

Diversity of the Endophytic Fungi Isolated From *Oxalis Corniculata* Linn



Microbiology

KEYWORDS : Endophytes, Colonization Frequency, *Oxalis corniculata* Linn, *Rhizopus nigricans* Var. *verticillatum*, *Hymenula affinis* Fautrey and Lambotte.

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ABSTRACT

Altogether 700 segments from 15 plants of *Oxalis corniculata* Linn. were collected and screened for the endophytic fungi on, stem, petiole and leaves. A total of 7 fungal species viz; *Aspergillus niger* Tiegh., *Aureobasidium pullulans* (de Bary) Arnaud Les., *Cladosporium epiphyllum* Person., *Cunninghamella blakesleeana* Lender, *Hymenula affinis* Fautrey and Lambotte., *Papulaspora* Preuss., *Rhizopus nigricans* Var. *verticillatum* isolated and identified based on the morphology of fungal culture and the types of spores. Quite a good per cent frequency was recorded on leaves as compared to petiole and stem. The diversity of endophytes was significantly documented in leaves than those of petiole and stem. Similar endophytes prevalence was recorded during monsoon followed by winter and summer.

INTRODUCTION:

Endophytic fungi, or endophytes, are microorganisms that colonize healthy plant tissue. They remain there for at least one cycle of their lives without causing any damage to the host plant through a symbiotic relationship of several fungi (Li et al., 2010; Qiu et al., 2010), and even bacterial endophytes (Andreote et al., 2009; Figueiredo et al., 2009) colonize plants. These endophytes can also protect the plant from many biotic and abiotic threats (Azevedo et al., 2000). They colonize intercellular and intracellular spaces as well as the interior of xylem and phloem cells (Hallmann et al., 1997). The relationship between endophyte and plant may have begun with the growth of higher vegetables hundreds of millions years ago, resulting from coevolutionary processes (Strobel et al., 1996; Strobel and Long, 1998). There is increasing effort to characterize and identify endophytic fungi isolated from medicinal plants. Many studies have shown that some medicinal properties of plants may be related to endophytic fungi hosted by these plants (Azevedo et al., 2002).

Oxalis corniculata Linn. is a member of Oxalidaceae Family and is commonly grow as wild in every where, it is a small annual or perennial procumbent or erect herb. Chemical composition of *Oxalis corniculata* contain Malic, tartaric and citric acids from leaves and stem. Leaves are a good source of vitamin C. Alcoholic extract of leaves is antibacterial. Medicinal uses of *Oxalis corniculata* are astringent, vermifuge, emmenagogue and antiseptic properties. Boiled with butter milk, it is a remedy for indigestion and diarrhoea in children. Fresh juice of plant cures dyspepsia, piles, anaemia and tympanitis. Leaves, cooling, refrigerating, stomachic, antiscorbutic and appetizing. No endophytic studies on this plant documented therefore the aim of present paper is to understanding the diversity of endophytic fungi in *Oxalis corniculata*, with the determination of per cent colonization frequency.

MATERIALS AND METHODS:

Plant materials and fungal isolates: In the present study, endophytic fungal diversity was undertaken by selecting Karnatak University Botanical Garden Dharwad. Geographically, Dharwad district is situated in between 14°15' to 15°5' North longitude and 74°49' and 76° 21' East latitude. There is a marked diurnal temperature difference. The temperature can be below as 20.2°C in June and high as 34.42°C in March. The annual rain fall is 600-850 mm. The climatic regions are semi - humid or humid. Soil is covered with a hard, compact crust having dark brown colour (Lakshman et al., 2013).

