Chapter I

INTRODUCTION

India has the richest ecosystem diversity, ranging from low land to high mountainous regions. For more than 400 million years, endophytic fungi have been associated with plants. They are widely distributed in plants, which are present in various geographical and climatic zones. Endophytic fungi exist as mycelial form in all living plants. Endophytic fungi were known to be present in wide range of hosts, like herbs to trees, mosses, ferns, lichens etc., (Stone et al., 2000). Therefore, it is considered as an important issue of biodiversity. Many studies had been carried out regarding endophyte diversity in different ecological conditions, yet they could not provide reliable information regarding the number of endophytes. Individual plants can harbour more than hundreds of endophytic fungal species and these endophytes contribute to the extreme diversity of fungi (Hawksworth, 2001). Compared to plants from other regions, tropical plants possess many undiscovered endophytic fungal diversity which act as store house for many novel metabolites of pharmaceutical importance (Strobel et al., 2004). Since 1990's extensive studies have been conducted in India, related to endophytic fungi. As per the available literature, more than 1000 plants have been collected and the diversity of endophytic fungi was studied. Hence additional practical efforts are required in the field of endophytic fungal research for the identification of more bioactive compounds.

Endophytic fungi describes the internal mycota of living plants in which fungi spend complete or part of their life cycles in the intercellular spaces of healthy plant tissues of the host without causing any visible symptoms (Petrini, 1991). This definition helps to understand the entire symbiotic interactions like parasitism, commensalism and mutualism between the fungi and their host plant. But their association with host plant is generally puzzling, varied and complex in nature. Detailed investigation of internal mycota is still uncovered due its wide distribution within and among individual host species (Stone et al., 1996). Genetic background, nutrient level and ecological conditions of the host plant are considered as an important factor for richest diversity of endophytic fungi and in turn benefits the host plant by inducing growth, increased resistance to disease and herbivore etc., (Firakova et al., 2007).

The distribution pattern of endophytic fungi varies from plant to plant. They are influenced by environmental conditions, such as temperature, humidity, illumination, geographic location, and vegetation. These conditions determine the distribution ranges of host plant, which in turn determines the genus and species of endophytic fungi, spore germination, growth, reproduction and metabolism during the entire life cycle (Song et al., 2007). Isolation of endophytic fungi from various plants growing in different ecological conditions could bring us variety of benefits such as, it helps to explore some unknown and enlarged diversity of fungi from plant ecosytem and their novel bioactive compounds.

Cucumis dipsaceus, commonly named as Arabian cucumber, Hedgehog gourd, Pepino diablito, Teasel gourd, Wild gourd etc., belongs to the family Cucurbitaceae. It is native to countries like Africa, Ethiopia, Kenya, Somalia, Tanzania, Uganda, Sudan and Southern Egypt (Chandran et al., 2013). It is an annual herb in which leaves are consumed as vegetable and fruit juice is topically applied to prevent hair loss. The cooked plant is also consumed in Kenya (Geethakumary et al., 2015).

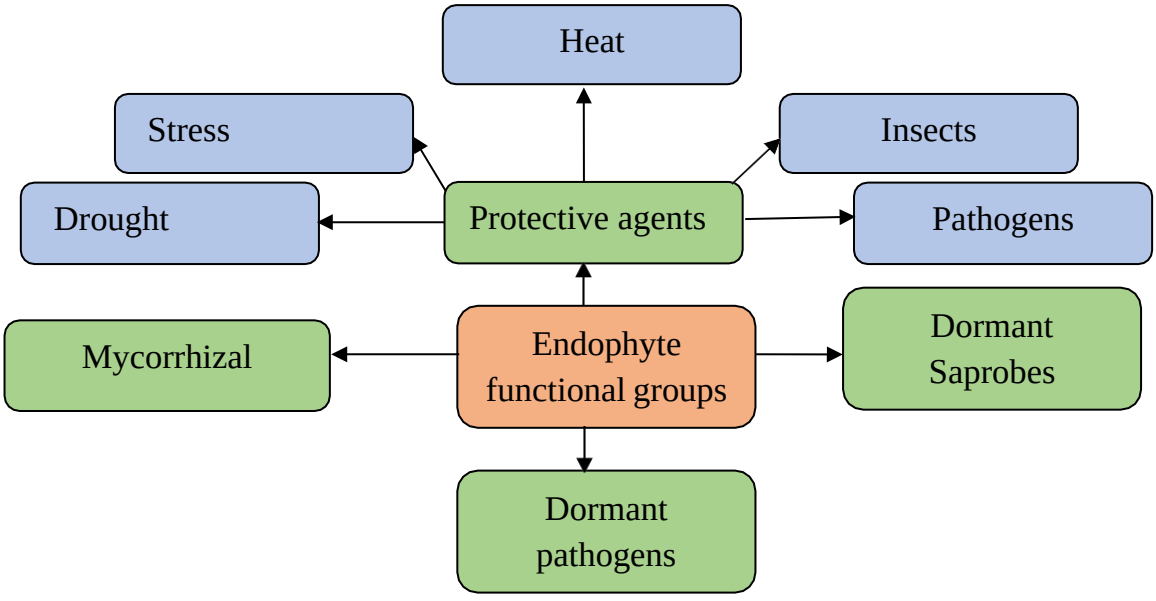
Considering the importance of isolation of endophytic fungi, the present work was carried out in *Cucumis dipsaceus*, a newly identified plant in Marudhamalai, Coimbatore, Tamilnadu. No previous reports are available on the endophytic profile of *C. dipsaceus* from this region. Hence, the present study has been undertaken to isolate and identify endophytic fungi from this plant.

REVIEW OF LITERATURE

A fungus is an eukaryotic microorganism which includes unicellular forms such as yeasts, molds and also multicellular fungi that produce fruiting forms known as mushrooms. They are the primitive members of the plant kingdom, slightly advanced than bacteria. Fungi occurs as heterotrophs, biotrophs, saprotrophs and necrotrophs. Next to insects, fungi are the second largest group of organisms found in tropical ecosystems (Webster and Weber, 2007).

Now-a-days studies on fungi were considered as important for addressing the global challenges. Fungal processes and their products lead to sustainability through efficient use of natural resources. Fungi are one of the natural sources for finding new drug candidate, antimicrobials and value added bio-based products. The knowledge about fungal morphology, reproduction and metabolism has given rise to uses of fungi in industry, agriculture, food, feed, medicine and health. In the process of evolution, fungi forms various relationship with plants (Lange et al., 2012). Plant-associated fungi are usually divided into five main functional groups: Mycorrhizal, Pathogenic, Epiphytic, Endophytic, and Saprotrophic fungi (Fig.-1).

Fig.1: Endophytic Fungi- Multiple functional roles



(Source: Porras-Alfaro and Bayman, 2011)

The symbiosis of fungi with plants started when the vascular plants appeared. The group, 'endophytes' forms one such special association with plants. The existence of endophytes has been traced to the fossil records which point out the endophyte-host association that may have developed from the time of emergence of first higher plant on earth.

ENDOPHYTES

The word endophyte refers to any organism that lives within or inside the plant. The origin of the term endophyte can be traced back to 1860s. It was coined by De Barry (1866). The term endophyte is used to differentiate the microrganisms residing within the host tissues, from epiphytes that live on plant surfaces. Diverse use of the term endophyte forced to find a new definition, namely "endophytes are the microorganisms that are residing inside the healthy parts of the plants without causing any visible infection or diseases, excluding pathogenic and mycorrhizal fungi (Petrini et al.,1982; Carroll, 1988). Some organisms that live as latent pathogens inside the host tissues and have an epiphytic phase in their life cycle are also endophytes. Endophytes include organisms like Bacteria and fungi (Fahey et al., 1991), Algae (Peters, 1991) and insects in plants (Feller, 1995). Accordingly, over the time, the use of the term endophyte has evolved and has been restricted in many ways such that it has now become more specific and meaningful. It has now evolved into a type of association that the fungus or bacterium has with its host.

Occurrence of endophytes

Endophytes have been recovered from plants of hot deserts, arctic, mangroves, temperate and tropical forest. They are found within angiosperms (Davis et al., 2003), non-vascular plants (Zhang et al., 2013), conifers (Kim et al., 2013) and ferns (Del Olmo-Ruiz and Arnold, 2014).

Colonization of endophytes

Bacteria and fungi are the major endophytes found in most of the plant tissues. Though bacteria being prokaryotes and fungi eukaryotes, their association with host plants is same. They may occur inter- or intra-cellularly, but their mode of colonization varies. For colonization of bacterial endophytes, plants release some exudates from roots which influence the microbial community in the rhizosphere. This exudate contains amino acids,

organic acids and proteins which help in recruiting bacterial endophytes from rhizosphere (Bulgarelli et al., 2012). Colonization depends on habitat, host and season. Their mode of entry is passive through wounds and other tissue openings or active, with the help of enzymes or vectors (ex. Insects). They have the ability to colonize both inter- and intra-cellularly (Van der Meij et al., 2018). But their colonization pattern is diverse (Vandenkoornhuyse et al., 2002) and frequently ends with pathogenecity (Schulz and Boyle, 2005).

Assemblages and adaptation of endophytes

After colonization, the assemblage of both bacteria and fungi inside the plants may vary based on the habitat of the host. They have been widely seen in various geographical and climatic zones. Recent molecular methods help to analyze the geographical distribution of microbial communities. Diversity and colonization pattern of endophytes increased during the vegetative period of the plants (Smalla et al., 2001) because asexual sporulation increases at the end of vegetative period. There are many physical factors affecting the microbial environment. Among them, temperature plays an important role. When microbes are exposed to low temperature, it resulted in decrease in protein synthesis, reduce membrane fluidity and cold denaturation of proteins.

Endophytes versus epiphytes

In contrast to endophytes, epiphytes live on external plant surfaces (Santamaria and Bayman, 2005). Epiphytes can be removed from plant tissues by surface sterilization usually with sodium hypochlorite and ethanol to break surface tension, whereas endophytes are not. Thus, an epiphyte that escapes from surface sterilization and grows in culture might be assumed to be an endophyte (Arnold and Lutzoni, 2007). Many microorganisms isolated from plants act both as endophytes and epiphytes. Epiphytes after penetration into plant tissues become endophytes. They are horizontally transmitted and act as epiphytes when internal tissues are exposed to the environment. Many fossilized tissues of stem and leaves with microorganisms act as evidence for the endophyte-host relationship. This association may be evolved from the time that higher plants appeared on the earth (Strobel, 2003). Among various microbes colonizing the plant, fungi are the most important group of endophytes.

Endophytic fungi

Higher plants are complex, multilayered and grow in various habitats. They possess wide internal spaces for microbes in their vegetative and reproductive parts. Such microbes are known as endophytes. Among them microfungi were found in great diversity. They are found in leaves, stem, root, bark etc. Therefore, higher plants act as store house for many undiscovered fungi. In the broadest sense, endophytic fungi are defined as fungi that colonize living plant tissues without causing any immediate open negative effects (Bacon and White, 2000). Endophytic fungi show their parasitism when the host is stressed (Firakova et al., 2007). The most important group of endophytic fungi is ascomycetes and anamorphic fungi (Arnold, 2007). Nearly 3,00,000 plant species are present in the earth. Each plant harbor one to 100's of endophytes. Thus the endophytes may be hyperdiverse (Huang et al., 2007).

Classification of endophytes

Endophytes have great influence on plant fitness, ecology and evolution. Basically endophytes are classified into two groups: - Clavicipitaceous (Class-1) and Non clavicipitaceous, based on their taxonomy, evolution, ecological function and host specificity. Later, six properties of endophytes such as tissue(s) colonized, colonization pattern, host range, *in planta* biodiversity levels, ecological functions and mechanism of transmission between host generations, made non Clavicipitaceous endophytes divided into three special groups class 2, class 3 and class 4 (Rodriguez et al., 2009).

Clavicipitaceous endophytes (CLASS 1)

Clavicipitaceae includes family of fungi (Hypocreales; Ascomycota) which are free living and symbiotic species associated with insects and fungi or grasses, rushes and sedges. Members of clavicipitaceae produce alkaloids which are toxic to animals and humans. It was first noted in late 19th century in seeds of *Lolium temulentum*, *L. arvense*, *L. linicolum* and *L. remotum*. Investigators found a toxic syndrome in animals after grazing the above plant species. An endophyte named *Neotyphodium coenophialum* is toxic to grazing animals (Bacon and White, 2000). Mycelium of clavicipitaceous endophytes can be seen in intercellular spaces of leaf sheaths, culms and rhizomes of barley (Dugan et al., 2002).

