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RESEARCH ARTICLE

Biodiversity of Endophytic Fungi and its Seasonal Recurrence from Some Plants

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ABSTRACT:

Plants have served humankind as source of fuel, food, clothing, shelter and medicine since the beginning of life. Many new modern techniques, methods, strategies and plant associations have been put into use to improve the quantity and quality of plant resources. Among the microbial populations in plants, endophytes play a key role in almost every aspects right from the production of bioactive compounds, and its role in medicinal applications. In the present study, the following are the list of plants from which the leaf samples have been collected *Mangifera indica* (L), *Psidium guajava* (L), *Catharanthus roseus* (L) G.don, *Citrus limon* (L). Burm.f., *Murraya koenigii* (L), *Hibiscus rosasinensis* (L). The inoculated leaf sample harboured a total of 50 isolate comprised of Zygomycotina (5), Ascomycotina (3), Hypomycetes (35) and Coelomycetes (7). Hymomycetes and Coelomycetes were the common fungus grown in all the leaf samples. Among the Hypomycetes and Coelomycetes fungi, two genera from Coelomycetes *Botryodiplodia theobromae* and *Pestalotiopsis breviseta* were frequently reccuring in all the months considered for the study.

KEYWORDS: Biodiversity, Endophytic Fungi, Hypomycetes, Coelomycetes.

INTRODUCTION:

Plants have served humankind as source of fuel, food, clothing, shelter and medicine since the beginning of life. Many new modern techniques, methods, strategies and plant associations have been put into use to improve the quantity and quality of plant resources. Microbial populations are key component of soil plant system where they are immense in a network of interactions affecting plant development[1]. Among the microbial populations in plants, endophytes play a key role in almost every aspects right from the production of bioactive compounds, and its role in medicinal applications. The endophyte-host plant interactions are variable and range from antagonistic to mutualistic[2].

The diversity of fungal endophytes are of gaining importance as they produce a variety of compounds which are useful to modern medicine, agriculture, industry, such as novel antibiotics, antimycotics, immunosuppressants and anti-cancer compounds[3].

An endophyte is an endosymbiont (any organism that lives within the body or cells of another organism, i.e. forming an endosymbiosis), often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease [4,5]. Endophytes are ubiquitous and have been found in all the species of plants studied to date; however, most of these endophyte/plant relationships are not well understood [6,7].

Endophytic fungi are crucial, quantifiable and integral component of fungal biodiversity, and influenced by community diversity of plants and its structure [8,9]. Endophyte represents the wide range of microbial diversity and adaptation which developed in unusual and sequestered habitats.

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Their diversity and functional habituation makes a new and exciting area of research for novel drug or medicine. Endophytic fungi influence the ecology, distribuition, biochemistry and physiology of the host plant[10]. Darwin (1872) suggested that diversity affects the ecosystem processes.

Mainly medicinal plants harbor more fungal endophytes than others[11]. Some of the fungal endophytes are common in many plant hosts and some are very specific to their host plant[3]. The functioning and stability of terrestrial ecosystems are determined by plant biodiversity and species composition[12]. Various protocols have been followed for the isolation of fungal endophytes from plants. The plant parts used for the endophyte isolation include leaves, stem, roots and bark. The method usually starts with the surface sterilizing agents like sodium hypochlorite, ethanol, mercuric chloride at different concentrations and varying time exposure dependent on the plant part chose [13]. This is followed by culturing the surface sterilized segments in nutrient media like Potato Dextrose Agar (PDA)[14], Rose Bengal Agar (RBA), Sabourauds Dextrose Agar (SDA), and Malt Extract Agar (MEA) etc. with appropriate antibiotic like Penicillin G, chloramphenicol or streptomycin etc. to prevent the growth of bacterial contaminants[15-17].

MATERIALS AND METHODS:

Sample collection:

As this study aims in studying the biodiversity of endophytic fungi, the plant leaf samples were collected from Anakaputhur, Kancheepuram District once in every 3 months (Mar-May, June- Aug, Sep- Nov, Dec-Feb) for one year to study its seasonal variation and to record Biodiversity. Anakaputhur is located at 12.9828°N 80.1264°E.The temperature is 28.4°C (24.0–32.8°C) and annual rainfall of 685 mm. The monthly mean relative humidity is 78.6%. The first showers of the South West monsoon occur during the middle of January- August. Showery weather continues through September and ceases in the middle of October, when the North East Monsoon occurs.