Collection of samples: The plant samples of *Oxalis corniculata* were collected from medium sized healthy plants. Plant materials were brought in closed sterile polythene bag to the laboratory and processed within 24 hours of collection. The plant parts used for isolation of endophytes in Leaf, Petiole, and Stem. Collected plant materials were washed under running tap water

to remove the adhering particles. Leaves were cut into sterile blade small segments measuring 0.5-1.0 cm by sterile blade. Surface sterilization was done by washing segments with 70% ethanol for 2 minutes, 2% sodium hypochlorites for 1-2 minutes, 70% ethanol for 30 seconds followed by 2 to 3 rinses of sterile distilled water. Segments were brought to laminar air flow and blotted in sterilized blotting paper before inoculating into the culture medium (Jayshree et al., 2011).

Inoculation of Explants: Surface sterilized plant segments were aseptically sampled. Leaf segments were randomly chosen separately. In each plant material petriplate 4-6 segments were placed on PDA and MEA media on separately. The inoculated petriplates were then placed for incubation.

Isolation and pure culturing: After 8-10 days inoculated petri plates were examined for fungal endophytes. The petri plates were screened to isolate the fungal endophytes. Fungi growing out from the cut ends of plant segments were transferred to fresh PDA media. After purifying the isolates for several times, final pure cultures were transferred to PDA slants in to the test tubes.

Identification of endophytic fungal isolates: The morphological identification of endophytic fungal strains is based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores, and reproductive structures. If, these features were discernible, measurements of all fungal characters were made in water mounts, and the slides were subsequently mounted in lactophenol and sealed with nail varnish. All experiments and observations were repeated at least twice. (Huang, et al., 2008). The endophytic fungal colonization frequency was calculated by using the formula of (Kumarsen and suryanarayanan, 1998).

$\% CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of tissues segments placed}} \times 100$

RESULTS

Data on the endophytic studies on *Oxalis corniculata* has produced 7 genera, belongs to 6 endophytic fungal species. Endophytes were identified based on a microscopic observation of mycelia, conidia, cultural characteristics (surface texture etc) and morphology of fruiting bodies. By using standard manuals (following the standard manuals by Burnett 2000; Gilman 2001; Nagmani et al., 2005; Subramaniyam 1983) endophytes are identified. On the examination of some cultures that they failed to sporulate, these were grouped as Mycelia sterilia, and divided into different morphospecies according to their cultural characteristics.

Altogether 700 segments were isolated, they belong to 7 genera and 6 species (Table 1). The most important endophytes were as

Aspergillus niger Tiegh., *Aureobasidium pullulans* (de Bary) Arnaud Les., *Cladosporium epiphyllum* Person., *Cunninghamella blacksleeana* Lender, *Hymenula affinis* Fautrey and Lambotte., *Papulaspora* Preuss., *Rhizopus nigricans* Var. *verticillatum*. All the isolated and identified fungus was stored in Botany Department Microbiology Laboratory Karnataka University Dharwad (BDMLKUD). The important cultured endophytes were photographed shown in (plate I and Plate II). In the present investigation, among the endophytes; *Cunninghamella blacksleeana* was dominant which is followed by *Aspergillus niger* Tiegh. The colonization frequency significantly higher *Cunninghamella blacksleeana* (73.26%) and low from *Aspergillus niger* Tiegh. (53.2%), *Hymenula affinis* Fautrey and Lambotte. (33.3%) which was followed by *Aureobasidium pullulans* (de Bary) Arnaud Les. (30.18%). In addition to three more endophytes are recorded viz; *Rhizopus nigricans* Var. *verticillatum*., *Papulaspora* Preuss., *Cladosporium epiphyllum* Person., However, highest colonization frequency were observed during the monsoon, this is mainly due to the leaves are mature and there was very little precipitation, endophytic species can be affected by season. Then it is followed by winter and summer seasons. Significant variation in the colonization frequency of endophytic species at different seasons observed, indicating the environmental factors such as rainfall and atmospheric humidity and their effect on host plant similar results were obtained by (Selvanathan et al., 2011). Therefore, surveys of endophytic fungal communities at different seasons have the different endophytic colonization frequency. In many instances leaves sampled during wet season harbor more endophytes than those screened during dry season shown in (Table 2). In the present study, all the seven endophytic fungi were isolated from the leaves, Petiole and Stem of *O. corniculata*. Highest numbers of endophytes were recorded in monsoon season followed by winter and summer seasons respectively. There results are consistent with earlier contribution of Selvanathan et al., 2011.