Non -clavicipitaceous endophytes

Non-clavicipitaceous endophytes are mostly associated with vascular and non-vascular plant species. Earlier, these endophytes had only single functional group but some researchers stated that they have three distinct functional groups. Non-clavicipitaceous explains mostly about diversity of endophytic fungi associated with leaves of tropical trees as well as high diversity of endophytes which can be seen in above ground tissues of nonvascular plants, seedless vascular plants, conifers, woody and herbaceous angiosperms ranging from tropical forests to boreal and arctic/antarctic communities (Arnold et al., 2000).

Non-clavicipitaceous includes fungi of family Ascomycetes, with a minority of Basidiomycetes. Dark septate endophyte, one of the fungal groups, was distinguished with the presence and absence of darkly melanized septa.

Effects of clavicipitaceous endophytes

Insects avoidance

Clavicipitaceous endophytes enhance resistance of hosts to insect feeding by producing alkaloidic mycotoxins, Loline and Peramine which are generally associated with resistance to insects (Patterson et al., 1991).

Mammalian herbivores avoidance

Clavicipitaceous endophytes have been reported to prevent host from feeding by mammalian herbivores, through mycotoxins like Ergot and Lolitrem alkaloids (Gentile et al., 1999).

Reduction of nematodes

Some studies indicated that clavicipitaceous endophytes have antinematode activity. Kimmons et al., (1990) stated that tall fescus (*Festuca arundinacea*) with an endophytic fungus *Acremonium coenophialum* has been shown to reduce nematode populations in field soils. Meng et al., (2012), reported that Benzopyranones from endophytic fungus *Hyalodendriella* sp., have antinematode property.

Increased resistance of host to disease

Clavicipitaceous endophytes produce indole derivative compounds, a sesquiterpene and a diacetamide compound which are responsible for inhibition of growth of other

pathogenic fungi. *Epichloe festucae*, an endophytic fungi have the capability to increase the resistance of host against various diseases (Kuldau and Bacon, 2008).

Enhanced ecophysiology of host plants

Clavicipitaceous endophytes help to enrich the ecological condition of host plants and allow plants to oppose abiotic stresses such as drought and metal contamination. For example, after the entry of endophyte, *Neothyphodium coenophialum* into the *Festuca arundinacea* tissue through root system, it helps the plant better acquire soil moisture and absorb nutrients, resulting in drought avoidance and faster recovery from water stress. In some cases, endophytes help to develop longer root hairs and enhance exudation of phenol-like compounds into the rhizosphere which help in absorption of soil phosphorus and enhanced aluminum tolerance (Malinowski and Belesky, 2000).

Effect of non clavicipitaceae on host palnt

Avoiding abiotic stress

In class II endophytes, individual isolates asymptomatically colonize the host tissue representing monocots and dicots. Class II endophytes help in avoiding abiotic stress in plants. Endophytic fungi isolated from host plants growing in geothermal soils (*Curvularia protuberate*), coastal beaches (*Fusarium culmorum*) and agricultural fields (*Colletotrichum* spp.) were reported to have the capacity of avoiding abiotic stress in host plants (Marquez et al., 2007).

Increase of biomass

Class II endophytes help to increase the host shoot and root biomass. Tudzynski and Sharon (2002) stated that biomass of root and shoot increased which might be due to the induction of plant hormones by the host or biosynthesis of plant hormones by the endophytic fungi present inside the host.

Protection from fungal pathogens

Many endophytes of class II protect hosts against fungal pathogens by the production of secondary metabolites. These endophytes explain about interactions with host defenses and fungal parasitism (Samuels et al., 2000). It provides resistance against fungal pathogens or competition between endophytes for their suitable environment.

Classification based on reproduction

Apart from this classification, some authors further classify fungal endophytes based on their host range, mode of reproduction, part of plant colonized, mode of transmission, source of nutrition and their symptoms noted on the host plant (Rodriguez et al., 2009). Fungal endophytes are classified based on the mode of reproduction, they may reproduce through sexual or asexual means (Brem and Leuchtmann, 2001).

Endophytes can be transferred to host plant either by vertical or horizontal transmission. Vertical transmission takes place when the endophytes are directly transferred from parent host plants to their progenies. Endophytes which occur in host plant frequently, for every generation is referred as true endophytes. When the transmission takes place through seeds they are known as seed transmitted endophytes (Saikkonen et al., 2002).

Horizontal transmission of endophytes takes place between two individual host belonging to same species or other. Here, the transmission takes place through airborne spores. Such endophytes are said to be spore transmitted endophytes. Those endophytes which exhibit weak pathogenicity against insect herbivores are mostly transmitted horizontally (Higgins et al., 2007).

Classification based on nutrition

Based on the source of nutrition, endophytic fungi were classified into biotrophs and necrotrophs. Biotrophic endophytic fungi obtain nutrition from living tissues of the host plant. Necrotrophic endophytes modify the host plant habitat and allow them to grow on dead tissues from which they can obtain nutrition through roots of the host plant. Endophytic fungi residing in plant tissues obtain carbon supply in the form of organic compounds from the host tissue for their food which in turn provide energy sources for the host plant (Lekberg and Koide, 2005). Due to evolutionary and ecological changes some endophytes which acted as biotrophs turned to necrotrophs under stress condition (Junker et al., 2012).

Classification based on appearance of symptoms

Based on the appearance of infection, endphytes have been classified as symptomatic (expressing symptoms) and asymptomatic (symptomless). Many true endophytes reside in host plants without causing any symptoms. They are huge in diversity and possess many

functional roles (Arnold and Lutzoni, 2007). Sometimes endophytic fungi cause infection in host plant but they are known as asymptomatic when the host plant is resistant to the fungi. But a sudden environmental change can influence the behavior of asymptomatic endophytes (Delaye et al., 2013).

Classification based on part colonized

Based on location of endophytic fungi in host plant part, they are classified as root endophytes, foliar endophytes etc. Many studies explain about localization of endophytes in various plant tissues. Some endophytic fungi that infect plant roots are known as root endophytes (Wilberforce et al., 2003) those colonize foliar part of the plant are known as foliar endophytes (Meyling et al., 2011).

Importance of endophytic fungi

Endophytic fungi has been considered as important for over a long period because of its pharmaceutical leads. It has the capacity to produce novel secondary metabolites. These metabolites act as antimicrobial, anticancer, antiviral agents, etc. The discovery of Taxol, a diterpenoid compound, the world's billion dollar anticancer drug was the first secondary metabolite from endophytes of *Taxus* sp. (Wani et al.,1971) that paved the way for endophyte studies. Nicolaou et al., (1994), suggested that *Taxus* sp., support certain endophytic fungi that may also synthesis Taxol. Taxol is used for treatment of ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well. Taxol is synthesized from endophytes of various *Taxus* sp., (Strobel et al., 1996), Bald cypress (Li et al., 1996), *Ginkgo biloba* (Kim et al., 1999) and *Podocarpus* (Sun et al., 2008).

Taxus sp., is slow growing tree therefore, the compound cannot be extracted. But the endophytic fungi can produce this compound in large quantity through fermentation processes. The price for the drug would also be reduced and the drug will be available to cancer patients (Stroble, 2003).

Ecology and biodiversity of endophytic fungi

Now-a-days endophytic fungi act as representative of fungal diversity. The number may increase over a period of time due to plant community and its structure (Sanders, 2004).

Apart from plants, they also colonize aquatic algae, mosses, ferns etc. Endophytic fungi can be seen in almost all the plant parts even in seeds (Hyde and Soytong, 2008).

Many fungi which are reported as endophytes can act as pathogens. They are present both in the healthy and diseased tissues, therefore some reasons behind their activity help in separation of endophytes, facultative pathogens and latent pathogens. Based on behavioral difference, fungi are classified into endophytic and latent pathogens. Some of the pathogenic fungi remain quite in host plant during the infection cycle and is known as "quiescent infections" and strains with impaired virulence can be considered as endophytes (Torres and White, 2014).

Localization of endophytic fungi

Endophytic fungal communities can vary in a single host species based on the type of host plant, plant density, nutrient availability in the soil and within the plant, their occurence at different sites, climatic zones, seasons, environmental conditions and their interaction with external microbiomes (Carroll, 1995; Gamboa et al., 2002).

Endophytic communities also vary in single host based on distance between the plant parts. Presence of endophytic fungi in reproductive structures have been less studied compared to other parts of the plant and they may show many functional differences (Gazis and Chaverri, 2010). For example, leaves, stems and roots of some wild and medicinal plants are colonized by distinct fungi that produce different ranges of secondary metabolites. This difference is due to exposure of inner tissues to different environment. Even in a single plant, vegetative structures show different endophytic fungal community (Gamboa and Bayman, 2001). More studies were needed on seasonal variation and succession to determine functional importance of changes in the mycobiome of the plant.

Host range

Endophytic fungi survives in various host plants from tropical, temperate, boreal forests, herbaceous plants from various habitats, including extreme arctic and alpine; ferns, angiosperms and gymnosperms (Torres and White, 2014). Endophytic fungi can be seen in almost all the plant species but now-a-days researchers widely accepted that endophyte free plants are also available and this is especially true for shrubs and trees (Gennaro et al., 2003).

Almost all the studies imply that, fungi and bacteria are the most frequently occurring endophytes. To study about host preferences of endophytes, usually endophytes inhabiting tropical plants were used (Arnold, 2005).

Host specificity

Host specificity denotes the preference by any microorganisms to a single host or group of related species. This specificity implies the chemical reaction that occurs between host and the endophyte (Holliday, 1998). 100's of endophytes can be isolated from a host, among them one or two definitely act as host specific endophyte (Tan and Zou, 2001). This specificity mainly occur due to ecological adaptation of host or endophytes but it can be influenced by various environmental conditions. Host specificity is one of the best examples for biological process which is known as ecological specialization. Different terminologies are used during these studies, they are host affinity, host association, host preference and host range.

Endophytic association showed complex biochemical production which may vary from host to host and from endophyte to endophyte. This variability occurs through mutation, genetic crossing or by unsubstantiated mechanisms such as by allowing transferring of information between themselves and host plants (Tan and Zou, 2001).

An endophyte form relation with two or more related host plant. But one host plant will be more preferred by a particular endophyte. It is known as host selectivity (Cohen, 2006). Host-preference is used to indicate a uniqueness of occurrence of endophytes to particular host, difference in endophytic community composition and relative frequencies from different host plants (Selim et al., 2012).

In foliar fungal endophytes (FFE) (Apigo and Oono, 2018) host specificity develops in four different dimensions. They are structural specificity (FFE abundance and evenness), network specificity (interaction strength), phylogenetic specificity (evolutionary relationships) and beta-specificity (spatial or temporal turnover). Structural specificity is a fundamental dimension which explains about abundance of endophytes among the host plants. Network specificity explains the strength of endophyte and their interaction with host. Phylogenetic specificity quantifies host specificity relative to the phylogenetic scale of the plant hosts in a community and Beta-specificity, however, quantifies the degree to which a given foliar fungal endophyte displays consistent host specificity across a range of contexts.

Taxonomy of endophytes

Many different species of fungi belonging to different classes and families have been recovered as endophytes. Certain classes of fungi have appeared very frequently. Some of them were Ascomycetes, Deuteromycetes, Basidiomycetes etc., (Saar et al., 2001). Class and species of fungi depends upon the host plant.

Ascomycetes as endophytes

The most common class occurring as endophytes is Ascomycetes (Stone et al., 2000). They invariably occur in various tissues and wide variety of plants. Paul et al., (2006) recovered 19 genera of endophytic fungi from *Taraxacum coreanum* of which 17 genera belonged to Ascomycetes such as *Alternaria, Apodus*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Fusarium*, *Leptodontidium*, *Leptosphaeria*, *Dothideales*, *Myrothecium*, *Nemania*, *Neoplaconema*, *Pestalotiopsis*, *Phaeosphaeria*, *Phoma*, *Plectosphaerella*, *Terfezia* etc. Persoh et al., (2010) recovered more number of Ascomycetes in the form Xylariaceae from epiphyte *Viscum album and their host Pinus sylvestris*.

Sandhu et al., (2014) isolated 10 endophytic fungi from *Ricinus communis*, among them seven isolates belonged to class Ascomycetes. Sometimes more number of endophytes isolated from same host belonged to Ascomycetes. Gonzalez-Teuber et al., (2018) isolated one hundred endophytic fungi from roots of *Chenopodium quinoa* and the fungal community was dominated by Ascomyctes. Taxonomically most of the endophytic fungi belong to ascomycetes and its anamorphs while some endophytic fungi belong to hypomycetes (Suryanarayanan et al., 2000), basidiomycetes (Martin et al., 2015) and least endophytes as zygomycetes.