Collection of plant samples:

The following are the list of plants from which the leaf samples have been collected. The names of the plants chosen for the study are listed below.

a. *Mangifera indica* (L)
b. *Psidium guajava* (L)
c. *Catharanthus roseus* (L) G.don
d. *Citrus limon* (L). Burm.f.
e. *Murraya koenigii* (L)
f. *Hibiscus rosasinensis* (L)

Isolation of endophytes:

Fresh leaf samples from the plants are collected and were placed in a sterile plastic bags and returned to the laboratory and were processed within 24 hrs of collection for the isolation of endophytic fungi.

Surface sterilization of plant material:

Samples were cleaned under running tap water and then air-dried. Surface sterilization was carried out according to the procedure of Suryanarayan and Thennarasan[18,19] with slight modifications. Leaves were surface sterilized by immersion in 70% ethanol for 1 min, 0.1% mercuric chloride solution for 3 mins and sterile distilled water for 1 min two times. The surface-sterilized leaves were cut into small pieces using a sterile blade and placed on sterile Potato Dextrose Agar plates amended with 120 mg/L of Chloramphenicol. The inoculation was carried under laminar wood chamber and after inoculation the plates were labelled accordingly and incubated for 25˚ C.

Composition of potato dextrose agar (PDA)

Potato	-	200 g
Dextrose	-	200 g
Agar	-	20 g
Distilled water	-	1000 ml
pH	-	5.6 ± 0.2

The media was prepared by adding the potato infusion (boiled filtrate of macerated potatoes) along with dextrose and agar. pH was adjusted and the media was sterilized by autoclaving at 121ºC, 15 lb pressure for 15 min. The media was then cooled and poured on to sterile petri dishes aseptically allowing for solidification.

Culturing and subculturing:

The Petri dishes were monitored every day to check the growth of endophytic fungal colonies from the leaf segments. After several days hyphae growing from the plant material were transferred to other plates, incubated again for 10 days, and periodically checked for culture purity. Continuous transfer of fungi was carried out as new colonies continued to appear for up to two or three weeks. Plates were then incubated and periodically ascertained for purity by hyphal tipping. The fungal isolates were numbered and stored in PDA slants.

Identification of the endophytes:

The endophytic fungi were identified according to their macroscopic (front and reverse side of fungal colonies) and microscopic characteristics such as the morphology of fruiting structures and spore morphology under a bright-field microscope (10X and 40X). The identification was also done by using wet mount technique in which the fungal colonies were stained using Lactophenol cotton Blue[20, 21]. Lactophenol Blue Solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal elements are stained intensely blue.

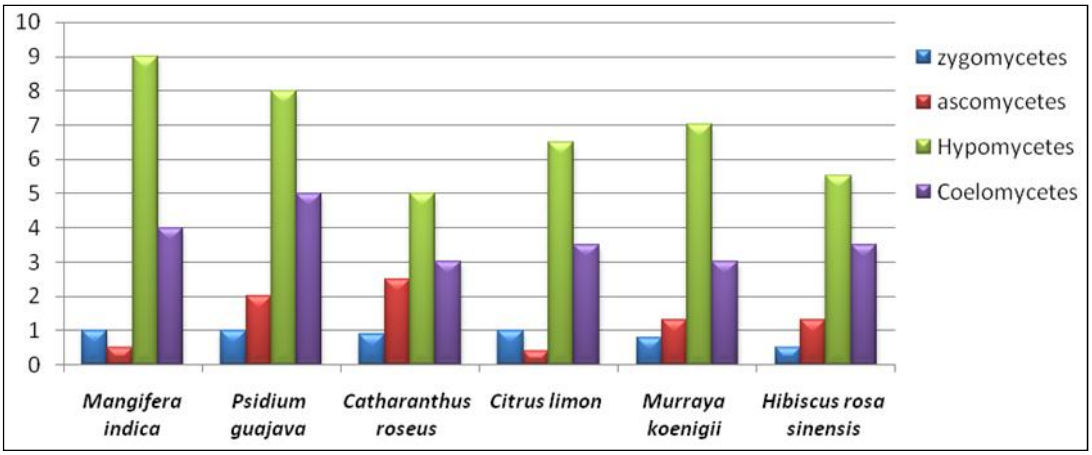
Composition of lactophenol blue

Cotton Blue	-	50 mg
Phenol	-	20 g
L(+)-Lactic Acid	-	20 ml
Glycerol	-	40 ml
Distilled water	-	20 ml