Description of Endophytic Fungi:

Species 1: *Aspergillus niger* Tiegh.

Colonies growing moderately on PDA or MEA, 3.5-4.5cm in 10 days, with abundant submerged mycelium, conidial heads carbon black, exudates lacks, conidial heads large and black, at first globose and then radiate or splitting in well defined columns in age, up to 700-800µm in diam; conidiophores arising directly from the substratum, smooth, non septet, thick walled, 1-2mm × 15-20µm; vesicles globose, walls thick, commonly 45-75µm in diam, occasionally longer, bearing two series of fully packed phialides, brownish; metulae mostly 20-30×5-6µm, often reaching 60-80×8-10µm, rarely septate; phialides 7-10×3-3.5µm; conidia globose, spinulose with colouring substance, black, 4-5µm; globose to subglobose (Plate II-1) sporangiophores, each terminating in a sporangium. Sporangiophores 1-2mm. high, the sporangia are globose 100-200µm in diameter (Plate I).

Table 1: Colonization frequency (%) of endophytes in *Oxalis corniculata* on different culture media

S.N	Endophytic fungi	Colonization frequency (%)						Total isolates
		MEA			PDA			
		L	P	S	L	P	S	
1	<i>Aspergillus niger</i> Tiegh.	13.3	6.66	-	20	-	13.3	53.2
2	<i>Aureobasidium pullulans</i> (de Bary) Arnaud. Les.	6.66	-	13.3	6.66	6.66	6.66	30.18
3	<i>Cladosporium epiphyllum</i> Person.	6.66	-	-	-	20	-	26.6
4	<i>Cunninghamella blacksleeana</i>	20	-	6.66	26.6	-	20	73.26
5	<i>Hymenula affinis</i> (Fautrey and Lambotte)	-	20	-	-	-	13.3	33.3
6	<i>Papulaspora</i> Preuss.	-	-	20	-	-	-	20
7	<i>Rhizopus nigricans</i> Ehrenberg.	-	-	-	6.66	13.3	-	19.96
TOTAL								256.5

* L-Leaf, P-petiole, S-stem

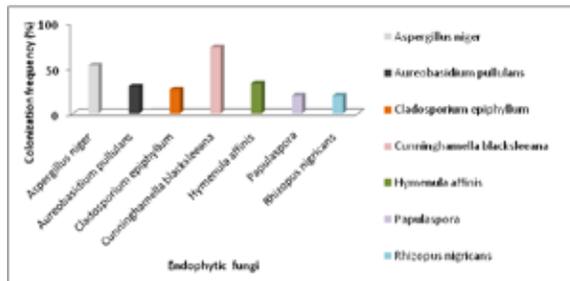
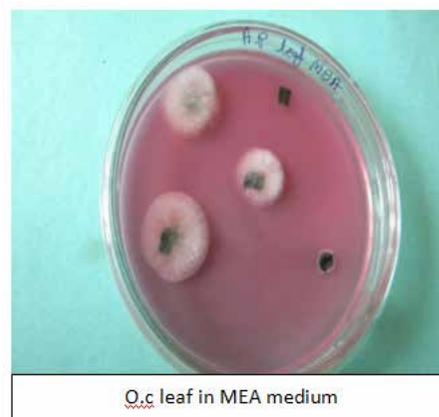


Fig. 1: Colonization frequency (%) of endophytes in *Oxalis corniculata* on different cultur media

Table 2: Percentage colonization of endophytic fungi in the *Oxalis corniculata* (Mar2011- Dec 2013)

