



Endophytic Mycodiversity of Sacred Tree - *Anthocephalus cadamba* in semi-arid region

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Abstract

A total of 112 fungal isolates were obtained from 180 samples of the leaf, stem and bark tissues of sacred tree *Anthocephalus cadamba* that grown in unusual semi-arid tropical region. The endophytic mycoflora were identified based on the colony morphology and sporulating structure in which 108 fertile fungal isolates belonging to 10 species and 4 sterile morphospecies. Most of the fertile fungi belonged to mitosporic and the isolates composition included hyphomycetes (33%), Coelomycetes (49%), ascomycetes (8%), zygomycetes (6%) and sterile fungi (4%). Species of *Phyllosticta* were recorded as most dominant fungal isolates in which *Phyllosticta hymanaeae* was dominant in the leaf and predominant in bark tissues, *Phyllosticta* sp. was recorded as prevalent fungal isolate in the stem tissues of *A. cadamba* during the study period. The endophytic mycodiversity in the study area was high in diversity and abundance. They were distinctly associated with host plant parts in which the tissues of leaf were harboured more endophytes than the stem and bark in colonization frequency, colonization rate, isolation rate and diversity indices. The present study revealed that the sacred tree *A. cadamba* is one of the ecological niche for sheltering endophytic mycoflora even grown in unusual semi-arid tropical region.

Keywords: *Anthocephalus cadamba* - endophytic fungi - semi -arid region

Introduction

Anthocephalus cadamba is a large deciduous tree that belonging to the family Rubiaceae and commonly known as Kadam. It's habitat all over India, especially in Assam, Bengal, southwards to Andhra Pradesh and Western Ghats. A fully mature kadam tree can reach up to 45 m (148 ft.) in height with straight cylindrical trunk. The bark is gray and smooth in young trees, rough and longitudinally fissured in old trees. Leaves are opposite, simple, elliptic-oblong; Flowers are in solitary globose head, orange or yellow and scented flowers. Plants associated with temples are sacred trees that possess supernatural power and are protected by the local people on religious basis and medicinal importance. In India, this tree is sacred to Hindus and mostly associated with Lord Krishna. Radha and Krishna are supposed to have conducted their love play in the hospitable and sweet-scented shade of the Kadam tree. The plants growing on arid land contain some functional components that protect them from their stress habitat in which the endophytes may also possess novel strategies for their survivability. The various parts of the Kadam tree is widely used in Ayurveda, Siddha and Unani system of medicine. In folk medicine it is used in the treatment of fever, uterine complaints, blood diseases, skin diseases, eye inflammation, diarrhoea, anaemia, leprosy, dysentery, stomatitis and also reported as anti-hepatotoxic, antimalarial, antimicrobial, wound healing, antioxidant, antihelmintic, analgesic, anti-inflammatory, antipyretic, diuretic and laxative.

Endophytes are the part of the microbial community found in all species of plant (Arnold *et al.*, 2000) that include bacteria, fungi, actinomycetes and mycoplasma (Bandara *et al.*, 2006) which live inside plant tissue for at least a part of their life cycle without causing any disease symptoms in the host (Petrini 1991). The most frequently encountered endophytes are fungi (Staniek *et al.*, 2008). Plants may serve as a reservoir of large number of endophytic fungi (Bacon and White 2000). Research of biodiversity of endophytic fungi has a long history and their diversity among plants has been found to be considerably large with reliable source of genetic diversity and novel undescribed species. Endophytic fungi are known to harbour compounds beneficial for plant health as well as human health which can produce various bioactive chemicals (Tejesvi *et al.*, 2011) that promote host growth and resistance to environmental stress (Saikkonen *et al.*, 2010) and also serve as potential sources of novel natural products for exploitation in medicine, agriculture, and industry (Strobel and Daisy 2003). Since

natural products are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. Endophytic fungi inhabits such a biotope. The isolation of novel secondary metabolites from the endophytes is an important aspect of present day research. The present study was carried out to determine the endophytic mycoflora associated with *A. cadambas*, a rarely occurring sacred tree in semi-arid region.