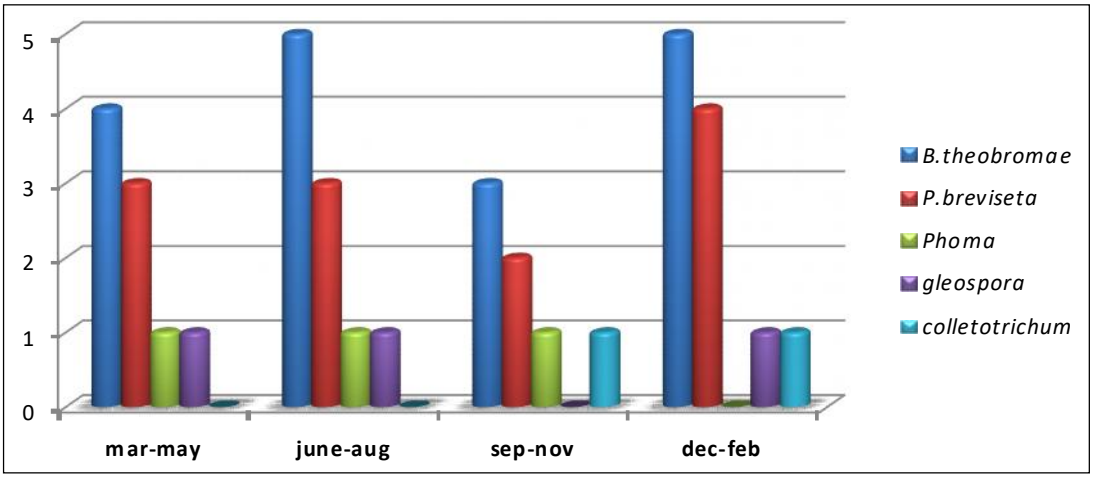
A drop of lactophenol blue solution was placed on a slide. Fungal culture was carefully teased using an inoculating needle. A coverslip was placed on the slide without any air bubble. After 5 min, slide was observed under a microscope with low power for screening in low intensity. The edges of the coverslip can be sealed with nail polish or permount to preserve the mount.

RESULTS:

The inoculated leaf sample harboured a total of 50 isolate comprised of Zygomycotina (5), Ascomycotina (3), Hypomycetes (35) and Coelomycetes (7). Hymomycetes and Coelomycetes were the common fungus grown in all the leaf samples **(**Fig.1**).** Among the Hypomycetes and Coelomycetes fungi, two genera from Coelomycetes *Botryodiplodia theobromae* and *Pestalotiopsis breviseta* were frequently reccuring in all the months considered for the study (Fig 2).



Hypomycetes and Coelomycetes were most dominant during the study from Mar-May, June- Aug, Sep- Nov, Dec-Feb, 2012-13
Fig. 1 Distribution of fungi in different host plants



***B.theobromae* and P.*breviseta* were dominanltly recurring during the study**.
Fig. 2 Frequency of the dominant fungi

Isolation of fungus:

From seventh day, observations were made each day for the growth of the most dominant fungi over the study. Among the various colonies identified (Fig 3), only the two most recurring fungi were transferred to a fresh PDA plates by hyphal tipping, and sub-cultured with the media containing plates which was amended with chloromphenicol under sterile conditions, to obtain the pure culture. The plates were incubated at room temperature (27°C) for about 3-4 days. The pure cultures

of the isolates were maintained on PDA slants.