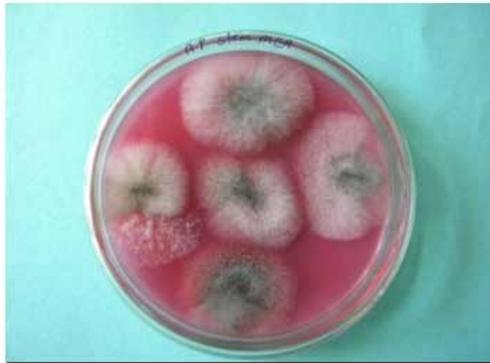
Sl. No	Endophytic fungi	Total colonization frequency (%)			
		Monsoon (June - Sept)	Winter (Oct - Jan)	Summer (Feb - May)	Mean Total
1	<i>Aspergillus niger</i> Tiegh.	31.5533	16.8867	16.4433	21.6278
2	<i>Aureobasidium pullulans</i> (de Bary) Arnaud. Les.	20.8867	16.4433	11.5533	16.2944
3	<i>Cladosporium epiphyllum</i> Person.	18.8833	13.5533	8.4400	13.6256
4	<i>Cunninghamella blacksleeana</i>	41.7767	18.2200	14.2200	24.7389
5	<i>Hymenula affinis</i> (Fautrey and Lambotte)	19.1067	17.3300	15.9967	17.4778
6	<i>Papulaspora</i> Preuss.	20.4433	11.9967	11.1067	14.5156
7	<i>Rhizopus nigricans</i> Ehrenberg.	21.3300	9.7733	13.3300	14.8111

PLATE - I

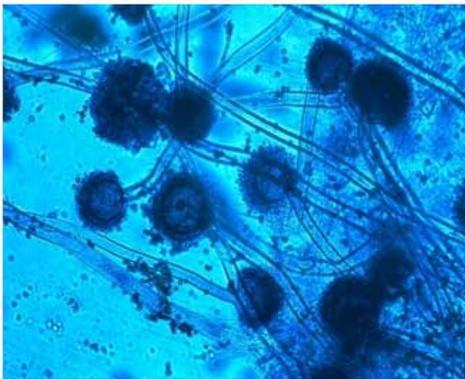




O.c stem in PDA medium



O.c stem in MEA medium



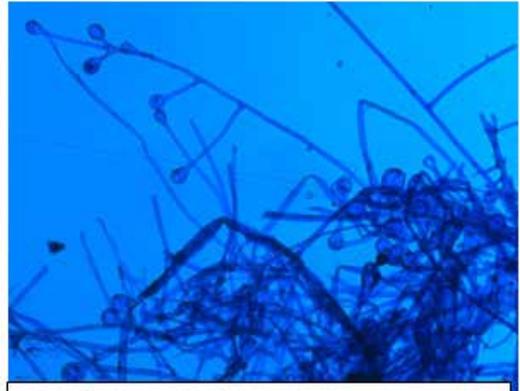
Aspergillus niger Tige



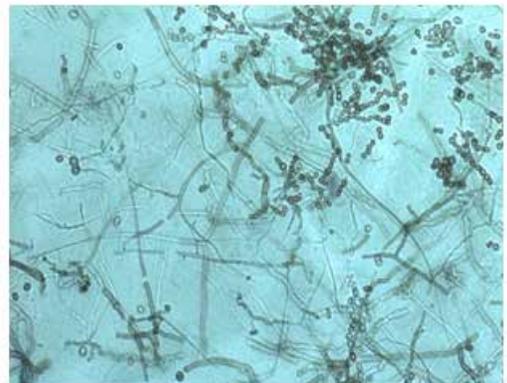
Pure culture



Aureobasidium pullulans



Cunninghamella blakesleeana



Cladosporium epiphyllum



hymenula affinis

Species 2: Aureobasidium pullulans (de Bary.) Arnaud, Les.

Colonies growing fast, reaching 4cm in 7 days at 24°C on MEA, mid to dark brown; Vegetative hyphae hyaline, up to 12µm wide; pigmented hyphae distinctly constricted at the septa; Conidiophores mostly 6-8µm wide, dark brown, with small lateral protuberances which become short, open ended necks of phialides; conidia hyaline ellipsoidal, often with in distinct basal apiculation, variable in size and shape, straight, mostly (7.5-)9-11X(3.5-)4-5.5(-7)µm, but may be bigger in old colonies; secondary conidia and endoconidia similar, but smaller (Plate II).