Reproduction of endophyte

In endophytes, reproduction takes place by two methods,

i) By vegetative growth

Some endophytes reproduce through vegetative means. They completely grow inside the plant tissues without forming any external structures on the host plant. Their reproduction takes place entirely inside the host plant. They develop hyphae into the

ovules of the host plant and reaches seed for infection. Therefore, endophyte transmission takes place via seeds. After the hyphal penetration, viability of seeds will be lowered compared to hyphae present in it (Selosse and Schardl, 2007).

ii) Via spores

Endophytic fungi develop mass of hyphal tissue from which reproductive structures (spores) arise. It is referred as stromatic tissues. They surround the developing inflorescence and infect the tissues. Mycologists refer this symptom as choke. As this infection takes place externally, transmission of endophytes occurs through horizontal mode. This type of transmission usually takes place in woody plants (Faeth and Fagan, 2002).

Role of endophytic fungi

Endophytic fungi form the mutualistic association with the host plant but they are much related to virulent pathogens. After many research on endophytic fungi, mechanism of their pathogenecity was well understood and concluded that diseases were caused directly by plant pathogenic fungi. The symbiosis process contains the absence of most cells or tissues, nutrient and chemical cycling between hosts and fungus, promoted photosynthetic capacity, induced longevity of tissue and promoted living of fungus (Brader et al., 2014). Some researchers made clear difference between pathogens and endophytes and proved that, they are completely different. In nature, endophytic fungi associated with plants helps to promote plant growth, increase nitrogen uptake in nitrogen deficit-soils, increase stress tolerance and reduce infection by nematodes.

Physiological role

Endophytic microbial community play an important role in physiology of the host plant. Endophyte infected plants are healthier when compared with endophyte free plants. This is due to the production of phytohormones like Indole Acetic Acid, Cytokinins etc., by the endophytes. Apart from this, endophytic fungi enhance the absorption of nutritional elements like nitrogen and phosphorus and regulate nutritional qualities in the form of cabon-nitrogen ratio of the host plant (Kaldorf, 2005). Host plants infected with endophytic fungi can withstand any unusual environmental changes and stress. Various beneficial aspects are offered by endophyte infected plants such as, drought acclimatization, improved resistance to insect pests and herbivores, increased competitiveness, enhanced tolerance to stressful factors (Zhang et al., 2006).

Ecological role

Endophytic microbes play an important role in ecological systems by having strong interaction with the plants. Biomass of the plants was low after the entry of insect herbivores in non host plants compared to endophyte infected plants. This represent that herbivores can take place in plant-microbe interaction directly or indirectly. Endophytes have some novel ecological functions and they can influence the plant community, biodiversity and their interaction with plants (Clay, 2001). Ecologically, endophytes have specific defense roles against pathogens. Certain endophytes produce toxic alkaloids which can deter herbivores (Braun et al., 2003). Alkaloids alter the feeding pattern of plant pathogens and maintain the community structure.

Effect of climatic factors on endophyte community

Water is essential for plant growth and survival. In addition presence of endophytes in plant help them to resist against drought. Endophytes can enhance production of secondary metabolites, diversity and distributions in plants. But certain environmental conditions make this situation to become critical. Studies on drought resistance mainly focus on physiology and genetics of the plant under various environmental conditions (Rampino et al., 2006). Microbial symbionts help in mediating the plant response against various stress factors. Fungal endophytes are the important plant symbionts that directly influence the plant drought resistance. When water deficit occur, the plants accumulate non structural carbohydrates which led to buildup of carbon based defenses, such as tannins which make colonization rate of endophytic fungi to become low during dry seasons. More colonization rate of endophytes in plants help to increase their biomass production, lower stomatal conductance and lower overall water loss relative to plants (Rodriguez et al., 2008).

Objectives of endophyte research

- Research on endophytic fungi, emphasize about autecology, synecology and biodiversity of endophytic fungi and pathogenic fungi in the environment.
- During endophyte studies, the host plants were randomly selected which explains the temporal and spatial distribution of endophytes.

- Ecological studies on endophytes help to understand their specific habitat and Infection frequencies for specific hosts.
- To understand the role of endophytic fungi in complex symbioses involving hosts, fungi, and insects.

Selection of plant material

Selection of plant material is an important step which provides opportunity to isolate endophytes from best plants in the world because an individual plant may harbor one to hundreds of endophytes. Therefore, some strategies were developed to quickly narrow down the search for the host plants (Strobel, 2003). Several criteria must be followed during plant selection which are as follows,

- Plants from unique environmental conditions with special habitat and unusual biology, with novel survival strategies should seriously be considered for study. Example, aquatic plants growing in harsh aquatic environment passes through many rocks and debris resist infections caused by normal water moulds. They can produce huge antifungal compounds that protect the plants from pathogenic fungi.
- Plants with good ethanobotanical history and their usage in traditional medicine can be considered for studies. Endophytes from these plants will be able to produce many antimicrobial and anticancer compounds as their host plants.
- Plants those are endemic to certain land mass.
- Plants growing in the areas of great biodiversity have potential to harbor diverse endophytes.
- Plants which are surrounded by infected plants but do not show any symptoms can be used for isolation of endophytes because the isolated endophytes have the capability to produce more antimicrobial compounds.
- Young plant tissue is more suitable for isolation of endophytes than older tissues which possess slow growing endophytes.

Now-a-days lot of efforts has been taken to identify novel compounds from natural sources with wide biological activities and applications. This led to an extensive research

on organic substances produced by various plants and microorganisms having diverse habit and habitat. A popular wild or medicinal plant with a rich ethanobotanical history is *Cucumis dipsaceus* Ehrenb. ex Spach.

Cucumis dipsaceus Ehrenb. ex Spach

Genus *Cucumis* L. is distributed worldwide. Only six species of *Cucumis* were reported from India. *Cucumis dipsaceus* (Plate-1), was reported first time in India in 2010. They were recorded in scrub forest of Marudhamalai foot hills, Coimbatore District, Tamil Nadu, India (Sarvalingam et al., 2010). It is native to Africa, Ethiopia, Kenya, Somalia, Tanzania, Uganda, Sudan and Southern Egypt.

SYSTEMATIC POSITION

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Dilleniidae
Order	:	Violales
Family	:	Cucurbitaceae
Genus	:	*Cucumis* L.
Species	:	*dipsaceus*
Bionomial	:	*Cucumis dipsaceus* Ehrenb. ex Spach

Common names

Arabian cucumber, Hedgehog gourd, Pepino diablito, Teasel gourd, Wild gourd etc.,

Distribution

Africa (Ethiopia, Kenya, Somalia, Tanzania, Uganda, Sudan, Southern Egypt) and Asia: India (Karnataka, Tamil Nadu, Kerala).

Botanical description

They are climbing herbs, annual, scabrous (Geethakumary et al., 2015) (Fig.2).

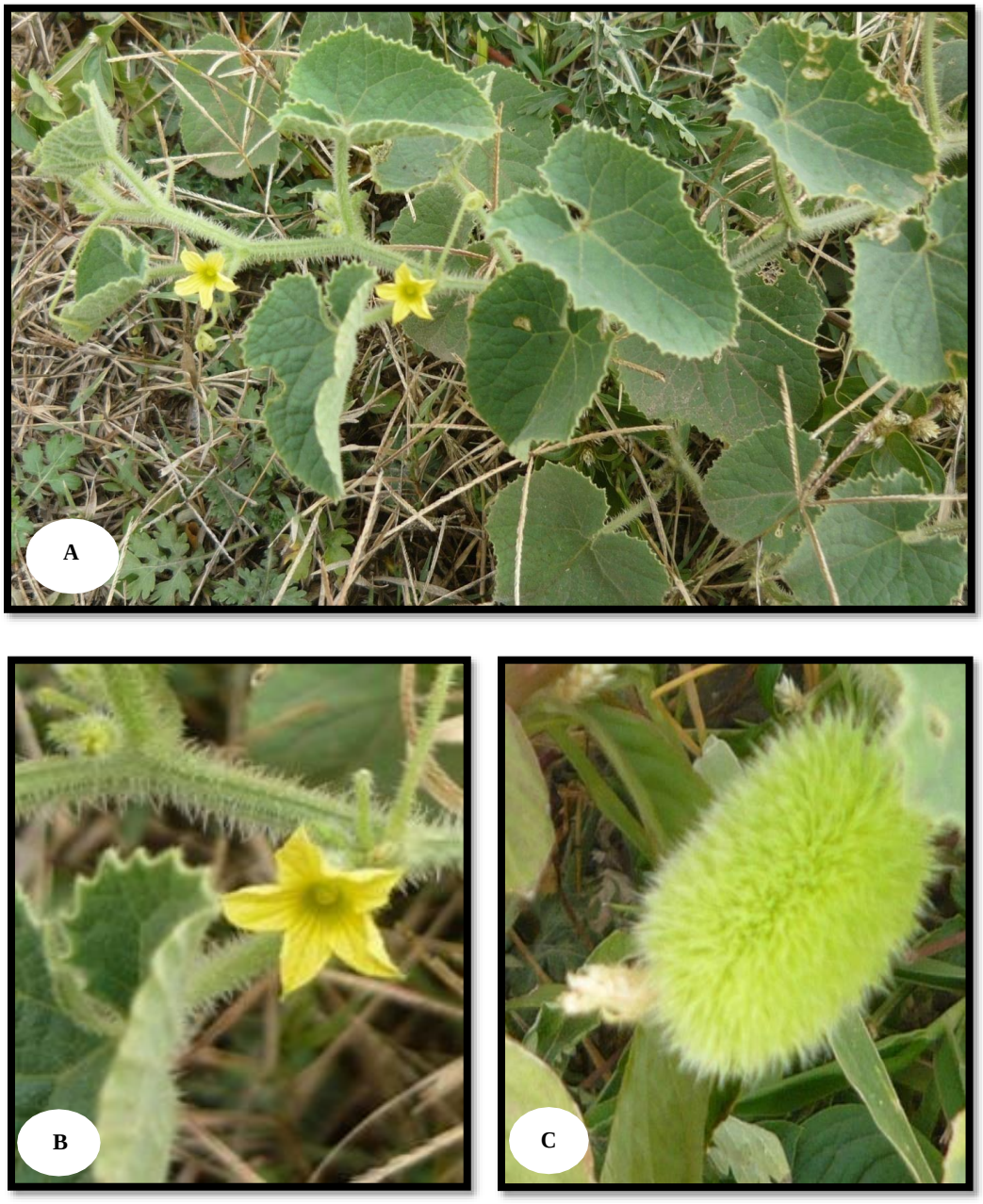
Stem

Weak, quadrangular, grooved, branched and hispid

Leaves

Ovate, shallowly trilobed, densely hairy on both surfaces, base cordate, apex acute to obtuse,

Plate-1: *Cucumis dipsaceus* Ehrenb. ex Spach



A- Twig of *C.dipsaceus* ; B- *C.dipsaceus* flower ; C- *C.dipsaceus* fruit

Petioles

1.5–4.5 cm long.

Tendrils

Simple, pubescent and utmost tip glabrous.

Inflorescence

Monoecious; male flowers axillary, 2–4 per axil; female flowers axillary, solitary.

Male flower

Pedicels 4 mm long; calyx lobes linear, apex acute; corolla yellow; tube campanulate; hispid outside, glabrous inside; stamens three; filament 1 mm long; anther lobes 2 mm long, straight, dorsifixed, ciliate.

Female flower

Pedicels 7 mm long, hispid; hypanthium are present; turbinate, hispidulous; calyx lobes linear 2 mm long; corolla lobes obovate 6–8 mm long, acute. Ovary 1 x 0.9 cm, oblong, densely aculeate, 3-locular; style1 mm long; stigma 3-lobed, papillate.

Fruit

Oblong, 5–7 x 3.5–4 cm, green, yellow when mature, many-seeded, densely aculeate.

Flowering

September – November.

Fruiting

November – January.

Ethanobotanical studies of *C.dipsaceus*

As the plants are native to Africa (Ethiopia, Kenya, Somalia, Tanzania, Uganda, Sudan, Southern Egypt), many local people and tribes of this country use *C.dipsaceus* as leafy vegetable and parts of the plant were used for medicinal purposes. Usage of *C.dipsaceus* in African countries was listed in Table-1.

