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Fungal endophytes of an aquatic weed *Marsilea minuta* Linn

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Abstract

Endophytic fungi are the organisms that colonize a plant without causing apparent harm to the host at any time in their life cycle. Endophytes from different angiosperms are widely studied however such studies from Pteridophytes are rare. In this study, the plant *Marsilea minuta*, an aquatic Pteridophyte belonging to the family Marsileaceae was explored for the presence of endophytic fungi. The plant was collected from four different places in and around Chennai (Avadi, Ambattur, Chengalpet and Guduvanchery). A total of 800 segment consisting leaflets, stolens, runners and roots (200 segments each) were investigated for the presence of endophytes. The study resulted in isolation of 574 colonies belonging to 17 fungal species among which 14 forms the Hyphomycetes, three forms the Coelomycetes and one form the non sporulating morphospecies. The data obtained were analysed statistically for relative density, colonization frequency, Species diversity indices like Gleason index, Shannon index, and Simpson's dominance index.

Keywords – Aquatic – Endophytic Fungi – Pteridophyte – Statistical analysis

Introduction

The term endophyte is defined by many authors, commonly as non-pathogenic organisms living within plant cell without causing disease symptoms or external structural alterations (Azevedo & Araujo 2007, Hyde & Soytong 2008, Sakalidis et al. 2011). Hirsch & Braun (1992) stated that the endophytes colonizing living plant tissues don't cause any immediate or overt negative impact. The role of endophytic fungi in ecological systems is varied and diverse. They support the host plant in drought tolerance and vigor (Stone et al. 2004). As mutualists they protect the plant against preying insects (Carroll 1991) and pests (Rocha et al. 2011). Further they help protect the host plants against oxidative stress by stimulating or enhancing the production of antioxidants (White & Torres 2010). Novel, biologically active metabolites of the host plant could be produced in large quantities from the associated endophytic fungi (Selim et al. 2011, Qadri et al. 2014, Tiwari et al. 2014). Thus, endophyte fungi could serve as a potential source for production of these metabolites and for isolation of new drugs (Kumar et al. 2005). Endophytes are ubiquitous in various plants (Arnold & Lutzoni 2007, U"Ren et al. 2012) which includes aquatic plants (Li et al.

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2010). Numerous studies have been carried out on the diversity of endophytes in vascular tissues of higher plants of terrestrial nature due to their diverse ecological roles. However, the endophytic diversity of aquatic Pteridophytes has been rarely studied.

Marsilea minuta L., an aquatic Pteridophyte belonging to the family Marsileaceae, is a small creeping fern with erect tetrafoliate leaves (Parrotta 2001). It is found throughout Africa, Madagascar and Comoros with wide occurrence in tropical Asia and as a weed in the southern United States. The bright green leaves are tender and are eaten as potherb in Senegal, Gambia and India. The weed is known to have traditional medicinal value and is used to stop nose bleeding, treat indigestion. The whole plant is used as sweet, astringent, coolant and expectorant. It is used in the treatment of psychopathy, diarrhoea, cough, skin diseases and fever as reported in Ayurveda. The reported medicinal properties of the plant includes anti- hepatitis and as anti-diabetic (Madhu et al. 2012). Many bioactivities of *M. minuta* have been identified (Parihar et al. 2003, Bhattamisra et al. 2007). Thus the present study aims to explore the endophytic population of the different plant parts of *Marsilea minuta* and further to study the fungal species diversity associated with geographical variation.

Materials & Methods

Sample collection

The healthy plants of *Marsilea minuta* were collected from four different places in and around Chennai (Avadi, Ambattur, Chengalpet and Guduvanchery) in sterile polythene bags and were processed within 24 hours.

Isolation of endophytes

The collected samples were washed thoroughly in running tap water. From the plant *Marsilea minuta*, 50 segments of different tissues of leaflet, petiole, rhizome and root each was cut into approximately $1cm^2$ segments were surface sterilised following the method of Dobranic et al. (1995). The segments were washed in 1% mercuric chloride for 10 seconds, sequentially in 70% ethanol for 30 seconds and finally rinsed with sterile water for 5 seconds. Ten segments of each plant tissues of different geographical locations were placed on Potato Dextrose Agar (PDA) medium amended with streptomycin.