Species 3 : Cladosporium epiphyllum Person.

Colonies greenish-black, large, thick; conidiophores at first erect, then falling, pale green; conidia very numerous, soon falling from the chain, at first one-celled, then two-to more-celled, olive-green, 10-22µ long×4-6µ thick (Plate II).

Species 4 : Cunninghamella blakesleeana Lender.

Colonies growing rapidly on PDA and MEA, white, later becoming yellow, loose, erect, 2-4cm in height; sporangiophores long, simple or regularly verticillately branched, lateral branches of the sporangiophores variable in length and number, usually less than 50µm long; terminal vesicals, globose to subglobose, 40-60µm; lateral vesicles usually smaller than terminal vesicles, 19-28µm; sporangioles hyaline, echinulate or smooth, ovoid ones 7.5-10µ; in diam (excluding spines), ellipsoidal ones 10-12.5×7-8µm(excluding spines) (Plate II).

Species 5 : Hymenula affinis Fautrey and Lambotte.

Conidia straight, somewhat dorsiventral near apex, apediculate, typically one-septate, 10.2×2.8µ(9-11.4×2.6-3µ) usually in a continuous smooth or slightly roughened, slimy-layer, from hyalin to pale salmon-colored on a globose agar. Conidiophores from simple to sparingly branched, septate. Mycelium hyalin. No chlamydospores (Plate II).

Genus 6 : Papulaspora Preuss.

Conidiophores and conidia lacking, reproductive units consisting of irregular clusters of cells(bulbils); bulbils without organization frequently pigmented; pale brown or orange, often appearing like microsclerotia; vegetative hyphae hyaline. In this genus no true spores are formed. The hyaline vegetative hyphae bearing "bulbils" are characteristic of the genus (Plate II).

Species 7 : Rhizopus nigricans Var. verticillatum .

Stolon creeping, recurving to the substrate in the form of arachnoid hyphae, which are strongly raised and distant from the substrate and implanted at each node by means of rhizoids (Plate II).

DISCUSSION:

Medicinal plants are one of the oldest forms of health care known, every plant on earth is known to harbor at least one endophytic microbe. These are one of the most unexplored and diverse group of organisms having symbiotic association with higher life forms and may produce beneficial substances for host (Weber,1981).

The leaves of the host plant exhibited highest endophytic association than the petiole and stem samples. endophytes isolated from leaf samples exhibited greater diversity and high colonization frequency compared to the endophytes of the other plant parts examined. *Oxalis corniculata* being a tropical plant and no report on biodiversity. A recent meta-analysis found that leaf endophytes are indeed more species-rich in the tropics than in temperate regions (Arnold *et al.*, 2007). Most of the leaf samples finding more number of endophytic diversity in the examined plants. One of the possible reasons for the differences in the colonization rates between plants is the structure and substrate which influence the colonization and distribution of endophytic fungi (Okane *et al.*, 1997). Similarly, Kumar and Hyde (2004) have reported that the colonization rate in the leaves was found to be significantly higher than those in other parts studied of the host. Present studies clearly exhibited that the number of endophytic fungi was higher in leaves followed by petiole and stem. Diversity of endophytes on a single plant often differ greatly in the dominant members of their endophytic

communities (Chaverri *et al.*,2010, Gazis *et al.*,2010, Hoffman *et al.*,2008, Pocasangre,2000), and may even show functional differences. As in case of Alfalfa plants of leaves, stems and roots are colonized by distinct fungi that produce different ranges of secondary metabolites (Weber *et al.*, 2006). Similar observation was noted in the present study even with a single plant different leaves may differ significantly in community composition (Gambao *et al.*,2001, Fisher *et al.*, 1996). Single leaves of a tropical forest tree, *manilkara bidentata*, showed fine scale variation of endophyte isolation rates and identity. In this respect, plants are genetic mosaics because each organ may have a unique combination of genes in its micro biome (Herre *et al.*, 2007.). However, some endophytes are restricted to single cell and tissues in the leaf endophytes in different tissues may not interact (Stone JK. 1987). The overall colonization frequencies differed with different organs. Similar results were obtained in the endophytic diversity of Thalavaipandian *et al.*, 2011.