Materials and methods

Collection of plant material

The leaf, stem and bark of *Anthocephalus cadamba* (Roxb.) Miq. (Rutaceae) were collected from the semi-arid tropical region of Aruppukottai Taluk, Virudhunagar District, Tamil Nadu, India and used for the investigation of endophytic fungal communities. The samples were taken 3-4 feet above the ground level of tree and brought to the laboratory in sterile zipper polythene bags and then inoculated within 3 hrs.

Isolation and identification of endophytic fungi

Isolation of endophytic fungi was standardized and modified based on the method described by Hallman *et al.*, (2007). The samples were washed with running tap water to remove dust, soil and debris adhering to them and surface sterilized with 70% ethyl alcohol and 4% sodium hypochlorite to remove adhering microorganisms. Finally the samples were rinsed with deionized double distilled sterile water to remove the surface sterilization agents and dried on sterile tissue papers in a laminar air flow chamber. The leaves and the inner bark tissue were cut in to segments in 3 x 3 mm² size and 3-5 mm thickened stem was also cut in to segments of 1 mm thickness under aseptic conditions using a sterile knife and scalpel. Five sterilized leaf, stem or bark segments were placed in a petridish containing Potato Dextrose Agar medium (PDA) supplemented with the antibiotic streptomycin sulfate 0.4 mg/ml to arrest bacterial growth. The petridishes were sealed with parafilm and incubated at 25±2°C for 15 days under dark conditions and monitored the growth of endophytic fungal mycelium in every day. After 15 days, individual fungal colonies growing out of the explants were sub-cultured on separate PDA petri plates for pure cultures at room temperature and identified in their sporulation stage from

8- 15 days. The fungi which failed to sporulate were designated as "mycelia sterilia". The morphological characters of the fungal isolates were observed and described according to the method of Photita *et al.*, (2004). Morphological identification was done according to the standard taxonomic key included colony diameter, texture, color, morphology of hyphae and conidia (Ainsworth *et al.*, 1973). Finally, the endophytic fungal isolates were transferred separately to PDA slants and maintained at 4°C for further study.

Statistical Analysis of endophytic fungi

The Colonization Frequency (CF%) was used to compare diversity and it was calculated by using the method of Hata and Futai (1996). The percentage of Colonization frequency of fungi was calculated as the number of segments colonized by an endophytic fungal species divided by the total number of segments analyzed. The Colonization Frequency (CF%) of a single endophytic species was calculated using Equation, (Gond *et al* 2007): $CF\% = (N_{col} / N_t) \times 100$ where, N_{col} = number of segments colonized by each fungus and N_t = total number of segments studied. Similarly the percentage frequency of dominant endophytes was calculated as the number of endophytic fungal colonies divided by total number of all endophytic fungal colonies (Mahesh *et al.*, 2005). The Colonization Rate (CR %) was used for the comparison of endophytic fungi in different tissues of given sample. The colonization rate was calculated as the total number of segments colonized by endophytic fungi divided by the total number of segments incubated for that plant sample, and expressed as percentage (Petrini *et al* 1982). The Isolation Rate (IR) of endophytic fungi was used to measure the fungal richness in different tissues. The isolation rate was calculated as the number of isolates obtained from segments divided by the total number segments, but not expressed as percentage (Petrini and Fisher 1988). The Relative Frequency (RF) of isolation, used to represent fungal density, was calculated as the number of isolates of one species or genera or groups divided by the total number isolates, and also expressed as percentage (Photita *et al.*, 2001). Different diversity parameters were calculated using PAST – Paleontological Statistics Software Packages, ver. 3.05. The Simpson's diversity (1-D) was used to estimate the abundance of endophytes and Shannon-Wiener Diversity index (H') and Fisher's alpha index were analysed to determine the species diversity of fungal endophytes colonized in leaf, stem and bark and the Evenness index that used to expresses the distribution of individual among the other endophytic fungi in every part of plant.