Table-1: Ethanobotanical status of *C.dipsaceus*

Country	Plant part	Uses	Reference
Erer Valley of Babile Wereda, Eastern Ethiopia	Fruits and Roots	Snake bite, Carnivore bite, Wound, Gallstone and Hepatitis	Belayneh et al., 2012
Asgede Tsimbila district, Northwestern Tigray, Northern Ethiopia.	Roots	Snake bite, Insecticide, Stomach pain/ Diarrhea, Tuberculosis.	Zenebe et al., 2012
Gemad District, Northern Ethiopia	-	Eyeblindness	Mesfin et al., 2013
Eastern Ethiopia	Fruit and leaves Leaves Fruit	Gonorrhoea Urinary retention Skin fungus	Belayneh and Bussa, 2014
Elgeyo Marakwet County, Kenya	Roots	Abdominal pain	Kigen et al., 2014
Amaro special district, Southern Ethiopia	Leaves	Pneumonia and Abdominal pain	Tekle et al., 2015
South Region of Ethiopia	Leaves	Malaria	Asnake et al., 2016
Tanzania	Leaves and Roots	Leaves and roots pounded and used as poultice for wound treatment	Mbunde et al., 2016
Gulomekeda District, Northern Ethiopia	Leaves	Common cold	Girmay and Teshome, 2017
Eritrea, EastAfrica.	Roots	Abdominal Helminthes and Diarrhea	Yemane et al., 2017

Pharmacological studies of *C.dipsaceus*

Many researchers (Chandran et al., 2013; Nivedhini et al., 2014; Lata and Mittal, 2015; Priya and Anusuba, 2018) stated that *C.dipsaceus* is an excellent nutraceutical supplement for human diet because of the presence of essential amino acids and important minerals. Research towards phytochemical constituents of fruits and leaves of *C.dipsaceus* showed the presence of all primary phytochemicals like tannin, alkaloids, saponins, flavonoids, resins, steroids and carbohydrates with good antioxidant activity. Lata and Mittal (2015) reported that, due to the presence of significant amount of phenolics and flavonoids in fruits, *C.dipsaceus* showed strongest antioxidant and free radical scavenging activity. These made *C.dipsaceus* to have strong hepatoprotective activity both under *in vitro* and *in vivo* conditions.

Priya and Anusuba (2018), identified the functional groups and phytoconstituents present in the leaves of *C. dipsaceus*. The results revealed the presence of functional groups like alcohols, phenols, alkanes, alkynes, alkyl halides, aldehydes, carboxylic acids, aromatics, nitro compounds and amines and some phytochemical compounds like Androstane, Squalene and phytol etc., which possess great biological activities.

Assessment of endophytic associations

Preliminary investigation on distribution pattern of endophytes in host plant tissue was carried out using microdissection method. It revealed disjunct and discrete patterns of fungal occupation occurring on a microscopic scale. It helps to understand the biology and distribution of endophytes within the host. Microscopic tools help to locate the mycelia *in vivo*. It acts as a fundamental tool for plant pathology, mycology and related disciplines. Many stains were used to visualize the endophyte mycelia inside the host plant, they are Rose Bengal (Jackson and Johnson-Cicalese, 1988), KOH-aniline blue (Verma et al., 2012), Lactophenol cotton blue (Kumari and Chandra, 2013; Lakra et al., 2013; Mishra et al., 2015), Acridine orange (Raja et al., 2016) sometimes various stains were mixed at certain composition like Naphthol yellow 0.1 g, Malachite green 0.5g, Fuchsin acid 0.1g (Restrepo, 2007), Trypan Blue (TB) and Sudan IV (Bernardi-Wenzel et al., 2010). Segments of the plant tissues were stained and observed under light microscope which shows localization, distribution of endophytes.

Many histological studies based on Rose bengal stain provides lighter colour to the mycelia. Therefore, Hignight et al., (1993) used methyl salicylate, an optical clearing agent on leaf segments before staining. Garcia et al., (2012), visualized fungal hyphae in intracellular and intercellular spaces in leaves of *Sapindus saponaria* L. using scanning electron microscope. Reyna et al., (2012) used transmission electron microscopy, scanning electron microscopy and Laser scanning confocal microscopy (LSCM) for detection of endophytes in leaves and petioles of *Oxytropis lambertii* (Fabaceae).

Sterilization protocols

Surface sterilization plays an important role in isolation of endophytes by killing the surface microbes. It was carried out by treating the plant tissues with general sterilizing agent for a period followed by sterile rinse. Sterilizing agent should kill the microbes without affecting the plant tissues and endophytic microbes. Procedure for sterilization may vary based on host plant and its tissue type. Isolation frequency of endophytes can be determined by careful investigation on effects of surface-sterilization procedures, isolation medium and sample unit size (Schulz et al., 1993). Selecting suitable sterilizing agent helps to recover optimal endophytes from particular host, species and tissues.

In general, surface sterilization consists of following steps, Pretreatment of plants helps to eliminate hydrophobic substances on plant surfaces and help sterilizing agent for better accessing. Therefore, thorough washing of plant material under running tap water was carried out to remove the adhering soil particles and majority of epiphytes. Usage of sterilizing agents completely wipes out the microbial community from plant surfaces followed by several rinse of plant material with sterile water under aseptic condition to remove the traces of sterilizing agent. Sterility checks should be done to find the effectiveness of sterilization. All the sterilization procedures should be carried out under laminar flow hood and between each step, the plant tissues should be blotted in tissue paper to avoid dilution of sterilizing agent.

Pretreatment

Pretreatment is usually carried out for leaf tissues because some of them possess wax like substance on its surface. Just washing with tap water is adequate for pretreatment. But in roots, sonication is required to dislodge the soil particles and organic matter from their surfaces before sterilization (Hallmann et al., 2006).

Sterilizing agent

Commonly used sterilizing agents are Sodium hypochlorite (NaOCl), ethanol, Hydrogen peroxide (H_2O_2) and Mercuric chloride ($HgCl_2$). Sometimes propylene oxide vapour and formaldehyde are used. NaOCl, an excellent oxidant, decomposes spontaneously in storage. It is ahouse hold chlorine bleach usually diluted with water at 2-10% concentration. Their percentage and exposure time on plant tissue should be specified. $HgCl_2$ should be used very carefully because of health reasons. It is used exclusively for plant tissues that cannot be otherwise sterilized (Hallmann et al., 2006). Effectiveness of sterilization can be improved by combining with suitable agents. Ethanol is a common wetting agent with some antibiotic property but it should not be used alone.

Surfactants

Surfactants such as Tween 20, Tween 80 and Triton X-100 combined with sterilizing agent, help to reduce the surface tension of the plant material. After usage, they were washed with sterile water or 70-95% ethanol for one minute. Some of the common sterilization agents and their protocols used in various studies were listed in Table-2.

Sterility check and optimization

Only if complete surface sterilization of the plant tissue is confirmed, then the isolated microorganisms can be assumed to be endophytes. Optimization of the surface sterilization procedure was carried out using three methods they are, (1) imprinting the surface sterilized plant tissue onto suitable media (Schulz et al., 1998) (2) culturing aliquots of water from the last rinsing of plant tissues onto suitable media and (3) dipping the plant segments into specified broth. Results obtained from all the three methods should be compared. If no microbial growth occurs on the medium, surface sterilization is complete.

ISOLATION OF ENDOPHYTIC FUNGI

For studying endophytic fungi in healthy plant tissues, staining method (Direct observation method), cultivation-based method (Bacon and White, 1994) and cultivation-independent (Gao et al., 2005) detection methods were used.

Table-2: Different sterilization protocol

Sterlizing procedure	Incubation time	Host/Tissue	Reference
Wash with running tap water 70% ethanol 1-3% NaOCl 70% ethanol	 0.5 min 1-3 min 0.5 min	Rhizomes and roots	Petrini et al., 1992
Wash with running tap water 96% ethanol 10% NaOCl 96% ethanol	 1 min 5 min 30 sec.	*Salix* leaves	Schulz et al., 1993
Wash with running tap water 99% ethanol 35% H_2O_2 96% ethanol	 1 min 5-120 min 30 sec.	*Castanea* shoots	Bissegger and Sieber, 1994
Wash with running tap water 75% ethanol 34%NaOCl 75% ethanol	 1 min 10 min 30 sec.	*Casuarina* seeds	Bayman et al.,1998
Wash with running tap water 75% ethanol 5%NaOCl 75% ethanol	 30 sec 3 min 1 min	*Cucumis sativus* L.	Yan et al., 2011

Sterlizing procedure	Incubation time	Host/Tissue	Reference
Wash with running tap water 2.5%NaOCl	 30 min	*Cucumber* Sp.,	Khan et al., 2012
Wash with running tap water 70% ethanol 1%NaOCl 70% ethanol	 2 min 5 min 0.5 min	*Lagenaria siceraria*	Karunai selvi, and Balagengatharathilagam, 2014.
Wash with running tap water 70% ethanol 1%NaOCl 70% ethanol	 5 min 5 min 5 min	*Cucumis melo* L. Fruits	Glassner et al., 2015
Wash with running tap water 70% ethanol 4%NaOCl Sterile distilled water	 5 sec. 90 sec. 10 sec.	*Cucumis maderaspatnus*	Nayak et al., 2016

In the direct observation method, endophytes which are living in plant tissues were directly observed under light and electron microscopy using different stains. It helps to observe all mycobiota of plant tissues especially biotropic fungi which cannot be cultured on growth medium. But the observed fungi possess only hyphal structures and therefore cannot be identified upto taxonomical level due to the lack of spore producing structures and spores. Therefore, it is not commonly used for endophyte diversity studies (Sun and Guo, 2012).

Cultivation based technique have been widely used in endophytic diversity studies (Rodrigues and Samuels, 1990). It helps to isolate endophytic fungi and further enter into their detailed studies like characterisation, population dynamics, species diversity or as inoculum to improve plant growth and health or screening for novel biologically active secondary metabolites (Ding et al., 2009). In cultivation dependent technique isolation procedure is critical and important. All the suitable media should be supplemented with antibiotics which help to eliminate microbes other than desired ones. The segments were incubated onto the suitable media, endophytes that emerge from plant tissues were transferred to fresh media (Su et al., 2010). Sporulating and non sporulating isolates were maintained separately and identified using their morphological characters. This technique is effective for rapid recovery of a large number of endophytic fungal species from plant tissues. Studies on endophytic fungal community of all plant species examined to-date have been assessed using this technique. During isolation process, the entire fungal communities inside the plant tissues cannot be recovered because some of the slow growing fungi or those do not grow readily on the media and species that is lost in competitive interaction during the culturing process cannot be isolated (Torres et al., 2011).

In cultivation independent method, molecular techniques have been widely used. It has the potential to overcome the problems of cultivation dependent method. In culture dependent process, endophytic fungi can be identified only when they sporulate on the media. Eventhough, many methods have been developed for sporulation process (Guo et al., 2000), high number of isolates donot sporulate in cultures. Classification of endophytic fungi relies only based on reproductive structures. Therefore, these non sporulating isolates could not be provided with taxonomic names. They were categorized under mycelia sterilia and referred as morphotype. Morphotype is based on their similarity in cultural characteristics such as colony colour, texture and growth rates. Hence, molecular

methods were required for identification and understanding the diversity of sterile mycelia. Many researchers (Guo et al., 2000; Wang et al., 2005; Wang and Guo, 2007; Sun et al., 2011) isolated more number of non sporulating isolates and divided the isolates into many morphotypes. These isolates were identified using ITS sequence analysis. Molecular techniques help to recognize fungal diversity in natural ecosystem. Schematic representation of above isolation procedures were shown in the Fig.2.