In conclusion; the understanding of the number of endophytic diversity associated with an *Oxalis corniculata* plant in leaves, petiole and stem exhibited a significant variation in colonization frequency of endophytic species. *Oxalis corniculata* significantly greater number of colonization frequency was documented on leaves. Our culture results showed that the culture media results shows as endophytes grows more in PDA compare to MEA media. Roots do not have endophytes association. In the contest of seasonal distribution the highest number of endophytes colonization during monsoon and it followed by winter and summer seasons was determined on the *Oxalis corniculata*.

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REFERENCE

- Andreote fd, azevedo jl, araujo wl ., 2009. Assessing the diversity Of bacterial communities associated with plants. *Braz j microbiol* 40:417-432.
- | Arnold, A.E., Henk, D.A., Eells, R.L., Lutzoni, F., Vilgalys, R., 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* 99:185–206.
- | Azevedo jl, maccheroni wjr, araujo wl, pereira jo., 2002. Microorganismos endofíticos e seu papel em plantas tropicais. In: Serafini la, barros nm, azevedo jl (eds) *biotecnologia: avanços Na agricultura e na agroindústria*. Caxias do sul: edusc. Pp: 233-268.
- | Azevedo jl, maccheroni wjr, pereira jo, araujo wl (2000) Endophytic microorganisms: a review on insect control and recent Advances on tropical plants. *Electron j biotechnol* 3: 40-65.
- | H. L. Burnett and Barry B. Hunter. 2000. Illustrated genera of Imperfect fungi Fourth edition.
- | Chaverri, P, Gazis, R., 2010. Perisporiopsis lateritia, a new species on decaying leaves of Hevea spp, From the Amazon basin in Peru, *Mycotaxon* 113:163–69.
- | Figueiredo jef, gomes ea, guimarães ct, lana ugp, Teixeira Ma, lima gvc, bressan w., 2009. molecular analysis of endophytic Bacteria from the genus bacillus isolated from tropical maize (zea mays L.). *Braz j microbiol* 40: 522-534.
- | Fisher, J., Faraboschi, P, and Desoli, G. 1996. Custom-Fit Processors: Letting Applications Define Architectures. In Proceedings of the 29th Annual International Symposium on Microarchitecture, pages 324-335, Paris, France.
- | Gamboa, M.A., Bayman, P., 2001. Communities of endophytic fungi in leaves of a tropical timber tree (Guarea guidonia: Meliaceae), *Biotropica* 33:352–60.
- | Gazis, R., Chaverri, P, 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (Hevea brasiliensis) in Peru, *Fungal Ecol*, 3:240–50.
- | Joseph C. Gilmen. 2001. A manual of soil fungi, Biotech book delhi | Hallmann j, quadt-hallmann a, mahaffee wf, klopper jw., 1997. Bacterial endophytes in agricultural crops. *Can j microbiol* 43: 895-914.
- | Herre, E.A., Mejia. L.C., Kylo, D., Rojas, E.I., Maynard. Z., 2007. Ecological implications of antipathogen effects of tropical fungal endophytes and mycorrhizae, *Ecology* 88:550–58.
- | Hoffman, M., Arnold. A.E., 2008. Geography and host identity interact to shape communities of endophytic fungi in cupressaceous trees, *Mycol. Res*, 112:331–44.
- | Jayashree M. Kurandawad and H. C. Lakshman, 2011. Studies on endophytic fungal diversity in some important medicinal plants of the botanical garden: a report, *Journal of Theoretical and Experimental Biology* (ISSN: 0972-9720), 8 (1 and 2): 45-51.