Results

Altogether 112 endophytic fungal isolates were obtained from 180 samples of leaf, stem and bark tissue of *Anthocephalus cadamba*. Among them, 10 species endophytic fungi belonging to 8 genera consisted of 4 hyphomycetes, 4 coelomycetes, 1 ascomycetes and 1 zygomycetes and non sporulating sterile morphospecies. The endophytic fungi were identified based on the colony morphology and sporulating structures. The fungal isolates were *Cladosporium cladosporioides*, *Curvularia lunata*, *Nigrospora sphaerica*, *Nigrospora sacchari*, *Colletotrichum truncatum*, *Pestalotiopsis versicolor*, *Phyllosticta hymanaeae*, *Phyllosticta* sp., *Chaetomium globosum* and *Mucor racemosus*.

The density of isolated individual endophytic fungal group belonged to hyphomycetes (33%), coelomycetes (49.1%), ascomycetes (8%), zygomycetes (6.3%) and 3.6% of non sporulating sterile form (Figure -1). The relative frequency of isolated genera consisted of *Cladosporium* sp. (7.13%), *Curvularia* sp. (8.93%), *Nigrospora* sp. (16.73%), *Colletotrichum* sp. (4.13%), *Pestalotiopsis* sp. (12.60%), *Phyllosticta* sp. (32.30%), *Chaetomium* sp. (8.07%), *Mucor* sp. (6.43%) and sterile form (3.67%) from leaf, stem and bark tissues of *A. cadamba* (Figure -2).

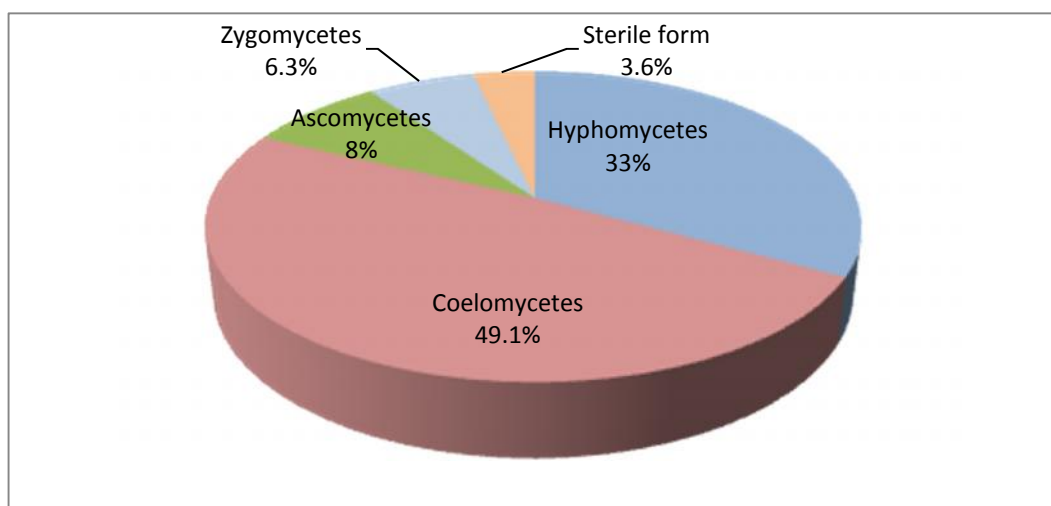


Figure -1 Relative Frequency of different groups of endophytic fungal isolates from *A. cadamba*.