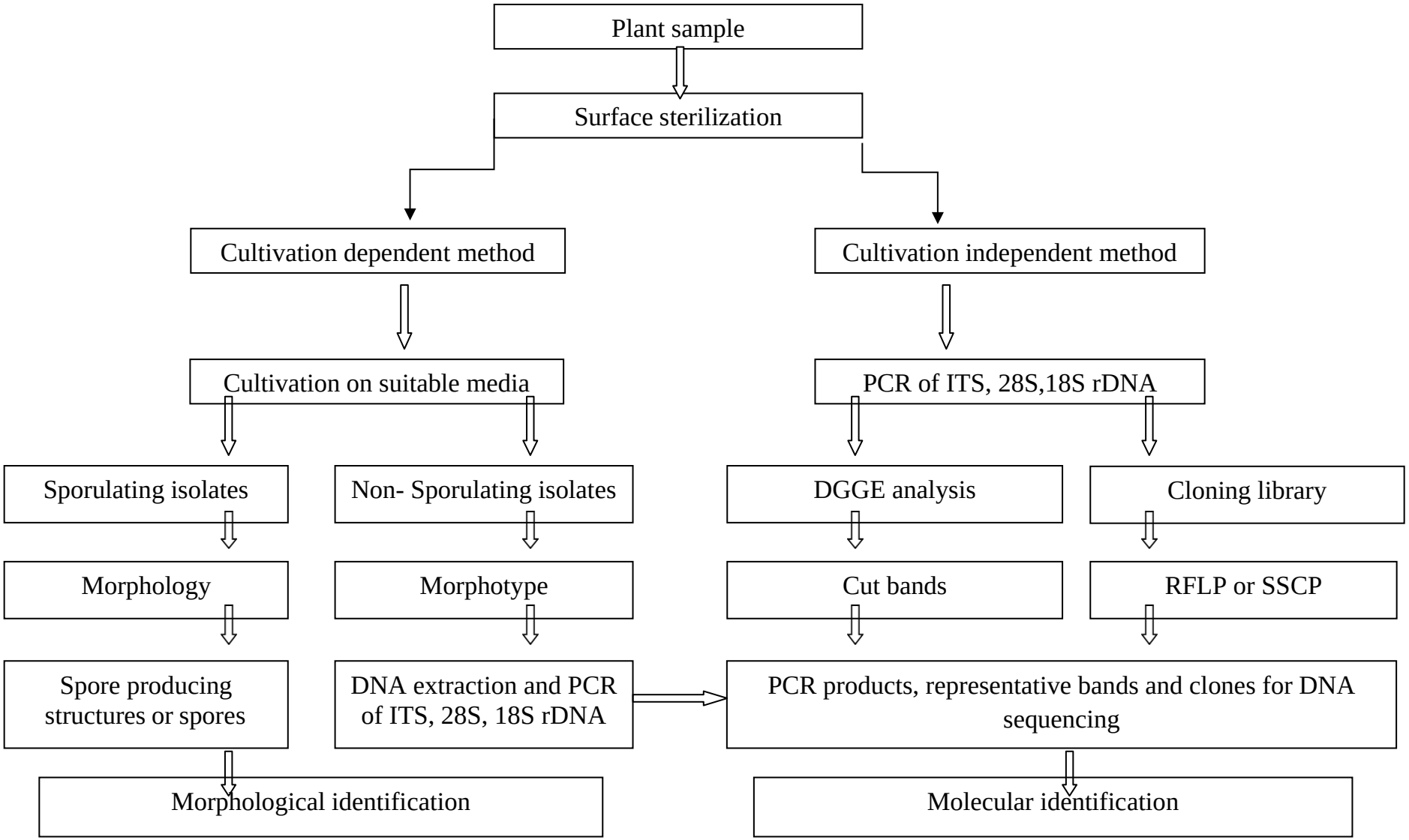
Apart from the above mentioned techniques, some other isolation procedures are also available they are,

Maceration technique generally employed for isolation of endophytic bacteria and sometimes for endophytic fungi especially for slow growing cultures (Sieber, 2002). It helps to isolate all possible endophytic fungi from plant tissues, including vascular bundles. For effective maceration, sterile water or saline solution is used for grounding the material. Depending on sample size and hardness of the material the mechanical device used for maceration may vary. After maceration the suspension is streaked onto suitable media. During maceration, plants have the ability to release toxins and enzymes which deactivate or kill endophytes. Therefore, the samples should be diluted immediately after maceration to decrease the concentration of toxic compounds (Hallmann et al., 2006).

Centrifugation method discovered to isolate endophytic bacteria from sugarcane stem. Later this technique is widely used for all plant materials and for isolation of endophytic fungi (Boyle et al., 2001). Centrifugation is commonly used to collect the intercellular fluids from plant tissues from which more number of organisms are isolated. Depending on sample size, small eppendorf to large sized test tube are used for centrifugation. The fluids are collected and streaked onto suitable media for recovery of endophytic bacteria or fungi. This process is time consuming but many samples can be centrifuged simultaneously (Hallmann et al., 2006).

Maceration and centrifugation methods give consistent results. But they have some limitations, viz (i) they are laborious, (ii) microorganisms which are deep inside the plant tissues that escape from sterilization process were misidentified as endophytes, (iii) sometimes hard sterilizing agents penetrate deep into plant tissues, which might kill the endophytes.

Fig-2: Schematic representation of isolation procedure



So the microbial diversity may be low from plants. To overcome these problems, alternative methods have been developed. Vacuum or pressure extraction technique was used to collect plant fluids (Hallmann et al., 1997). Maceration and centrifugation technique helps to isolate endophytes from conducting elements and intercellular spaces of plant, but were not able to isolate microbes present intracellularly.

In vacuum pressure technique, sterile water was washed through the vascular system at low pressure to collect the endophytic microorganisms. According to the type of tissue, the pressure should be gradually increased to prevent the damage. Though the method was time saving, some plants like young Cucumber and tomato does not accept because the plant part of these plants get collapsed at low pressure (Hallmann et al., 2006).

Media and incubation

Usually common mycological media are used for isolation and subculturing of endophytic fungi. Selecting suitable culture media for isolation of endophytes is very important. Different workers have used different culture medium for isolation of fungal endophytes such as Water agar (Dar et al., 2015), Potato dextrose agar (Ahmed et al., 2012; Sandhu et al., 2014; Dar et al., 2015; Mani et al., 2015; Fareed et al., 2017), Rose bengal agar (Dar et al., 2015), Malt extract agar (Mani et al., 2015; Fareed et al., 2017), Sabouraud's dextrose agar (Lu et al., 2012; Mani et al., 2015; Fareed et al., 2017), Czapeks dox agar (Fareed et al., 2017) and Synthetic nutrient agar (Boyle et al., 2001). To increase the diversity of endophytic fungi, various media are used with different pH and temperature, based on host plant and their tissues.

Antibiotics, fungicides or specific nutrients are added to media to suppress growth of certain microbial groups. For isolation of endophytic fungi, media supplemented with chloramphenicol, streptomycin and tetracycline are commonly used. Incubation of plates with plant segments may vary based on the type of host or its tissue. Endophytic fungi take long time for emergence, therefore incubation time will be more and the media may dry out. Incubation temperature range from 18-25˚C (Torres and White, 2012).

Isolation of endophytic fungi from leaves

Most of the plant associated fungi reported to date were identified using their fruitbodies produced on the host plant. Most of the plants like liverworts, mosses, seed free vascular plants, conifers, and angiosperms possess symbiotic relation with fungi especially

with the above ground tissue of plant, mainly leaves. Foliar fungal endophyte is a fundamental aspect of plant biology. All plant species surveyed to date possess one or more endophytes within the leaves (Stone et al., 2000).

Endophytic fungi involve in various plant phenotypic traits such as drought tolerance, leaf chemistry, tolerance to heavy metals in soils etc. (Clay and Schardl, 2002). In leaves, endophytes were transmitted horizontally. They undergo specialized localization within plant tissues. Horizontally transmitted endophytes do not show any direct effect on plants they inhabit. But they show some ecological effects like enhancing thermotolerance and salinity tolerance to temperate plants, increased resistance to foliar pathogens and increased host resistance to severe drought (Arnold and Lutzoni, 2007).

Occurrences of endophytic fungi in leaves of *Azadirachta indica* were reported by many researchers. Diversity of endophytic fungi may vary based on host habitat. Rajagopal and Suryanarayanan (2000) recovered only five endophytic fungi of which four were sterilia mycelia and one *Fusarium avenaceum*. In contrast, Verma et al., (2007) isolated 233 isolates from leaves, stem and bark of neem. Leaves showed 45% of isolates than stem and bark, whereas, Tenguria and Khan (2011) recovered 85 endophytic fungi belonging to 10 genera with 5% of sterilia mycelia. Occurrence of foliar endophytes in leaves of tropical trees varied based on environment, tissue type and age of the host plants.

Sometimes diversity of endophytic fungi may vary within a sample. Hata et al., (2002) reported the diversity of endophytic fungi within leaf samples of *Pasania edulis*, most important tree of the warm temperate forests in southern Kyushu. Endophytic fungi were recovered from leaves collected from natural location and from nursery. *Phomopsis* sp., *Phyllosticta* sp. and *Colletotrichum* sp. were frequently isolated but their occurrence vary in leaf tissue. This might be due to the infection process of these fungi from spores on leaf surfaces and competitive interactions of the endophytes within leaves (Rakotoniriana et al., 2008).

Krishnamurthy et al., (2009); Kim et al., (2013); Maheswari and Rajagopal (2013); Rampadarath et al., (2018), conducted a systematic study on the endophytic fungi of various host plants to understand their biology and distribution pattern during different seasons. Climatic conditions affect endophytic fungal diversity and variations in their

occurrence (Jalgaonwala and Mahajan, 2015). Wet conditions were generally favorable for fungal sporulation. Therefore, more colonization of endophytes took place in wet season than the summer.

Isolation of endophytic fungi from stem

Compared to foliar endophytes, diversity of stem inhabiting fungal endophyte remains understudied. Like leaves, they also present in plants of tropical and temperate regions. Lu et al., (2012) studied the diversity of endophytic fungi from stem tissues of *Actinidia macrosperma* and identified 11 isolates with major species like *Acremonium furcatum, Cylindrocarpon pauciseptatum*, *Trichoderma citrinoviride*, *Paecilomyces marquandii* and *Chaetomium globosum* which possessed some biological activity.

Gautam et al., (2013) isolated endophytic fungi from *Cannabis sativa* stem, leaves and petiole tissues. Among them, stem tissues showed highest colonization of 84.94%. *Aspergillus* and *Penicillium* were recorded as most frequently occurring genera. Sandhu et al., (2014) recovered 10 fungal species from stem tissues of *Ricinus communis*, among which *Aspergillus* was found to be frequently occurring genera. Tolulope et al., (2015) screened endophytic fungi from three traditional medicinal plants of Nigeria and identified 10 isolates from stem tissues. *Aspergillus niger*, *Macrophomina* sp., *Trichoderma* sp. and four different *Penicillium* species were recorded as major endophytes.

Many researchers studied diversity of endophytic fungi in stem tissues of various host plants. *Aspergillus* sp., *Penicillium* sp. and sterile mycelia were found to be dominant endophytes.

Endophytic fungi from family Cucurbitaceae

Cucurbitaceae family contains 97 genera and 940-980 species. Commonly known as Cucurbits and gourds, they are distributed widely in tropical, subtropical and temperate regions. Morphologically they are climbers, lianas, vines and rarely trees (Jeffrey, 1980). It includes several economically important cultivated plants such as Cucumber (*Cucumis sativus*), Melon (*C.melo*), watermelon (*Citrullus lanatus*), luffa (*Luffa cylindrica*), pumpkin (*Cucurbita moschata*) and wax gourd (*Benincasa hispida*). Important chemical constituents of Cucurbitaceae are Cucurbitacins, Saponins, especially triterpene saponins

and non-proteinogenic aminoacids. It has vitamin A, K and C, large amount of potassium, high levels of thiamin, niacin, vitamin B6, iron, magnesium and phosphorous. Its a very good source of dietary fiber. Members of Cucurbitaceae have multipurpose function in humans and animals (Shang et al., 2014).

Kim et al., (2007) recovered endophytic fungi from leaf, stem and root tissues of *Cucumis sativus* and *Cucurbita pepo* using malt extract agar supplemented with chloramphenicol. They identified 4 genera namely *Coniochaeta, Fusarium oxysporum*, *Fusarium* sp., *Talaromyces* sp. *Fusarium* sp., were frequently isolated from all parts of the plant. Yan et al., (2011) recorded more number of endophytic fungi from *Cucumis sativus*. They are *Acremonium* sp., *Actinomucor* sp., *Aspergillus* sp., *Aureobasidium* sp., *Cercospora* sp., *Chaetomium* sp., *Colletotrichum* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium* sp., *Humicola* sp., *Paecilomyces* sp., *Phyllosticta* sp., *Stagonospora* sp., *Trichoderma* sp., and *Ureobasidium* sp.

Waqas et al., (2012) studied the diversity of endophytic fungi in cucumber plants using hagem media and isolated two important species namely, *Phoma glomerata* and *Penicillium* sp. Reddy et al., (2014) assessed the diversity of endophytic fungi from various Cucurbitaceae plants (Cucumber, Bitter gourd, Ridge gourd, Snake gourd, Pumpkin and Ash gourd) collected from different districts (Coimbatore, Virudhunagar, Madurai, Dindugal and Ramnad) of Tamil Nadu. About 18 isolates have been confirmed based on morphological and molecular characters. They are *Chaetomium* spp., *Alternaria* spp., *Trichoderma* spp., *Aspergillus* sp. and some mycelia sterilia.

Su and Niu, (2018) isolated 21 *Talaromyces* strains from 10 Cucurbit plants using potato dextrose agar media. *T. cnidii*, *T. pinophilus*, *T. radicus* and *T. wortmannii* were frequently isolated and two new species of *Talaromyces* were recorded namely *T. cucurbitiradicus* from pumkin root and *T. endophyticus* from cucumber stems.