The petri dishes were incubated in a light chamber for a period of three weeks at 28±2˚C temperature by maintaining dark -light cycle (Suryanarayanan 1992). The light chamber had a bank of three 4-feet Philips day light fluorescent lamps. The segments received 2200 lux of light through the Petri dishes lid as measured by a Lutron (Germany) 1×-10^1 Lux meter. The incubation temperature was 28 ± 2˚ C. The petri dishes were observed periodically and the growing fungi were transferred to fresh PDA slants. To prevent the rapidly growing fungi from inhibiting the slow growing species, the former were removed following isolation (Bills 1996). Sporulating isolates were identified to species level based on their microscopic and macroscopic morphology using standard manuals (Ellis 1971, 1976, Sutton 1980, Udayaprakash 2004). The sterile isolates which could not be assigned to any taxonomic group were sorted described as ˙sterile form´ in analysis of the results (Frohlich et al. 2000, Suryanarayanan et al. 2000)

Statistical Analysis

The density of colonization (rD %) of single endophytes species and the colonization frequency of endophytic fungi in the segments of *Marsilea minuta* were calculated by the method of Fisher & Petrini (1987).

$$\text{Relative density of Colonization} = \frac{\textit{Total no of individuals of a fungi recorded}}{\textit{Total number of segments screened}} * 100$$

$$\text{Colonization frequency} = \frac{Number\ of\ segments\ colonized\ with\ \geq 1\ isolate}{Total\ number\ of\ segments\ \ screened} * 100$$

Species diversity indices

The species diversity indices were calculated in accordance to Kumar & Hyde (2004)

$$\text{Gleason index } [H_G] = N_{p-1}/\ln N_i$$

Where Ni is the total number of fungal isolates, Np is the total number of species to which these fungi belong

$$\text{Shannon index } [H_S] = -\Sigma_j (p_j \ln p_j),\ j = 1\ldots\ldots Np,$$

Where j is the order given to the individual fungal species, pj is the proportion of individuals belonging to a particular species (i.e. j^{th} species) to the total number of individuals isolated.

$$\text{Simpson dominance index } (D) = 1/\Sigma j\ (pj^2)$$

Jaccard's Similarity index

Jaccard's Similarity Coefficients were calculated for all possible pairs of host tissues to compare the endophytic assemblages according to the formula:

$$\text{Similarity coefficient} = \frac{C}{A+B-C}$$

where, A and B are the total number of fungal species isolated from any two tissue type and C the number of fungal species found in common between them (Arnold et al. 2000).

Results

Endophytic fungi composition

The segments of leaflet, petiole, rhizome and root of *Marsilea minuta* were processed for isolation of endophytic fungi from the plant samples collected from four different places in and around Chennai (Avadi, Ambattur, Chengalpet and Guduvanchery). A total of 574 isolates were isolated from 800 segments of the plant. Altogether 17 fungal species were identified based on the morphology of the fungal culture and characteristics of the spore. Among the fungal species fourteen forms belongs to hyphomycetes and three forms to coelomycetes. The non-sporulating cultures were grouped together as sterile forms. The sporulating species accounted for 91.99% of the total isolates, while non-sporulating contributed 8.01%.

The leaflet segments were harboured with 158 isolates belonging to 13 species. The leaflet segments were dominated by the species *Colletotrichum* sp. (43.03%) followed by *Curvularia lunata* (25.31%) and *Drechslera halodes* (16.45%). *Curvularia lunata* was isolated from leaflet segments of Ambattur, Chengalpet and Kovipathagai, while the occurrence of *Colletotrichum* sp. was constrained to the leaflet segments from Guduvanchery and *Drechslera halodes* to leaflet segments of Kovipathagai site.