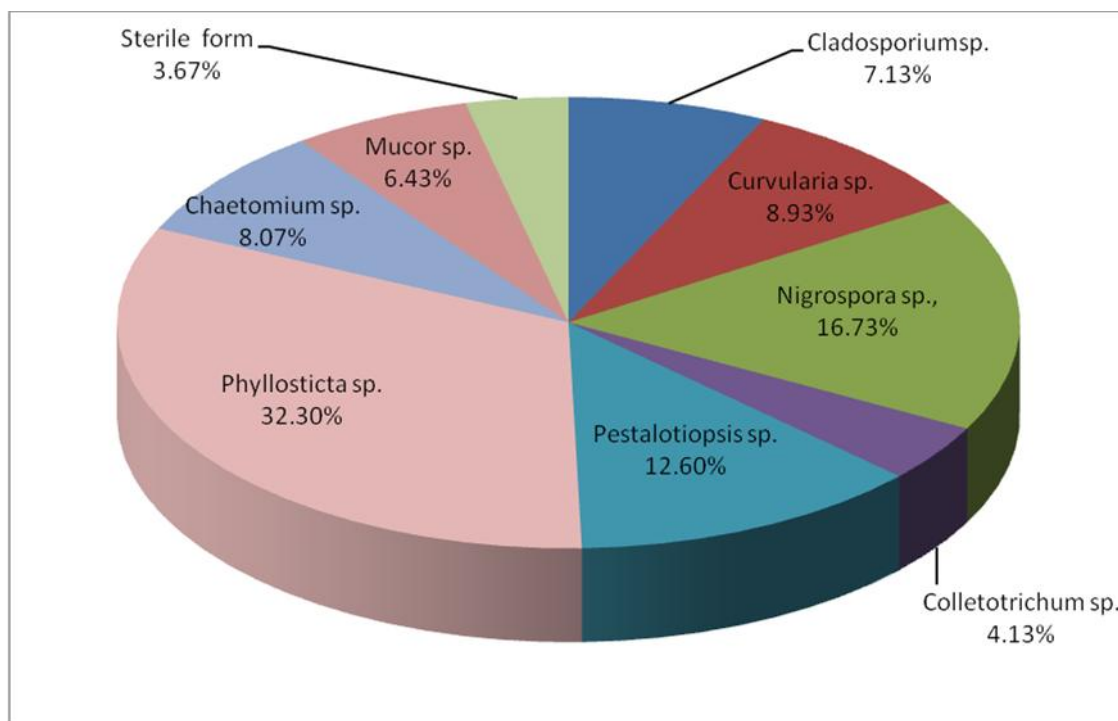


Figure 2 - Relative Frequency of endophytic fungal genera isolates from *A. cadamba*.

In this study, the overall colonization frequency of endophytic fungal isolates in leaf was found to be 73.4% as highest when compared to stem (60%) and bark (53.4%) tissue. The colonization frequency of *Cladosporium cladosporioides* was 5% in leaf and stem respectively and 3.3% in bark. In *Curvularia lunata*, the colonization frequency was 6.7% in leaf and 5% was found in stem and bark respectively. Among the species of *Nigrospora*, the colonization frequency of *Nigrospora sphaerica* was 10% in bark, 6.7% in leaf and absent in stem whereas the colonization frequency of *Nigrospora sacchari* was 8.3% in leaf, 6.7% in stem and absent in bark. The isolates of *Pestalotiopsis versicolor*, the colonization frequency was 8.3% in leaf and stem respectively and 6.7% in bark. In *Phyllosticta hymanaeae*, the highest colonization frequency of 11.7% was reported in leaf and bark respectively and 10% in stem but in *Phyllosticta* sp., maximum of 11.7% in stem, 10% in leaf and 5% in bark. In *Chaetomium globosum*, 5% in leaf, 6.7% in stem and 3.3% in bark as colonization frequency. The colonization frequency of *Mucor racemosus* was 5% in leaf, 6.7% in bark and absent in stem whereas sterile forms were 1.7% in leaf and bark respectively and the stem had 3.3%. (Table -1 & Figure -3). *Phyllosticta hymanaeae* was found to be the dominant species in leaf (15.9%)

and bark (21.9%) tissues. *Phyllosticta* sp. was observed as the most dominant endophytic fungal isolates (19.4%) in the tissue of stem (Table -1 & Figure -4).