Identification of endophytic fungi

Though, histological techniques were useful in detecting endophytes in plant tissues, it cannot be identified up to genus level. Endophytes can be readily identified based on their morphological characters such as colony or hyphae, the characters of the spore or reproductive structures (Barnett and Hunter, 1998). Most of endophytic fungi belong to the

ascomycetes and asexual fungi (Huang et al., 2001). Some endophytes failed to sporulate and categorized under mycelia sterilia. In such cases, each endophyte was separately inoculated in different media like, Potato dextrose agar (PDA), Corn meal agar (CMA), V-8 agar or water agar to achieve optimum conditions for sporulation (Zhang et al., 2006). Once fructifications (asexual or sexual) are formed, their microscopic details can be examined and compared to known genera and species. Some endophytes do not sporulate even under optimum conditions. Such non identifiable mycelia sterilia rises the importance of using modern molecular techniques. Ribosomal DNA sequence analyses with specific primers help to resolve the identification problem of mycelia sterilia (Lacap et al., 2003).

MATERIALS AND METHODS

Sampling

Leaf and stem tissues of *Cucumis dipsaceus* were collected from Marudhamalai hills, Coimbatore district. Healthy and mature plants were carefully chosen for sampling. Samples were collected in sterile polythene bags and processed within 24 hours of collection.

Standardization of sterilization protocols

Five different sterilization protocols referred by Yan et al., (2011), Khan et al., (2012), Karunai Selvi and Balagengatharathilagam, (2014), Glassner et al., (2015) and Nayak et al., (2016) were engaged with slight modification in the concentration of sterilizing agent and treatment time in order to standardize the surface sterilization of plant parts (Table-3).

Table-3: Different protocols used for standardization of surface sterilization

Treatments	Chemicals	Concentration (%)	Time	Reference
I	Ethanol NaOCl	75 02	1 min 3 min	Yan et al., 2011
II	NaOCl Ethanol	2.5 75	5 min 5 min	Khan et al., 2012
III	Ethanol NaOCl	80 03	30 sec 5 min	Karunai Selvi and Balagengatharathilagam, (2014)
IV	Ethanol NaOCl	70 02	5min 5min	Glassner et al., (2015)
V	Ethanol NaOCl	70 04	5 min 10 min	Nayak et al., (2016)

NaOCl- Sodium hypochlorite

Sterility check

Efficiency of sterilization was assessed by impregnation technique (Schulz et al., 1993). Surface sterilized whole leaf was inoculated into Potato dextrose agar media and incubated for seven days to check the contaminants. Simultaneously, 1ml of last rinsed

water of surface sterilization was streaked onto Potato dextrose agar media to find the microbial community present in it.

Histological studies of endophytic fungi

Microdissection

Localization of endophytes in *C.dipsaceus* stem tissues were observed using hand sectioning method (Transverse section) followed by staining with Lactophenol cotton Blue and Trypan blue. The sections were observed under light microscope for detection of endophytes. Fungal colonies and its hyphae appeared in blue colour (Lakra et al., 2013).

Composition of stains

Lactophenol cotton blue

Cotton blue	-	0.05g
Phenol crystals	-	20g
Glycerol	-	40ml
Lactic acid	-	20ml
Distilled water	-	20ml

Trypan blue

0.05% of trypan blue powder in Lactoglycerol.

Leaf clearing technique

Leaf clearing procedure was adopted with slight modification from Phillips and Hayman (1970). Leaf materials were cut into small pieces and heated with 2-10% potassium hydroxide (KOH) solution at different temperatures ranging from 40-90˚C for half an hour to one hour. This process helps to remove cytoplasm and most of the nuclei from leaves. Most of the chlorophyll content have been removed and freshly prepared KOH solution was changed at regular intervals until colour of the leaves become pale. The leaf samples were rinsed with water at least for three times and acidified with diluted hydrochloric acid. The leaf materials were stained with trypan blue stain for 5 to 60 min. Excess stains were removed using clear lactoglycerol.

Isolation of endophytic fungi

After proper sterilization process, leaf and stem samples were cut into small pieces under aseptic conditions. Highly sterile conditions were maintained for isolation of endophytic fungi. Leaves were cut into segments (3-5mm ×1-1.5cm long segments) with or without midrib. Isolation of endophytic fungi was done according to the method prescribed by Su et al., (2010). Leaf and stem segments were blotted on sterile blotting paper to absorb water present on their surfaces. The segments were placed on petridishes containing various media like Potato Dextrose Agar (PDA), Czapeks Dox Agar (CDA), Malt Extract Agar (MEA) and Sabouraud dextrose Agar (SDA) amended with 50µg/ml of chloramphenicol to suppress the growth of bacteria. Inoculated plates were incubated for 3 weeks at 28±2˚C in both dark and light conditions. Fungal isolates emerged from plant segments were transferred to fresh media without the addition of antibiotics to obtain pure cultures for identification.

Composition of media used for isolation of endophytic fungi

Potato Dextrose Agar (PDA)

To prepare PDA, 200g of potato was cut into pieces and boiled in distilled water to obtain potato extract. The infusion was filtered, 20g of dextrose was added and the volume of media was made up to 1000ml using distilled water. pH was adjusted to 6.5. After adding agar, media was autoclaved for 15minutes at 121˚C.

Czapeks Dox Agar (CDA)

To prepare CDA, Sucrose – 30g, Sodium nitrite -2.0g, Dipotassium hydrogen phosphate - 1.0g, Magnesium sulphate - 0.5g, Potassium chloride - 0.5g and Ferrous sulphate - 0.01g was added to distilled water and boiled for few minutes to dissolve the ingredients completely. The volume of media was made up to 1000ml using distilled water. pH was adjusted to 7.3±0.2. After adding agar, media was autoclaved for 15minutes at 121˚C.

Malt Extract agar (MEA)

To prepare MEA, Malt extract -30g and Mycological peptone - 5.0g was added to distilled water. After dissolving the chemicals volume of media was made upto 1000ml with distilled water. pH was adjusted to 5.4±0.2. After adding agar (15g), media was autoclaved for 15minutes at 121˚C.

Sabouraud Dextrose Agar (SDA)

To prepare SDA, Glucose – 20g and Peptone -10g was added to distilled water. After dissolving the chemicals volume of media was made upto 1000ml with distilled water. pH was adjusted to 5.6±0.2. After adding agar (20g), media was autoclaved for 15minutes at 121˚C.

Identification of endophytic fungi

The potential fungal isolates were identified using their colony and morphological characters. To study morphological characters, fungal hyphae were stainined with lactophenol cotton blue and observed under microscope. They were further authenticated by Agharkar Research Institute, Pune, India.

Parameters studied

During isolation process, following parameters were noted (Maheswari and Rajagopal, 2013).

$$\text{Colonization rate} = \frac{\text{Total number of segments infected by fungi}}{\text{Total number of segments incubated}}$$

$$\text{Colonization frequency} = \frac{\text{Number of plant segments colonized by single endophyte}}{\text{Total number of segments observed}} \times 100$$

$$\text{Isolation rate} = \frac{\text{Number of isolates obtained from plant segments}}{\text{Total number of segments incubated}}$$

RESULTS

Diversity of endophytic fungi in *C.dipsaceus* was studied to find the potential isolate that have the capacity to produce biologically active substances. Plant samples were collected from the foothills of Marudhamalai, Coimbatore, Tamilnadu. Plant samples were identified and authenticated (Annexure-I) by Botanical survey of India (BSI/SRC/5/23/2014-15/Tech./663), Coimbatore, TamilNadu, India.

Biodiversity of endophytic fungi in *C.dipsaceus*

Standardization of sterilization

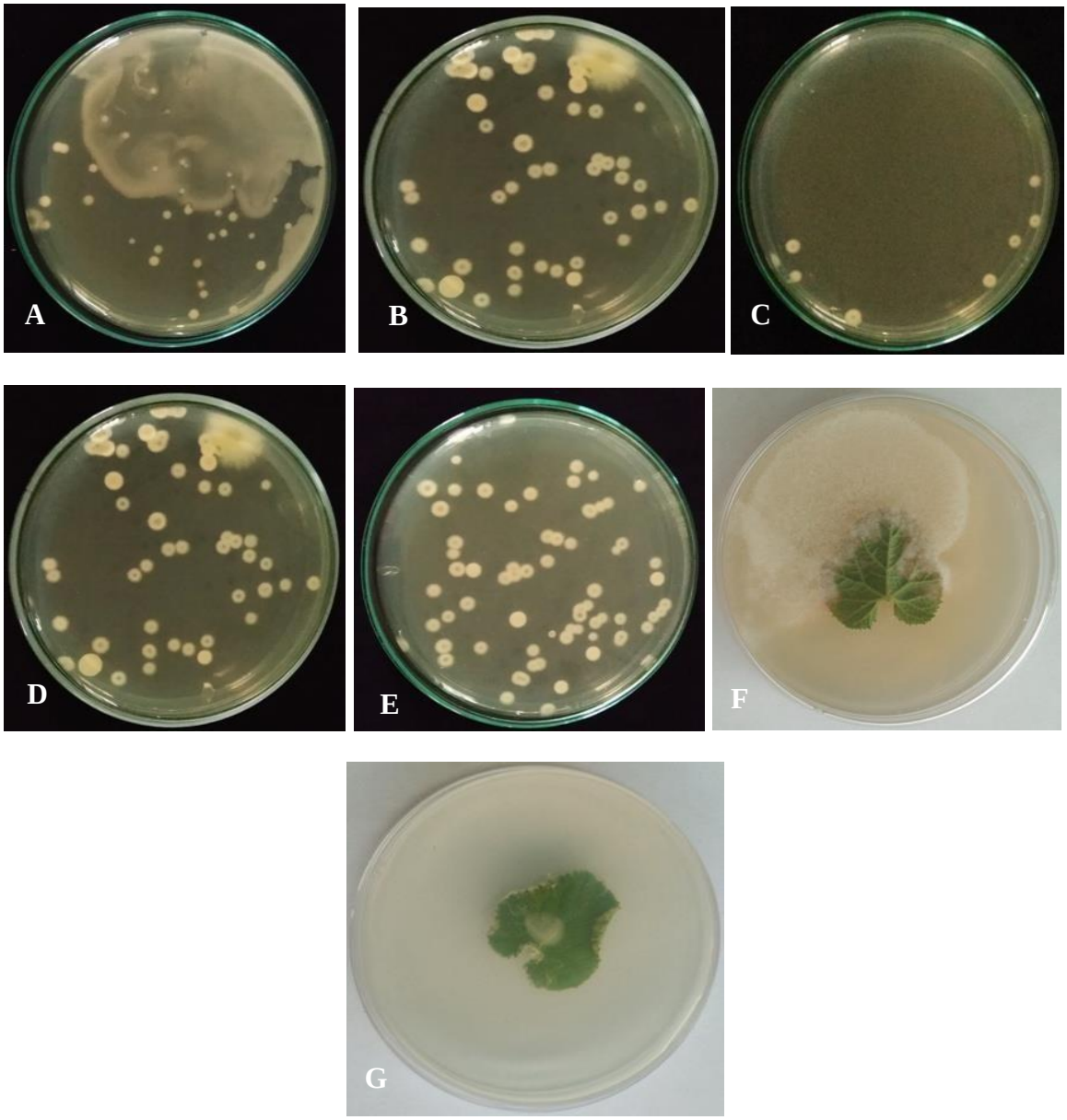
Five treatments listed in Table-3 were used to standardize the surface sterilization process. Among them, procedure adopted from Karunai Selvi and Balagengatharathilagam, (2014) showed effective sterilization. Here the plant material was washed with tap water followed by rinsing with 80% ethanol for 30sec. and NaOCl for 5 min. They were again rinsed with 80% ethanol and finally washed with sterile distilled water. Efficiency of surface sterilization using various concentration of sterilizing agents and imprint technique was shown in Plate- 2.

Histological studies on endophytic fungi

Localization and abundance of endophytic fungi in the tissues of *C.dipsaceus* was examined through microdissection. Microscopic examination revealed that they harbor some endophytic fungal colonies in transverse section at low magnification (10X) and 40X. Colonies were observed in inner layers of plant tissues (Plate-3).

Pretreatment of leaves with 6% KOH at 80˚C for one hour cleared the pigments without affecting them. Acidification and staining with trypan blue and lactophenol cotton blue greatly increased the accuracy of estimation of endophytic fungi. Fungal hyphae running in intercellular tissues of plants were examined at lower magnification (Plate-4).