Segments of the petiole resulted in the isolation of 262 colonies belonging to 8 species. The petiole segments colonized in high proportion by *Curvularia lunata* (38.18%) followed by *Colletotrichum* sp. (18.32%) and *Drechslera halodes* (13.73%). Similar to leaflet segments, the occurrence of *Curvularia lunata* was high in petiole segments of Ambattur, Chengalpet and Kovipathagai, and *Drechslera halodes* was restricted to petiole segments of Kovipathagai site. The occurrence of *Colletotrichum* sp. was found in petiole segments of Chengalpet site in addition to Guduvanchery.

A total of 120 isolates belonging to 13 species was isolated from rhizome. The endophytic fungal community of rhizome segments was dominated by *Curvularia lunata* (45%) followed by *Phoma* sp. (13.33%) and *Fusarium* sp. (13.33%). The occurrence of *Curvularia lunata* was high in rhizome segments from Ambattur, Chengalpet and Kovipathagai sites, while *Phoma* sp. was found in rhizome segments of Chengalpet and Guduvanchery site and *Fusarium* sp. was found in rhizome segments of Chengalpet and Avadi sites.

Root segments yielded a mere 34 isolates belonging to 5 species which was comparatively the lowest. The endophytic population of roots was dominated by *Curvularia lunata* (52.94%) and Mycelia Sterile (29.41%). *Curvularia lunata* was found to occur in high frequency in Ambattur, Chengalpet and Kovipathagai. The occurrence of sterile forms was restricted to the root segments of Guduvanchery and Kovipathagai. The isolation frequency (IF %) was calculated for the sites studied and is tabulated in Table 1.