Table -1 The Colonization Frequency (CF%) and Dominant Endophytic Fungi (DF%) in *A. cadamba*

Endophytic fungi	Leaf			Stem			Bark		
	*NI	CF (%)	DF (%)	*NI	CF (%)	DF (%)	*NI	CF (%)	DF (%)
Hyphomycetes									
<i>Cladosporium cladosporioides</i>	3	5	6.8	3	5	8.3	2	3.3	6.3
<i>Curvularia lunata</i>	4	6.7	9.1	3	5	8.3	3	5	9.4
<i>Nigrospora sphaerica</i>	4	6.7	9.1	-	--	--	6	10	18.6
<i>Nigrospora sacchari</i>	5	8.3	11.4	4	6.7	11.1	--	--	--
Coelomycetes									
<i>Colletotrichum truncatum</i>	3	5	6.8	2	3.3	5.6	--	--	--
<i>Pestalotiopsis versicolor</i>	5	8.3	11.4	5	8.3	13.9	4	6.7	12.5
<i>Phyllosticta hymanaeae</i>	7	11.7	15.9	6	10	16.7	7	11.7	21.9
<i>Phyllosticta</i> sp.	6	10	13.6	7	11.7	19.4	3	5	9.4
Ascomycetes									
<i>Chaetomium globosum</i>	3	5	6.8	4	6.7	11.1	2	3.3	6.3
Zygomycetes									
<i>Mucor racemosus</i>	3	5	6.8	--	--	--	4	6.7	12.5
Sterile form	1	1.7	2.3	2	3.3	5.6	1	1.7	3.1
Total	44	73.4	100	36	60	100	32	53.4	100

*NI – Number of Isolates

Endophytic fungi may inhabit all available tissues, some endophytic fungi preferred to colonize in the leaves, and other endophytic fungi may colonize the tissue of other organs of the plant. A total of 112 isolates of endophytic fungi were recovered from 120 segments of leaf, stem and bark tissue of *A. cadamba* in which 44 isolates were recovered from the leaves, 36 from stem and 32 from bark tissue of *A. cadamba*. The high range of colonization rates (76.7 to 91.7%) of endophytic fungi were obtained in the present study. The highest colonization rate of 91.7% was found in leaf and the lowest colonization rate of 76.7% was obtained in bark tissues (Table-2 & Figure -5). The isolation rates of endophytic fungi were recorded as 0.53 to 0.73 in which the low rate of isolation (0.53) was found in stem and high rate of isolation (0.73) was recorded in leaf (Table-2 & Figure -6). The colonization and isolation rates of endophytic fungi in leaf was higher followed by stem and bark tissues in the present study.

