drechslera halodes	-	6.1	-

Table 1 Continued.

Endophyte	Host Plants		
	Eichhornia crassipes	***Nymphaea nouchali***	***Vallisneria spiralis***
Drechslera hawaiiensis	3.6	3.2	1.0
Fusarium oxysporum	3.7	6.0	-
Nigrospora oryzae	6.7	15.0	5.1
Penicillium sp.	2.6	4.2	2.1
Ascomycetes			
Chaetomium indicum	5.7	7.1	5.1
Chaetomium globosum	-	3.0	3.9
Sporormiella minima	3.1	2.1	-
Coelomycetes			
Pestalotiopsis microspora	6.1	5.1	5.4
Phyllosticta sp.	2.0	4.0	-
Colletotrichum sp.	-	3.2	-
Sterile mycelia			
Sterile mycelia	-	2.1	-

Apart from environmental factors, there appeared to be some selection mechanism at work with reference to host-endophyte association (Rajagopal 1999). This was evident when diversity indices (Simpson and Shannon Wiener index) for the endophytic fungi were calculated. Even though the three hosts growing in same type of ecosystem showed different endophytic fungi diversities (Table 2), among the 3 hosts, *Nymphaea nouchali* showed highest Simpson and Shannon wiener index (0.854 and 2.7). This indicated that this host had more diversity and species are distributed equally. The Simpson index is least for *Vallisneria spiralis* (0.842 and 2.3), which indicates least number of endophytes isolated from this host (Table 2).

Table 2 Simpson and Shannon Wiener Index study on the three hosts of hydrophytes

Host plant	Simpson Index	Shannon Wiener Index
Eichhornia crassipes	0.854	2.7
Nymphaea nouchali	0.966	3.0
Vallisneria spiralis	0.842	2.3

Endophytic fungi are the excellent source of major chemical compounds including alkaloids, flavonoids, terpenoids, phenols etc. (Suryanarayanan 2017, Suryanarayanan et al. 2012, Tan & Zou 2001, Rajagopal et al. 2015). In the present investigation an in depth study of four endophytic fungi crude extract isolated from three hosts were tested for bioactive compound production and their activity. The endophytes used in the present studies are *Chaetomium indicum*, *Curvularia lunata*, *Nigrospora oryzae* and *Pestalotiopsis microspora*. All the four dominant endophytic fungal extracts were tested for the presence of various chemical groups like alkaloid, flavonoid, diterpenid and phenol in their extract respectively. The results are presented in (Table 3). All the four endophytes

produced all the chemicals tested. Feng et al. (2017) (Feng et al. 2017, Zhang et al. 2012, Ruby et al. 2011) and few others have reported that a number of flavonoids, phenolics, alkaloids, diterpenes produced by endophytic fungi have been discovered from endophytic fungi in plants, which showed excellent biological properties such as anti-microbial, insecticidal, cytotoxic and anti-cancer activities. Hence, production of such chemical compounds by these endophytic fungi would enhance their host resistance to microbes and insects. However, such a syllogistic conclusion is not indefensible as some more evidences are required sustaining this surmise.

Table 3 Various chemical groups present in endophytic fungi isolated from hydrophytes

Chemical Groups Present	**Endophyte**			
	Nigrospora oryzae	***Chaetomium indicum***	***Pestalotiopsis microcarpa***	***Curvularia lunata***
Alkaloid	+	+	+	+
Flavonoid	+	+	+	+
Diterpenoid	+	+	+	+
Phenol	+	+	+	+

Fungi have to penetrate and enter the host tissue to lead the endophytic mode of life. Hence, endophytic fungi must have the potential to produce cell-wall degrading enzymes such as pectinolytic and cellulolytic enzymes, which are essential strategy for endophytic fungi to damage and penetrate the host tissues to lead an endophytic mode of life. Many of the foliar endophytic fungi isolated from the forest trees of Western Ghats produced extracellular enzymes like amylase, cellulases, chitinases, laccases, pectinases, proteases etc. (Kumaresan & Suryanarayanan 2002). Fisher et al. (1992) also have shown that several endophytic fungi produce such enzymes. In the present study also, it was found that *Chaetomium indicum*, *Curvularia lunata*, and *Pestalotiopisis microspora* produced all the enzymes in culture when qualitatively studied (Table 4). *Nigrospora oryzae* produced all enzymes except L-asparaginase (Table 4).