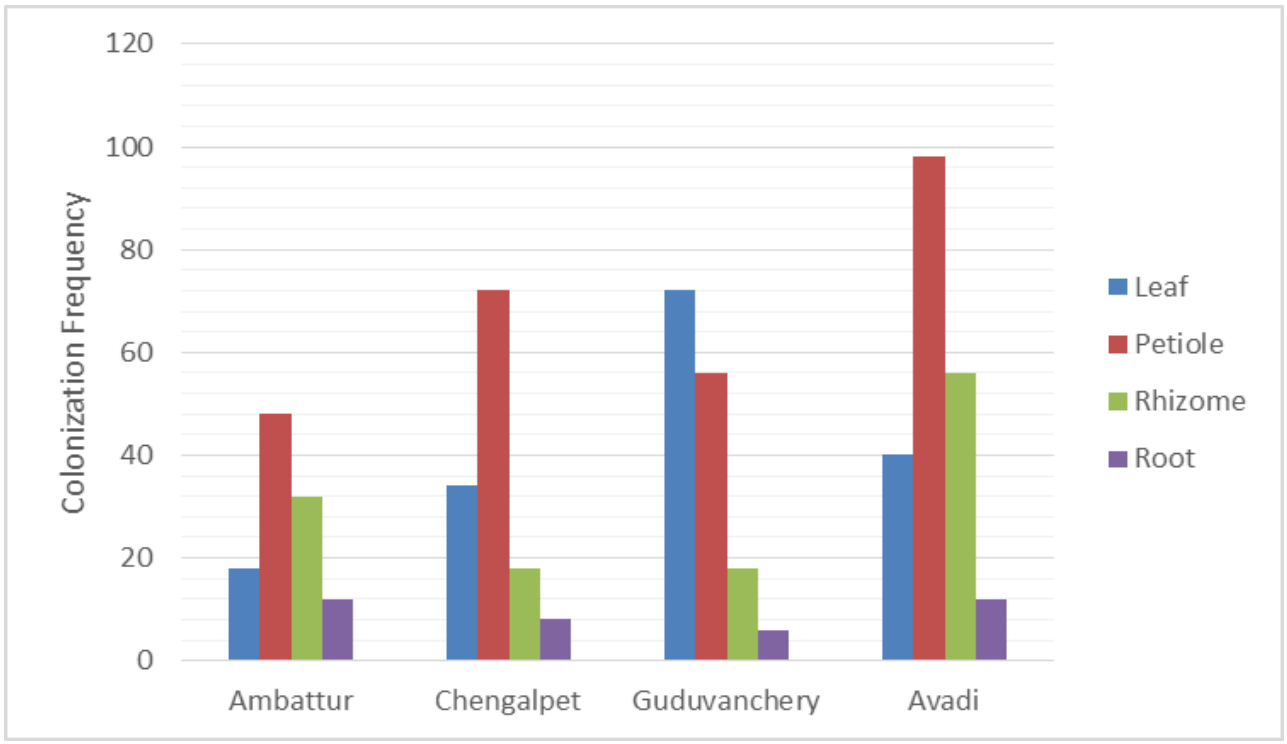

Fig. 1 – The colonization frequency of endophytic fungi from various plant parts

The colonization frequency of endophytic fungi from various plant parts is showed in fig. 1. It could be observed that the colonization percentage was comparatively low for root segments with maximum colonization percentage of 12% in Ambattur and Kovipathagai root segments. High colonization frequency of >45% in all petiole segments was observed. The highest frequency of 98% was observed with petiole segments from Avadi. Leaflet and Rhizome segments had moderate frequency of colonization.

Jaccard's species diversity:

The Jaccard's species diversity of the endophytic fungal diversity isolated from the plant *Marsilea minuta* is presented in Table 2. The data obtained was subjected to statistical analysis such as Gleason, Shannon and Simpson's Dominance index to evaluate the richness, evenness and dominance of the endophytic fungal species isolated. It was observed that the overall endophytic fungal community had Gleason, Shannon and Simpson's index value to be 2.67, 1.94 and 4.88 respectively. It was found that the overall endophytic fungal diversity had low evenness with the Pielou's evenness of 0.31. This is due to the occurrence of certain fungi in low numbers.

Table 1 Relative density of Colonization (rD %) of Endophytic fungi from four sites in and around Chennai

	Relative density of Colonization (%)															
	Ambattur				Chengalpet				Guduvanchery				Avadi			
	L	P	R	RT	L	P	R	RT	L	P	R	RT	L	P	R	RT
Acremonium sp.	0	0	0	0	7.14	0	25	0	0	0	0	0	0	0	0	0
Alternaria alternata	22.22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aureobasidium pullulans	0	0	0	0	0	0	0	0	2.77	0	0	0	0	0	0	0
Botryodiplodia thoebramae	11.11	0	0	0	0	23.33	0	0	0	0	0	0	0	0	0	0
Colletotricum sp.	0	0	5.26	0	0	10	0	0	94.44	85.71	0	0	0	0	0	0
Curvularia clavata	0	0	5.26	0	0	0	0	0	0	0	0	0	0	0	0	0
Curvularia lunata	22.22	95.83	63.15	83.33	85.71	36.66	25	50	0	0	0	0	30	24.48	50	50
Curvularia tuberculate	0	0	5.263	0	0	0	0	0	0	0	0	0	0	0	0	0
Drechslera australiensis	0	0	0	0	7.14	0	0	0	0	0	0	0	0	0	0	0
Drechslera halodes	0	0	0	0	0	0	0	0	0	0	0	0	65	36.73	17.85	0
Fusarium sp.	0	0	0	0	0	0	25	0	0	0	0	0	5	12.24	25	16.66
Nigrospora sphaerica	0	0	5.26	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium funiculosum	11.11	0	5.26	0	0	0	0	50	0	0	0	0	0	0	0	0
Penicillium sp.	22.22	0	0	16.66	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	0	30	25	0	2.77	10.71	77.77	0	0	0	0	0
Scolecobasidium humicola	0	0	5.26	0	0	0	0	0	0	0	0	0	0	0	3.57	0
Sterile form	11.11	4.16	5.26	0	0	0	0	0	0	3.57	22.22	100	0	24.48	0	33.33
Torula sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	2.04	3.57	0

Ambattur was represented by the highest number of endophytic fungal species (11 species) followed by Chengalpet with 8 species. Guduvanchery and Avadi were represented by 6 fungal species each. The number of isolates was high in Avadi (206 isolates) followed by Guduvanchery (152 isolates), Ambattur (116 isolates) and Chengalpet (100 isolates).