- | Kumar, D.S.S., Hyde, K.D., 2004. Biodiversity and tissue recurrence of endophytic fungi in Tripterygium wilfordii, *Fungal Diversity* 17:69-90.
- | Kumaresan, V., Suryanarayanan, T.S., Johnson, J.A., 1998. Foliar fungal endophytes from two species of the mangrove Rhizophora. *Canadian Journal of Microbiology*, 44:1003-1006.
- | Lakshman H. C., and Jayshree M. Kurandawad., 2013. 'Diversity of the endophytic fungi isolated from spilanthus acmella linn. - a promising medicinal plant. *International Journal of Pharma & Bio Sciences* . Apr-Jun2013, Vol. 4 Issue 2, pB-1259-B-1266. 8p.
- | Lakshman, H.C., & Inchal, R.F., 2012. Indigenous Medicinal plants and their practical utility, Edt; H.C. Lakshman and R.F. Inchal, New India Publishing Agency, New Delhi. ISBN: 978-93-81450-11-6 pg 163 Ref pg no26 and 27.
- | Li hy, zhao ca, liu cj, xu xf., 2010. Endophytic fungi diversity Of aquatic/riparian plants and their antifungal activity in vitro. *J Microbiol* 48: 1-6.
- | Nagmani. A., I. K. Kunwar, C. Manoharachary. 2005. Hand book of soil fungi. I. K. International Pvt. Ltd. | Okane, I., Nagagiri, A., Ito, T., 1997. Preliminary study of endophytic fungi in evergreen plants from Ishigaki and Iriomote islands. *Osaka Res. Comm* 18: 45-51.
- | Pocasangre, L., Sikora, R.A., Vilich, V., Schuster, R.P., 2000. Survey of banana endophytic fungi from Central America and screening for biological control of the burrowing nematode (Radopholus similis), *InfoMusa* 9:3–5.
- | Qiu m, xie r, shi y, chen h, wen y, gao y, hu x., 2010. Isolation and Identifi cation of endophytic fungus sx01, a red pigment producer from Ginkgo biloba l. *World j microb biot* 26: 993–998.
- | Selvanathan Srimathi., Isaivani Indrakumar, Muthumary Johnpaul., 2011. Biodiversity of the Endophytic fungi Isolated from Calotropis gigantea (L.) R. Br. Recent Research in science and Technology., (ISSN : 2076-5061), 3 (4): 94-100.
- | Stone, J.K., 1987. Initiation and development of latent infections by Rhabdocline parkeri on Douglas fir, *Can. J. Bot.* 65:2614–21.
- | Strobel ga, hess wm, ford ej, sidhu rs, yang x., 1996. Taxol from Fungal endophytes and the issue of biodiversity. *J industrial microbial* 17: 417-423.
- | Strobel ga, long dm., 1998. Endophytic microbes embody Pharmaceutical potential. *Amer soc microbiol news* 64: 263-268.
- | Subramanian, C. V., 1983. Hyphomycetes, Taxonomy and Biology, Academic Press London Vol I & II 930.
- | Thalavaipandian, A., Ramesh, V., Bagyalakshmi., Muthuramkumar, S., Rajendran, A., 2011 Diversity of fungal endophytes in medicinal plants of courtallam hills, Western Ghats, India. *Mycosphere* 2(5): 575-582.
- | Tunlid a, talbot nj., 2002. Genomics of parasitic and symbiotic fungi. *Curr opin microbiol* 5: 513–519.
- | Weber, R.W.S., Anke, H., 2006. Effects of endophytes on colonization by leaf surface microbiota, In *Microbial Ecology of Aerial Plant Surfaces*, ed. Mj Bailey, AK Lilley, TM Timms-Wilson, pp. 209–222. Oxfordshire, UK: CABl. | Weber, J. 1981. A natural control of Dutch elm disease. *Nature*, London, 292: 449-451. |