Plate -2: Optimization of sterilization



A-Treatment I: Ethanol-70%, NaOCl- 2%; B- Treatment II: Ethanol-75%, NaOCl- 2.5%; C-Treatment III: Ethanol-80%, NaOCl- 3%; D- Treatment IV: Ethanol-70%, NaOCl- 2%; E- Treatment V: Ethanol-70%, NaOCl- 4%; F and G- Leaf imprignation technique

Plate-3: T.S of *C.dipsaceus* stem showing endophyte colonies

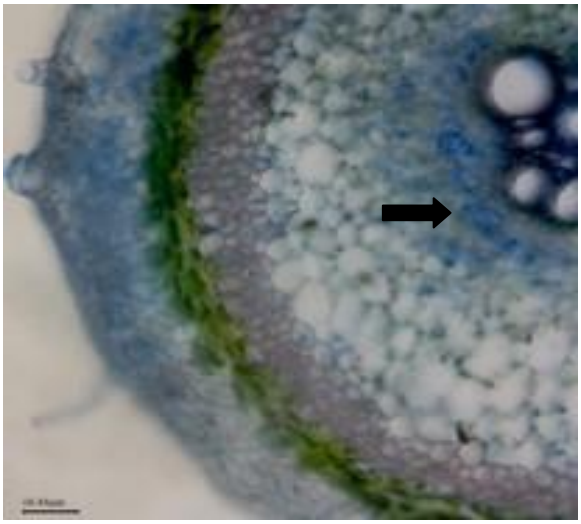
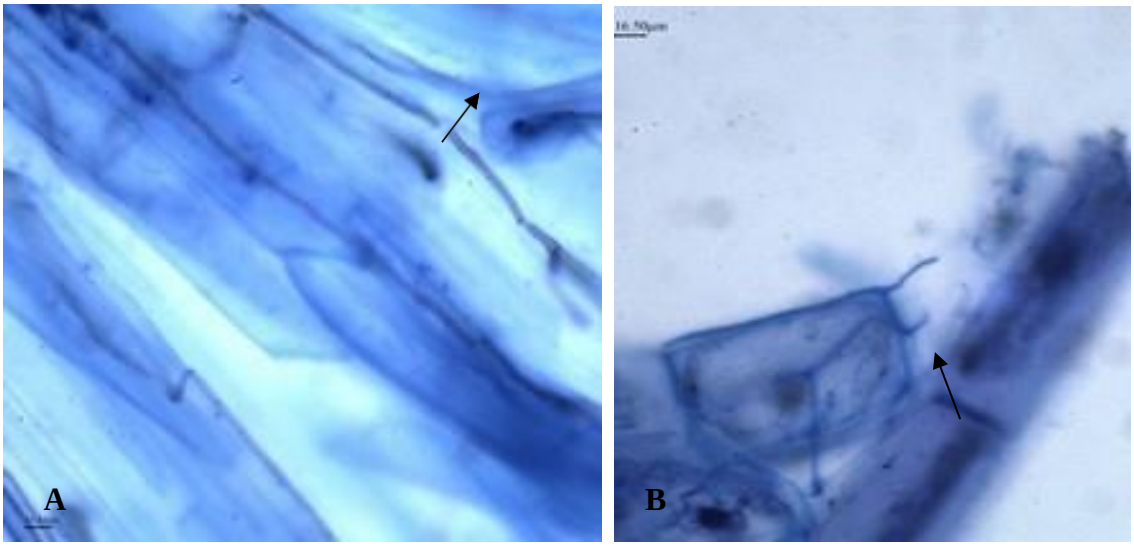


Plate-4: Localization of endophytes using leaf clearing technique



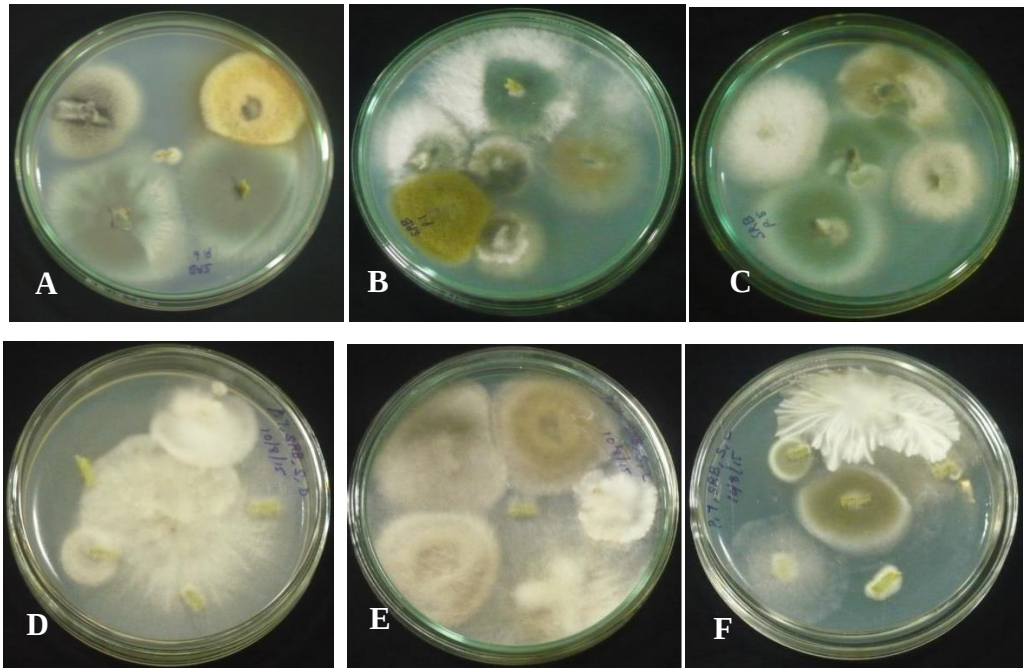
A- Section stained with Lactophenol cotton blue

B- Section stained with Trypan blue

Isolation and identification of endophytic fungi

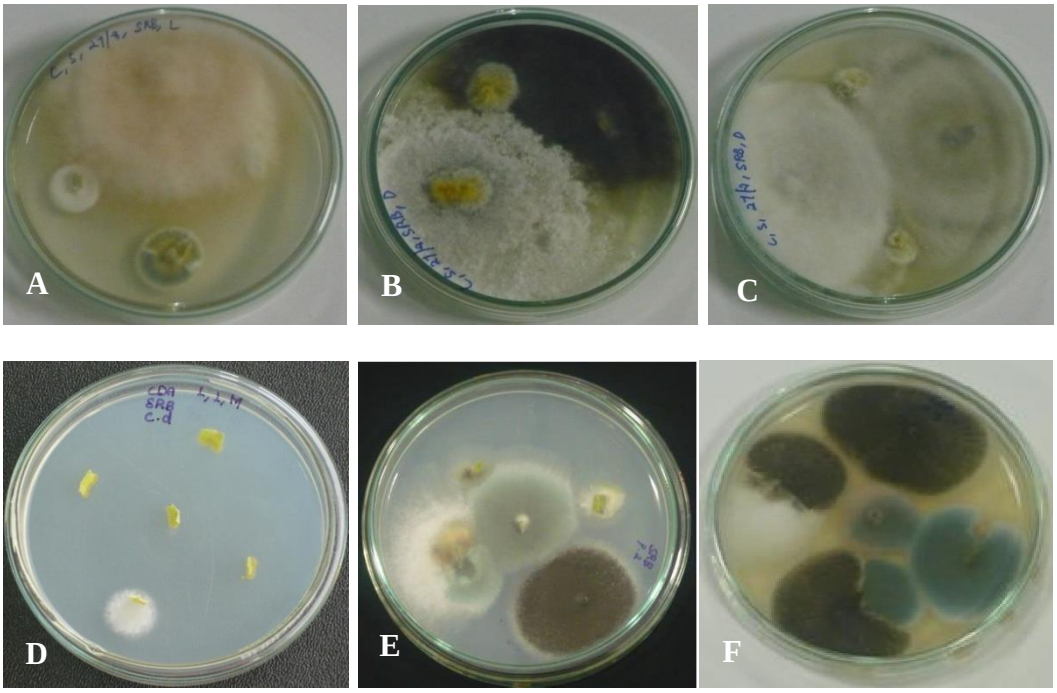
Thirty plants of *C.dipsaceus* were collected from Marudhamalai foothills to study the endophytic fungal diversity. Totally 500 segments (250 leaf and 250 stem segments) were screened for presence of endophytic fungi. Emergence of endophytic fungi from leaf and stem tissues using various media were recorded (Plate: 5-8).

Plate-5 :Emergence of endophytic fungi on PDA media (Leaf and Stem)



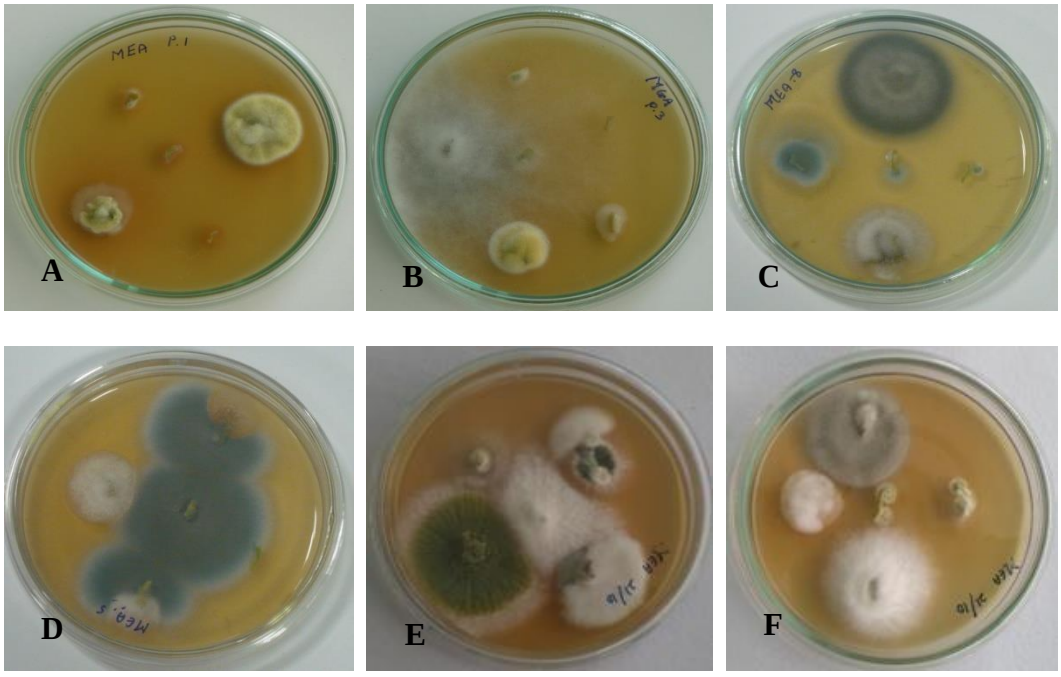
A-C: Isolation from leaves; D-F: Isolation from stem

Plate-6:Emergence of endophytic fungi on CDA media (Leaf and Stem)



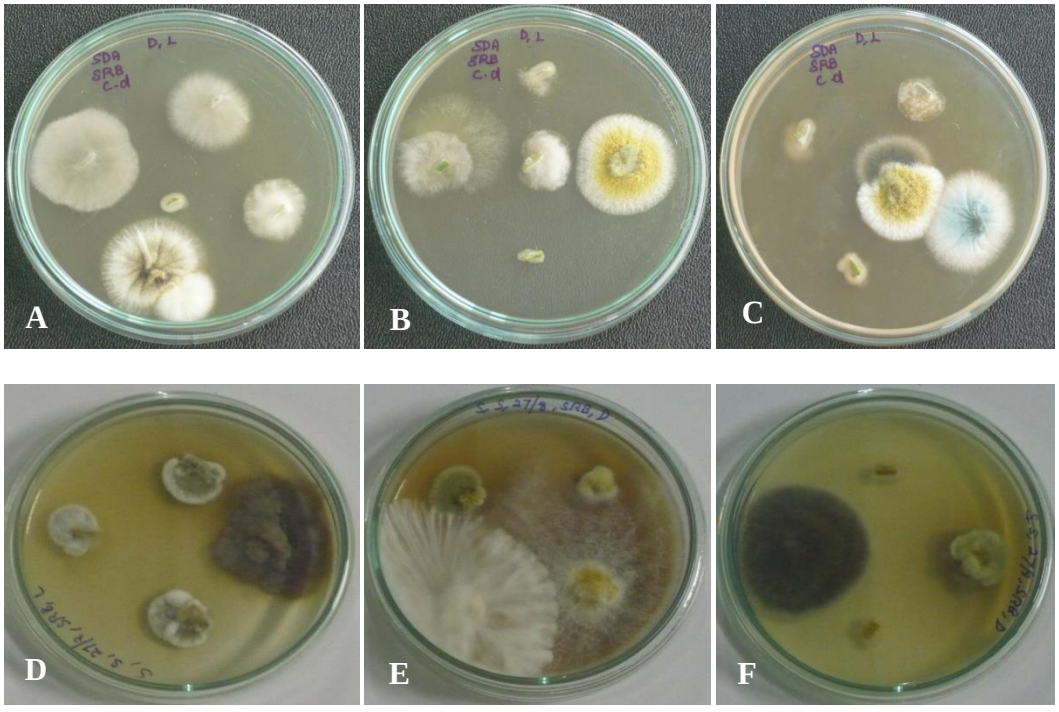
A-C: Isolation from stem; D-F: Isolation from leaves