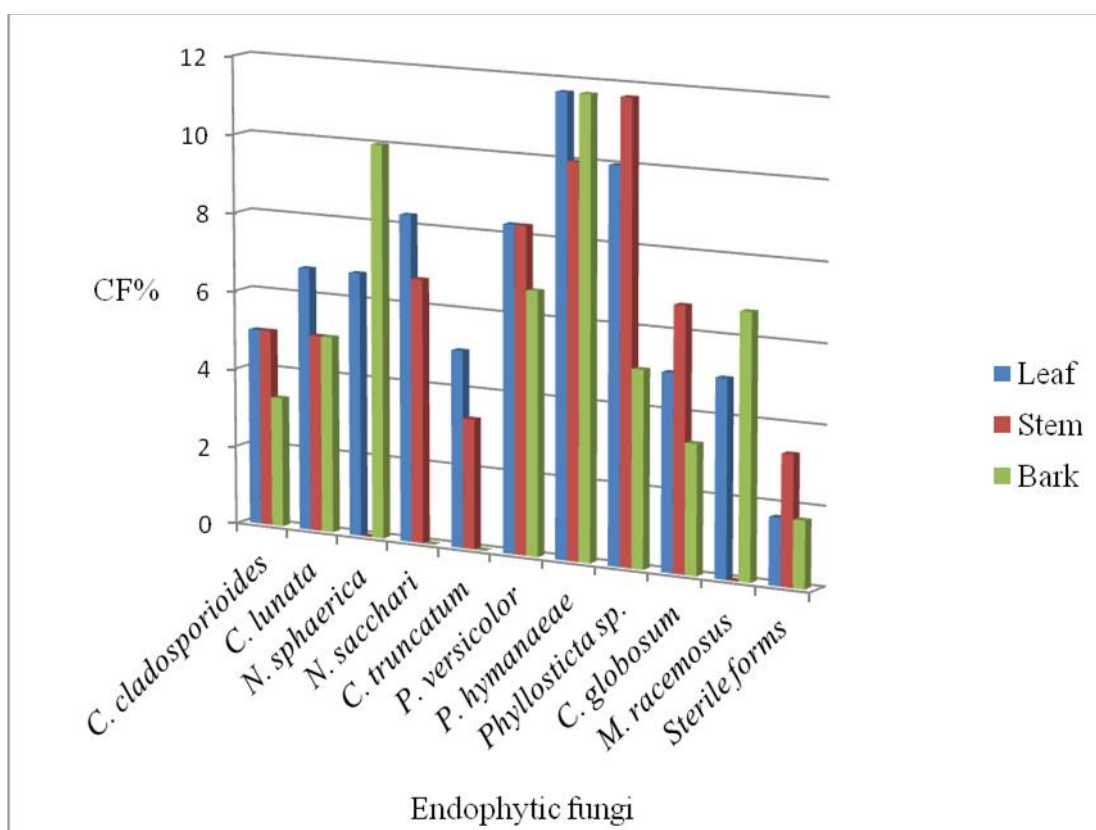


Figure -3 Colonization Frequency (CF%) of endophytic fungal isolates from leaf, stem and bark tissues of *A. cadamba*.

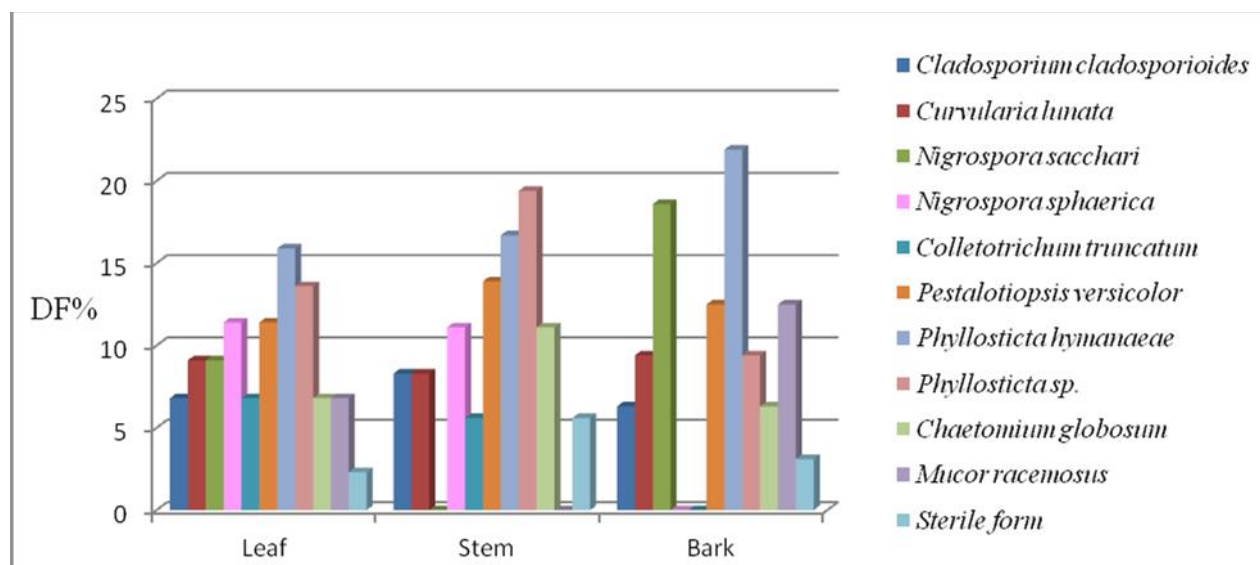


Figure -4 Dominant endophytic fungi (DF%) isolated from leaf, stem and bark tissues of *A. cadamba*

Table -2 The Colonization Rate (CR %), Isolation Rate (IR) and Diversity Indices of Endophytic Mycoflora in *A. cadamba*.