Table 4 Qualitative analysis of extra cellular enzymes produced by endophytic fungi isolated from hydrophytes

Endophyte	**Amylase**	**Protease**	**Cellulase**	**Laccase**	**L- asparaginase**
Curvularia lunata	+	+	+	+	+
Nigrospora oryzae	+	+	+	+	–
Chaetomium indicum	+	+	+	+	+
Pestalotiopsis microspora	+	+	+	+	+

Sun et al. (2011) reported that endophytic fungi have to necessarily produce a variety of enzymes to overcome the host barriers of plant to enter and counter the host defense mechanism. The capacity to produce cell wall degrading enzymes such as cellulolytic and pectinolytic enzymes is an essential strategy for endophytic fungi (Arumugam 2016). Several studies on endophytic research have shown to produce these enzymes (Arumugam 2016, Rajagopal 1999). Endophytic fungi are known to produce several bioactive compounds in culture (Suryanarayanan 2017, Tan & Zou 2001). But very scant attention has been given on the influence of endophytic fungi metabolite on cell division in root meristem and induction of root growth. Luginbuhl & Muller (1982) showed that culture filtrates of *E. purpurascens* and *A. pullulans* positively affect ivy seed germination. Our

study showed varied results, *Chaetomium indicum* and *Pestalotiopsis microspora* significantly increased the radical and plumule length in ADT-36 rice variety (Table 5). Whereas, *Curvularia lunata* and *Nigrospora oryzae* inhibited the growth of radical and plumule growth (Table 5). Another indication of the production of bioactive compounds by four endophytic fungi of current study was that the culture filtrate of *Chaetomium indicum* and *Pestalotiopsis microspora* significantly increased the mitotic index of onion root meristems whereas culture filtrates of *Curvularia lunata* and *Nigrospora oryzae* reduced the cell division (Table 6). Rajagopal (1999) reported that the culture filtrates of neem endophytes influenced onion root mitosis positively. It is interesting to note that those endophytic fungi that induced root elongation in rice also increased the mitotic index in onion root mitosis (Tables 5, 6). To conclude, three hydrophytic plants were shown to harbor fungal endophytes and for the first time endophytic distribution in the leaf of *Vallisneria spiralis* was studied. Again for the first time, it was shown that the endophytes of hydrophytes produce bioactive compounds that could influence cell division and root elongation in plants.

Table 5 Germination of AD8 rice variety as influenced by culture filtrate of endophytic fungi (length in mm)

Endophyte	Radicle±SD	Plumule±SD
Control	15.5±4.0	14.0±2.0
Curvularia lunata	12.9±3.0	13.8±2.1
Nigrospora oryzae	14.0±2.9	14.1±2.0
Chaetomium indicum	23.5±3.0	17.1±3.1
Pestalotiopsis microspora	21.6±2.4	15.0±2.9

Table 6 Effect of endophytes on mitosis in onion root meristem

Endophyte	Prophase		Metaphase		Anaphase		Telophase		Total
	MI±SD	%	MI±SD	%	MI±SD	%	MI±SD	%	
Control	3.7±0.8	62.0	1.2±0.5	21.1	1.0±0.5	11.8	0.9±0.4	5.1	**6.8**
Curvularia lunata	2.0±0.3	33.0	1.4±0.4	28.0	1.6±0.5	25.0	1.1±0.4	14.0	**6.1**
Nigrospora oryzae	2.2±0.2	44.0	1.6±0.4	29.0	1.8±0.5	19.0	1.0±0.4	8.0	**6.6**
Chaetomium indicum	2.4±0.4	39.0	2.0±0.6	35.0	1.9±0.4	14.0	1.7±0.5	12.0	**8.0**
Pestalotiopsis microspora	2.2±0.4	40.0	2.1±0.5	35.0	2.0±0.5	12.0	1.5±0.4	13.0	**7.8**

MI = Mitotic index, SD = Standard deviation, % = percentage of dividing cell

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