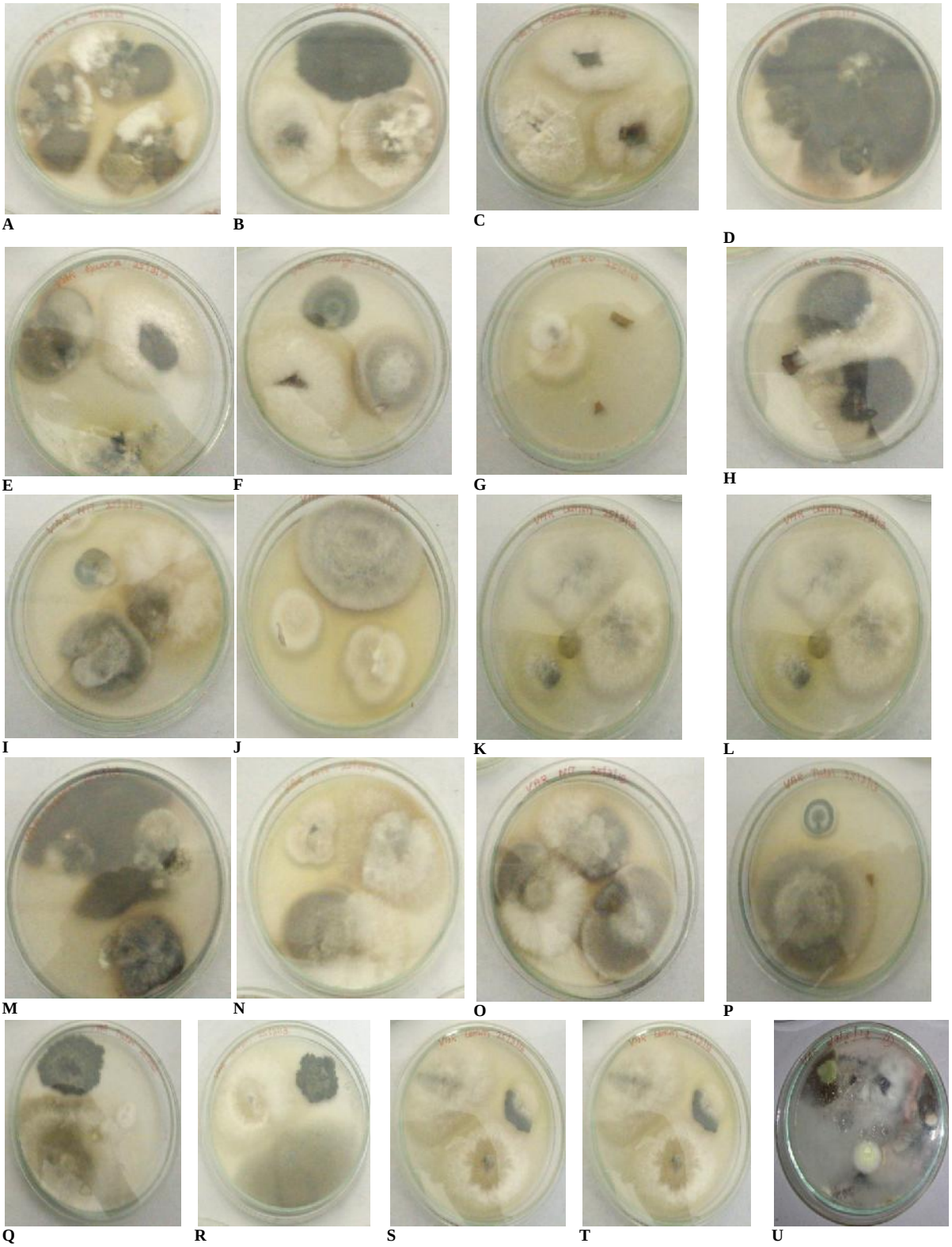


Fig. 3. Endophytic fungi isolated from different host plants

Groups of endophytes isolated from *Mangifera indica, Psidium guajava, Catharanthus roseus Citrus limon, Murraya koenigii* and *Hibiscus rosa sinensis.*

Identification of endophytic fungus:

Macroscopic appearance of the fungal colonies, their morphological appearance and the mechanism of spore production and characterization of the spores were noted using the standard mycological manuals. The identification of molds was based on the shape, method of production of arrangement of spores (conodial ontogeny). Microscopic appearance was noted following wet mount preparation by lactophenol cotton blue staining and the microscopic slides were mounted observing them under 4X, 10X and 40X objectivities[20,21]. The group of some Hypomycetes fungi were identified and it was found to be *Aspergillus niger*, *A.terreus*, *A.japonicus*, *A.flavus*, *A.nidulans*, *Pencillium* spp and *Trichoderma* spp (Fig.4). The pure cultures were examined periodically for sporulation and identification. The taxonomy identification revealed that the two most dominant fungi are *Botryodiplodia theobromae*, and *Pestalotiopsis breviseta*, which belongs to Coelomycetes. The other groups of Coelomycetes (Fig.5) which was identified are *Phoma* species, *Phyllosticta* species and *Gleospora* species. Water mounting is used for the identification of coelomycetes.