Source	No. of segments colonized by fungi	No. of Fungal Isolates	CR (%)	IR	Shannon –Wiener Diversity Index(H)	Evenness Index	Simpson Diversity Index (1-D)
Leaf	55	44	91.7	0.73	2.313	0.9184	0.8946
Stem	50	36	83.3	0.6	2.115	0.9209	0.8704
Bark	46	32	76.7	0.53	2.065	0.8761	0.8594

In this study, the distribution of endophytic fungi in leaf, stem and bark tissues were investigated for the assemblage, tissue specificity, abundance and diversity. The number of endophytic isolates of *Phyllosticta hymanaeae* was found to be higher in leaf and bark than in the stem. The isolates of *Phyllosticta sp.* was found to be higher in stem than in the leaves and bark. *Nigrospora sphaerica* and *Mucor racemosus* were observed in leaf and bark and not in stem. The endophytic fungi *Nigrospora sacchari* and *Colletotrichum truncatum* were found in leaf and stem and not in bark.

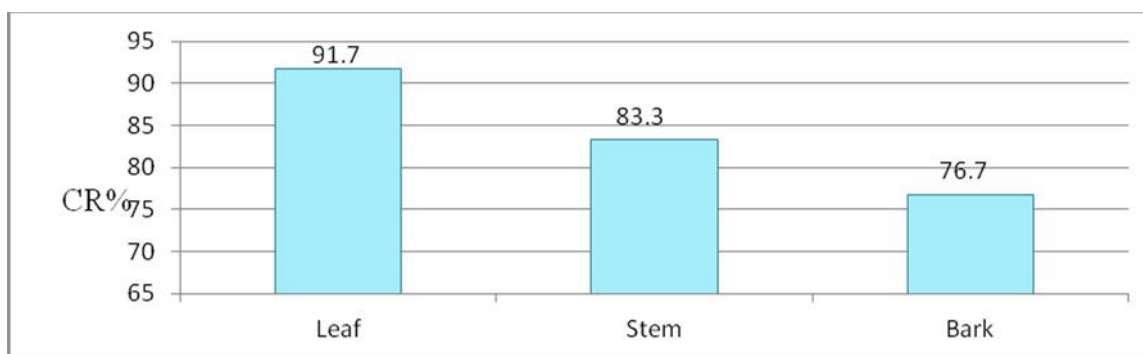


Figure -5 Colonization rate (CR%) of endophytic fungal isolates from leaf, stem and bark tissues of *A. cadamba*

Among the parts of plant, the leaf possessed the maximum diversity of endophytic fungi (Shannon –Wiener Diversity (H): 2.313) followed by stem (H: 2.115) and bark (H: 2.065). The Evenness index was highest in stem (0.9209) and lowest in bark (0.8761). The result of Simpson Diversity Index (1 - D) revealed that the leaf exhibited the maximum abundance of endophytic fungi (0.8946) followed by stem (0.8704) and bark (0.8594) (Table-2).

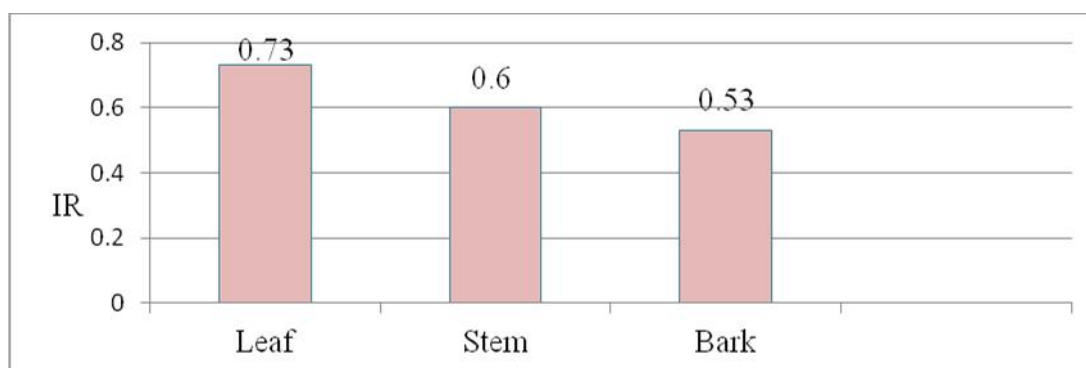


Figure - 6 Isolation rate (IR) of endophytic fungal isolates from leaf, stem and bark tissues of *A. cadamba*