The similarity between species isolated from different plants parts of *Marsilea minuta* from four different sites in and around Chennai is presented in Table 3. It could be observed that plant parts of same site had high similarity of species. Among the sites, Avadi had high similarity of species within its plant parts and Chengalpet had comparatively low similarity of species within its plant parts. The root segments showed no similarity of species with other plant parts in many cases.It could also be observed that low similarity of species was observed within plant parts of different regions.

Table 2 The statistical analysis of the richness, evenness and dominance of endophytic fungal community isolated from the plant parts of *Marsilea minuta.*

	Site	**Ni**	**Np**	**H_G**	**H_S**	**H_{Si}**
Leaflet	Ambattur	18	6	1.72	1.73	5.4
	Chengalpet	28	4	0.9	0.51	1.08
	Guduvanchery	72	3	0.46	0.25	2.39
	Avadi	40	3	0.54	0.79	1.38
Petiole	Ambattur	48	2	0.25	0.17	1.34
	Chengalpet	60	4	0.73	1.29	3.46
	Guduvanchery	56	3	0.49	0.49	4
	Avadi	98	5	0.87	1.39	2
Rhizome	Ambattur	38	8	1.92	1.37	1.11
	Chengalpet	8	4	1.44	1.38	1.33
	Guduvanchery	18	2	0.34	0.52	1.52
	Avadi	56	5	0.99	1.23	1
Root	Ambattur	4	2	0.72	0.69	1.94
	Chengalpet	12	2	0.40	0.45	3.69
	Guduvanchery	6	1	0	0	2.88
	Avadi	12	3	0.80	1.01	2.57
All segments	Overall	574	18	2.67	1.94	4.88

Where *Ni*-Number of isolates; *Np*-Number of species; H_G-Gleason index*;* H_S-Shannon index; H_{Si}-Simpson's dominance index

Discussion

Studies on the endophytic community associated with aquatic Pteridophytes are scarce. The endophytic diversity from plant parts of *Marsilea minuta* collected from various sites were evaluated in the present study. The present study is one of its kind in reporting the endophytic fungal diversity from *Marsilea minuta,* an aquatic Pteridophyte. Altogether 17 species was identified which include the sterile mycelia.

Differences in endophytic fungal species was observed in the plant collected from different sampling sites. Some species were found abundant in particular region, while few species were restricted to a particular sampling site. It was observed that *Colletotrichum* sp. which was dominant in Guduvanchery, was meagre in other sites, *Curvularia lunata,* the most dominant fungi recorded in this study was completely absent in Guduvanchery. Collado et al. (1999), Gallery et al (2007) have reported such variations occurring due to differences in geographical location of the host. The comparison of diversity between two regions has been drawn by calculating the similarity index between the populations (Danilov & Ekelund 1999, Washington 1984). The statistical analysis showed that the diversity of the endophytic community varied greatly between various sites in the present study. The reason for variation in number of species and their diversity according to the site of collection of the plant has been elaborately explained in previous literatures (Petrini et al. 1992, Sieber 2007).