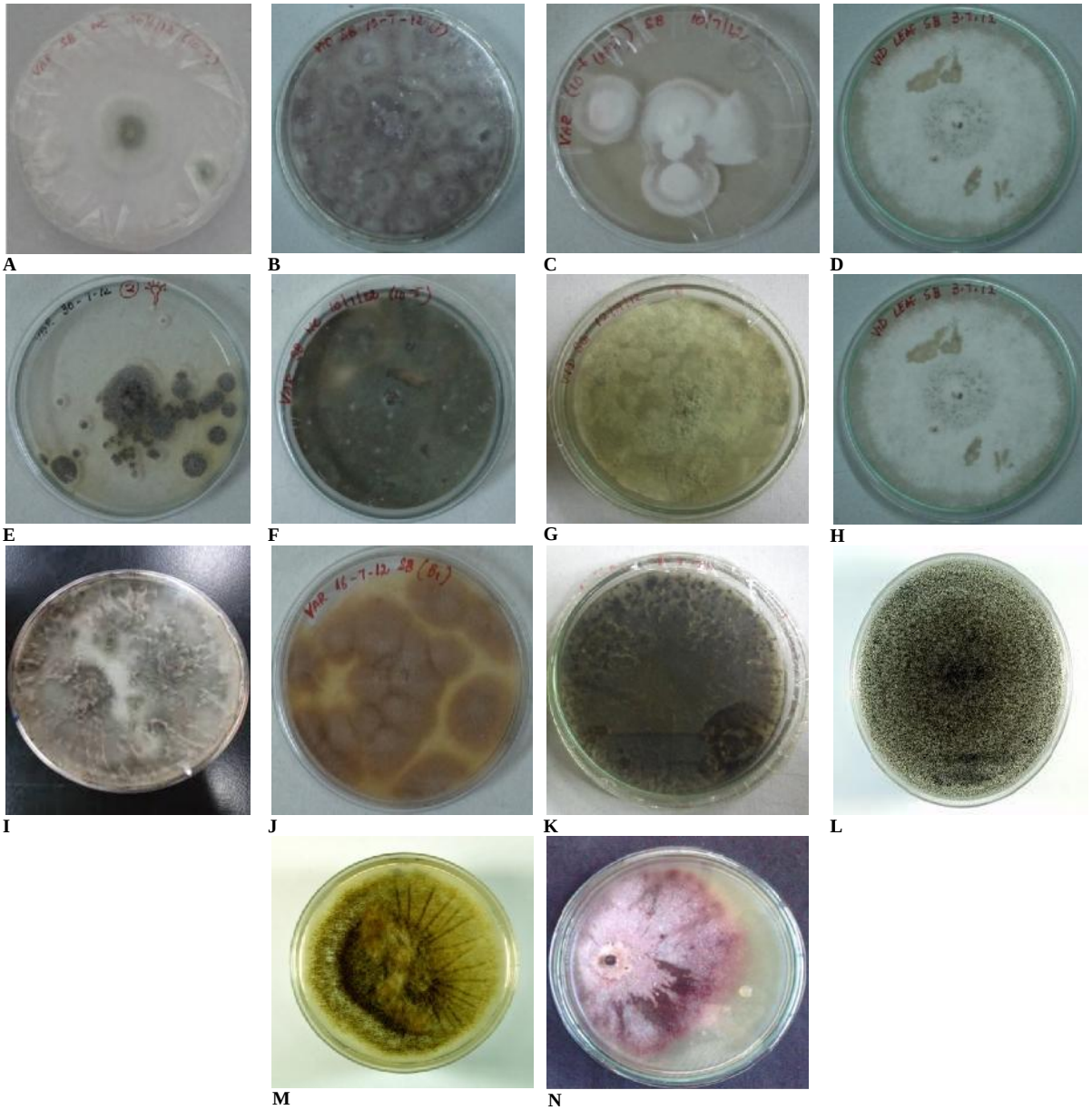


Fig.4 Hypomycetes among Endophytes

A) *Penicillum* sp., B) *Nigrospora* sp., C) *Cladosporium* sp., D) *F.solani.*, E) *A.japonicus*, F) *A. nidulans*, G) *P.chrysogenum*, H) *F.oxysporum*, I) *Trichoderma* sp., J) *A.terreus*, K) *Curvularia* sp., L)) *A. niger*, M) *A.flavus*, N) *Fusarium* sp