Discussion

Endophytes may increase host fitness in harsh environments (Redman *et al.*, 2002). This is especially true of plants in arid environments (Faeth and Hammon 1997) and also in semi-arid region. Plants associated with temples are sacred trees that are protected by the local people on religious basis and medicinal importance. Medicinal plants are reported to harbour endophytes

(Strobel 2002) and have a capacity to protect their host from infectious agents and also provide adaptability to survive in adverse conditions. Many studies have shown that some medicinal properties of plants may be due to endophytic fungi living inside the plants (Azevedo *et al.*, 2002). Endophytic fungi from medicinal plants could be a rich source of functional metabolites (Huang *et al* 2008). 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 and Daisy 2003).

In this study, the sacred tree *A. cadamba* which even grown in unusual semi-arid, yielded 112 endophytic fungal isolates from 180 samples of leaf, stem and bark tissue segments. Most of the fungal isolates belonged to mitosporic in which coelomycetes (49.1%) was predominant over other fungal groups was supported by Nalini *et al.*, (2014) in Medicinal Plants of Western Ghats, India. Among the isolated fungal genera, *Colletotrichum* and *Phyllosticta* are generalists endophytes and have been reported from several plant hosts (Suryanarayanan *et al* 2002) and the remaining genera such as *Cladosporium* in *Coffea arabica* (Oliveira 2014), *Curvularia* in *Ipomoea carnea* (Tayung *et al.*, 2012), *Nigrospora* in *Crataeva magna* (Nalini *et al.*, 2005), *Pestalotiopsis* in *Terminalia arjuna* (Tejesvi *et al.*, 2005), *Chaetomium* in *Eugenia jambolana* (Manila *et al* 2014) and *Mucor* in *Vitex negundo* (Desale and Bodhankar 2013) were also reported as endophytes.

The colonization of the endophytic fungi is ubiquitous yet selective in nature. Okane *et al.*, (1998) reported that the composition and frequency of a colonization related with the place and the host condition and also differs within the tissue or organs of a host plant (Kumar and Hyde 2004). In this study, the overall colonization frequency of endophytic fungal isolates in leaf was found to be 73.4% as highest when compared to stem (60%) and bark (53.4%) tissue. Among the species of *Phyllosticta*, the *Phyllosticta hymanaeae* had shown the highest colonization frequency of 11.7% in leaf and bark respectively and *Phyllosticta* sp. also showed the highest frequency of 11.7% in stem. In this study, the species of *Phyllosticta* were dominant in leaf, stem and bark tissues. This result was supported by Pandey *et al.*, (2003).

Endophytic fungi may inhabit all available tissues, but the leaves of tropical plants are densely colonized by endophytes (Suryanarayanan *et al.*, 2002). The highest number of isolates (44), colonization rate (90%) and isolation rate (0.73) of endophytic fungi were found in leaf as like the similar results have been reported by Kumar and Hyde (2004) and Gangadevi and Muthumary (2007). In diversity indices also, the leaf possessed the maximum species diversity (Shannon –Wiener Diversity Index (H): 2.313) and the maximum abundance (Simpson Diversity Index (1 – D): 0.8946) of endophytic fungi. Maheswari and Rajagopal (2013) suggested that high colonization of endophytes in leaf tissue may be due to their anatomical structure and supply of nutrient elements on which the endophyte depends.

Conclusion

The present study clearly revealed that the endophytic fungi was higher in leaf followed by stem and bark in all statistical analysis and they were distinctly associated with host plant parts of *A. cadamba*. The fungal group Coelomycetes was found to be dominant in which the *Phyllosticta* spp. was identified as dominant fungal genera. This study revealed that the sacred tree is also one of the ecological niche for endophytic fungi.

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