Table 3 Jaccard's Similarity index between species isolated from different plant parts of *Marsilea minuta* from four different sites in and around Chennai

	L-A	P- A	R-A	Ro-A	L-C	P-C	R-C	Ro-C	L-G	P-G	R-G	Ro-G	L-Av	P-Av	R-Av	Ro-Av
L-A	1															
P-A	0.33	1														
R-A	0.27	0.25	1													
Ro-A	0.33	0.33	0.11	1												
L-C	0.12	0.25	0.1	0	1											
P-C	0.12	0.2	0.2	0	0.16	1										
R-C	0.11	0.2	0.09	0	0.2	0.33	1									
Ro-C	0.14	0.2	0.25	0	0.25	0.2	0.2	1								
L-G	0	0	0.12	0	0	0.4	0.2	0	1							
P-G	0.12	0.25	0.22	0	0	0.4	0.16	0	0.66	1						
R-G	0.14	0.33	0.11	0	0	0.2	0.2	0	0.33	0.66	1					
Ro-G	0.16	0.5	0.12	0	0	0	0	0	0	0.33	0.5	1				
L-Av	0.12	0.25	0.1	0.25	0.25	0.16	0.4	0.25	0	0	0	0	1			
P-Av	0.22	0.4	0.18	0.16	0.16	0.12	0.28	0.16	0	0.14	0.16	0.2	0.6	1		
R-Av	0.1	0.2	0.18	0.16	0.16	0.12	0.28	0.16	0	0	0	0	0.6	0.66	1	
Ro-Av	0.28	0.66	0.22	0.25	0.25	0.16	0.4	0.16	0	0.2	0.25	0.33	0.5	0.6	0.33	1

L- Leaflet; P- Petiole; R-Rhizome; Ro-Root; A-Ambattur; C-Chengalpet; G- Guduvanchery; Av-Avadi

The fungal species which were isolated as endophytes exhibited tissue specificity and differences in endophytic fungal community among various plant parts. The occurrence of *Scolecobacidium humicola, Curvularia tuberculata, C. clavata* and *Nigrospora sphaerica* were restricted to petiole segments. While leaflet segments was the only host for *Drechslera australiensis, Aureobasidium pullulans* and *Alternaria alternata.* These observations indicate that the fungal species have affinity to specific tissue of the host and are able to utilize or survive within the substrate (Photita et al. 2001, Rodrigues 1994). It was observed that petiole segments harbored more number of colonies followed by leaflets, rhizome and root. This is due the proximity of petiole segments to the soil. The presence of endophytes in leaves are due to the exposure. Specificity in host plant part by endophytic fungi has been reported by Ganley & Newcombe (2006) and Huang et al. (2008).

The statistical analysis of the data provided thorough insight of the variation in diversity. The richness and evenness were statistically evaluated to indicate the plant parts or regions rich in particular species. Gleason index is sensitive to the richness of the fungal community. High Gleason index observed in leaflet and petiole of Ambattur and Rhizome of Chengalpet refers to low richness of the fungal species. Shannon index is a measure of both richness and evenness. Higher values describes that the species are equally distributed within the sample while values closer to 0 indicate that the species are not equally distributed within the population (Jost 2006). High values of Shannon index were observed in many parts of the plant indicating that the endophytic fungal community were rich and even. It was observed that the Shannon index for the overall plant was higher than plant parts, which might be due to the fact that no other plant part has complete diversity of all endophytic fungal species.

The root segments from Guduvanchery had Shannon index value of 0, which indicates dominance of single species (Sterile form). Leaflet segments of Guduvanchery and petiole segments of Ambattur had low Shannon index as they were dominated by *Colletotrichum* sp. and *Curvularia lunata* respectively. Simpson's dominance index which indicates the evenness of a

fungal species, was found to be high in petiole of Guduvanchery and leaflet of Ambattur. The results indicate that these segments had an even distribution of fungal species.

Only three Coelomycetes namely, *Phoma* sp., *Colletotricum* sp., and *Botryodiplodia thoebramae* were isolated in the present study. Though in general the endophytic population is dominated by the Coelomycetes, their diversity was low in the present study. The possible reason for the host plant failing to harbour these species is the environmental stress which may have hindered the colonisation of Coelomycetes, as the sampling sites are largely polluted regions. This variation may be partly due to environmental conditions which include humidity, temperature, rainfall and inoculum conditions (Photita et al. 2001, Santamaria & Bayman 2005). The study deals with four different geographical locations. However, expansion of the study from different aquatic plants of different nature will result in more diverse fungal species.

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