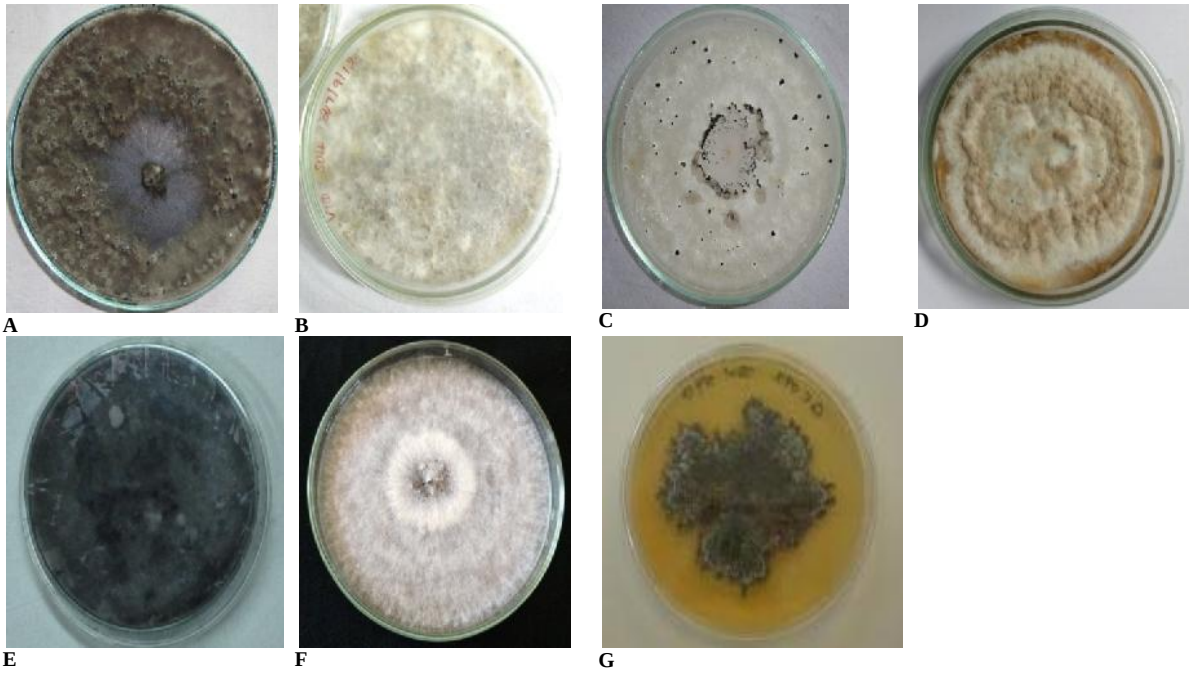


Fig.5 Coelomycetes among endophytes
A) *B.theobromae*, B) *Phoma* sp., C) *P.mangiferae*, D) *P.breviseta*, E) *Gleospora sp.*, F) *Phyllosticta* sp.1., G) *Phyllosticta* sp 2.

DISCUSSION:

In the present world, plant resources have been exploited in various means for food,forage, construction of dwellings, making household implements, sleeping mats, fire and shade, etc. The diversity of life on earth has renumerated our nation with its bounty of nature enriched with variety of different types of ecosystem, that India is one among the world's 15 nations that are exceptionally rich in species diversity[22]. Endophytes are organisms that inhabit plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to their host. Fungal endophytes are ubiquitous and have been isolated from algae, pteridophytes, gymnosperms and angiosperm members. Endophytic fungal diversity is more in tropical forests where woody angiosperm diversity is higher[23]. In the present study the plants has been collected from Anakaputhur, Kancheepuram District, (*Mangifera indica*, *Psidium guajava*, *Catharanthus roseus Citrus limon* , *Murraya koenigii* , *Hibiscus rosasinensis*) belongs to different families played a major part for the isolation of various endophytes, which belonged to Hypomycetes and Coelomycetes. Sterile forms have often been isolated as endophytes from many plants[14]. Lacap *et al.,* (2003) also reported that sterile mycelium prevails in most of the endophytic research studies[24].

Among the endophytes isolated, Hypomycetes and Coelomycetes were found to be dominant in the study. In the seasonal recurrence *Botryodiplodia theobromae* and *Pestalotiopsis breviseta* were the two fungi which were dominantly present. In PDA the colonies grew well up to 7cm diameter after five days and good sporulations were obtained in about 7 to 10 days incubation.

CONCLUSION:

Altogether the present Investigation infers the seasonal recurrence of endophytes present in the selected plants. It was found that Hypomycetes and Coelomycetes were the two major groups of fungi present in the study. It was very clear that *B.theobromae* and *P. breviseta* were the two most recurring fungi during the study. Further, the fungi can be used for the production of secondary metabolites and various other biotechnological methods.

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