Plate-7: Emergence of endophytic fungi on MEA media (Leaf and Stem)



A-C: Isolation from leaves; D-F: Isolation from stem

Plate-8: Emergence of endophytic fungi on SDA media (Leaf and Stem)



A-C: Isolation from leaves; D-F: Isolation from stem

Both sporulating and sterile forms were isolated. A total of 22 isolates were obtained from plant tissues (Table-4). The overall fungal composition includes 16 isolates in stem and 18 isolates in leaf. They were authenticated by Agharkar Research Institute, Pune, India (Annexure-II). Among the morphologically identified organisms, non sporulating dematiaceous form of fungi and *Paecilomyces* were further subjected to molecular identification using 18S rRNA sequencing. *Aspergillus* was the dominant genera with 8 species viz, *A.aculeatus*, *A.flavus*, *A.fumigatus*, *A.nidulans*, *A.niger*, *A.ochraceus*, *A.terreus* and *A.ustus.*

Table-4: List of endophytic fungi from *Cucumis dipsaceus*

S.No.	Endophytic fungi	Family	Source
1.	*Aspergillus* sp.	*Aspergillaceae*	S and L
2.	*A.aculeatus*		L
3.	*A.flavus*1		S and L
4.	*A.flavus*2		S and L
5.	*A.fumigatus*1		L
6.	*A.fumigatus*2		S
7.	*A.nidulans*		S and L
8.	*A.niger*		S and L
9.	*A.ochraceus*		S
10.	*A.terreus*1		S and L
11.	*A.terreus*2		S and L
12.	*A.terreus*3		S and L
13.	*A.ustus*1		L
14.	*A.ustus*2		S and L
15.	*A.ustus*3		S
16.	*Chaetomium* sp.	*Chaetomiaceae*	L
17.	*Chaetomium globosum*	*Chaetomiaceae*	L
18.	*Melanospora zamiae*	*Ceratostomataceae*	S and L
19.	*Nodulisporium gregarium*	*Xylariaceae*	S and L
20.	*Penicillium javanicum*	*Trichocomaceae*	S and L
21.	*Talaromyces radicus*	*Trichocomaceae*	L
22.	*Purpureocillium lilacinum*	*Ophiocordycipitaceae*	S and L

S- Stem; L- Leaf

Isolated endophytic fungi belong to families like *Aspergillaceae*, *Ceratostomataceae*, *Ophiocordycipitaceae*, *Trichocomaceae*, *Chaetomiaceae* and *Xylariaceae*. All the isolated endophytic fungi belong to Phyla Ascomycota. Using molecular technique, non sporulating dematiaceous fungi and *Paecilomyces* were identified as *Chaetomium globosum* and *Talaromyces radicus*. Organisms name followed by number denotes that same organisms with different morphological structures. During isolation process, some of the parameters like colonization rate, frequency and isolation rate were studied and shown in Table-5.

Table-5: Colonization and isolation rate of endophyti fungi from *Cucumis dipsaceus*

Parameters	Stem	Leaf	Total
No.of segments	250	250	500
No.of segments colonized by fungi	192	232	424
No. of isolates	16	19	35
Colonization rate	0.76	0.92	0.84
Isolation rate	0.06	0.07	0.07

Colonization rate of stem and leaf tissues were 0.76 and 0.92 respectively and isolation rate was 0.06 and 0.07 respectively. Colonization frequency was more in *Aspergillaceae* followed by *Ceratostomataceae, Chaetomiaceae, Ophiocordycipitaceae*, *Trichocomaceae* and *Xylariaceae* (Table-6). Isolation rate on both the tissues indicated that about 95% of organisms were common in leaf and stem tissues. Some of endophytes were specifically found in stem or leaves.

Table-6: Colonization frequency of endophytic fungi

Endophyte	No. of stem segments colonized	CF(%)	No. of leaf segments colonized	CF(%)
Aspergillaceae				
Aspergillus sp.	12	4.8	13	5.2
A. aculeatus	-	-	20	8.0
*A.flavus*1	16	6.4	12	4.8
*A.flavus*2	11	4.4	11	4.4
*A. fumigatus*1	-	-	18	7.2
*A.fumigatus*2	22	8.8	-	-
A.nidulans	9	3.6	13	5.2
A.niger	12	4.8	14	5.6
A.ochraceus	13	5.2	-	-
*A.terreus*1	17	6.8	16	6.4
*A.terreus*2	11	4.4	11	7.2
*A.terreus*3	6	2.4	15	6.0
*A.ustus*1	-	-	24	9.6
*A.ustus*2	9	3.6	6	2.4
*A.ustus*3	11	4.4	-	-
Chaetomiaceae				
Chaetomium sp.	-	-	17	6.8
Chaetomium globosum	-	-	8	3.4
Ceratostomataceae				
Melanospora zamiae	19	7.6	5	2.0
Xylariaceae				
Nodulisporium gregarium	7	2.8	9	3.6
Trichocomaceae				
Penicillium javanicum	11	4.4	11	4.4
Talaromyces radicus	-	-	3	1.2
Ophiocordycipitaceae				
Purpureocillium lilacinum	6	2.4	7	2.8

Characteristics of isolated endophytic fungi

Aspergillus sp.

The colonies of *Aspergillus* sp. showed rapid growth on Potato dextrose agar media at 28˚C. They reached 4-5cm in diameter within 10-15 days. They were less flocculent, with dark green coloured mycelium. They have the ability to produce red pigment. Soon as they mature, pigments turned red to pale white. Conidial heads were abundant and 5-10µm in diameter. Conidiophores were 2-3mm in length and 10-20 µm in diameter (Plate-9.1).

Aspergillus aculeatus

Colonies of *A.aculeatus* were 5-7cm in diameter within 10 days of incubation at 28˚C on Potato dextrose agar media. They were dark brown in colour and pale white on reverse side. Colonies were rough with thick white mat below the conidiophores. Conidiophores were 5-7mm in length and 1-2mm in diameter. Conidia heads were spherical to globose with 4-6µm in diameter (Plate-9.2).

*Aspergillus flavus*1

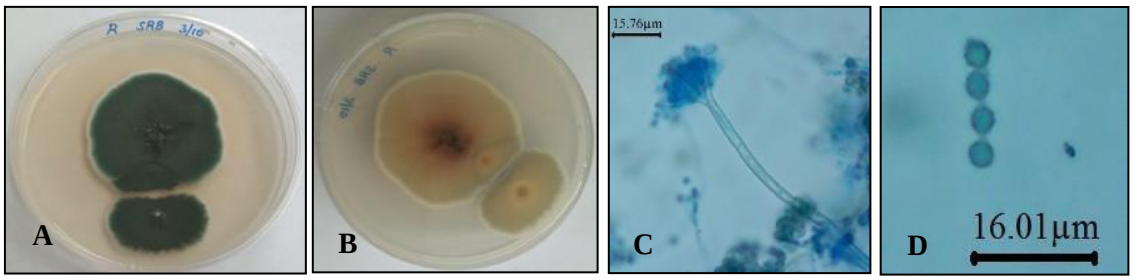
*Aspergillus flavus*1 were more flocculent when cultured on Potato dextrose agar. Colonies were greenish yellow in colour with pale yellow colour on reverse side. They showed rapid growth of about 4-5cm in diameter within 10 days. Conidiophores were 4-8mm in length and 2-3mm in diameter. Vesicles were wide with hemispherical and rough conidial heads of about 3-4µm in diameter (Plate-9.3).

Aspergillus flavus 2

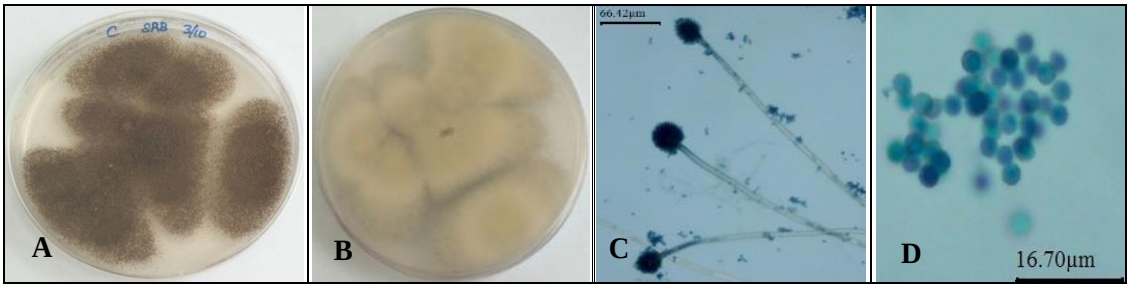
The colonies of *A.flavus* 2 became 4.5cm within 10 days at 27˚C when they were grown on Potato dextrose agar. They were dark green in colour with pale white colour on reverse side. Conidiophores were 5-9mm in length and 2-3mm in diameter with smooth conidia. Vesicles were spherical in shape. Conidial heads were 3-4.5µm in diameter (Plate-9.4).

Plate-9: Morphological characters of endophytic fungi

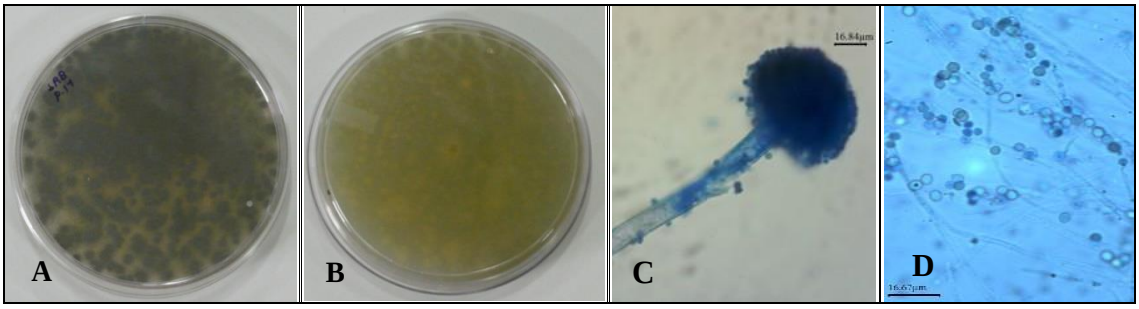
***Aspergillus* sp.**



Aspergillus aculeatus



***Aspergillus flavus*1**



***Aspergillus flavus* 2**

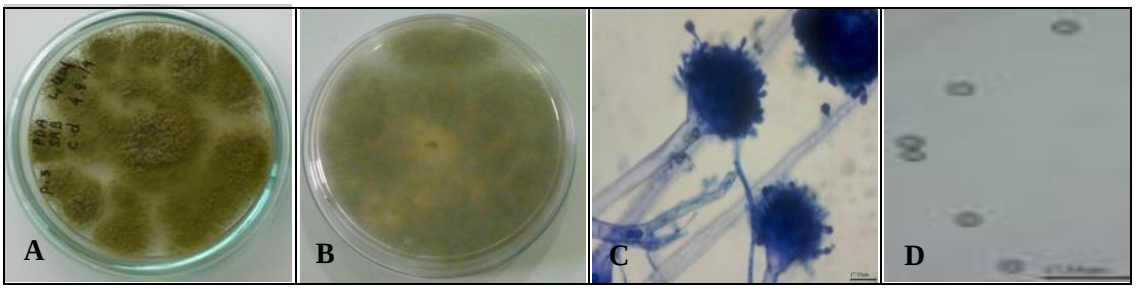


Plate-9.1, 9.2, 9.3 and 9.4 : A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

***Aspergillus fumigatus*1**

*Aspergillus fumigatus*1 produced light greenish coloured colonies on Potato dextrose agar. Under morphological status conidia were in the form of powder with uniseriate head of 3-4µm in diameter. Conidiophores were arranged in the form of bunches, 4mm in length and 2mm in diameter (Plate-9.5).

***Aspergillus fumigatus* 2**

The colonies of *A.fumigatus* 2 grew rapidly on Potato dextrose agar at 28˚C within 10 days. Colonies were grey in colour with hyaline on the reverse side. They have the capacity to produce semi liquid like exudates. Conidiophores were 6-7mm in length and 1-2 mm in diameter with uniseriate conidial heads (Plate-9.6).

Aspergillus nidulans

Colonies of *A.nidulans* were green in colour at the center surrounded by small orange coloured granule like structures. On the reverse, colonies were orange red in colour. They showed slow growth on Potato dextrose agar and reached only 2cm in diameter within 12 days. Conidiophores were smooth with hemispherical vesicles. Exudates were yellowish brown in colour. Conidia were short, globose with 2-3µm in diameter. Ascopores were globose, smooth walled, orange red in colour with 0.5- 1µm in diameter (Plate-9.7).

Aspergillus niger

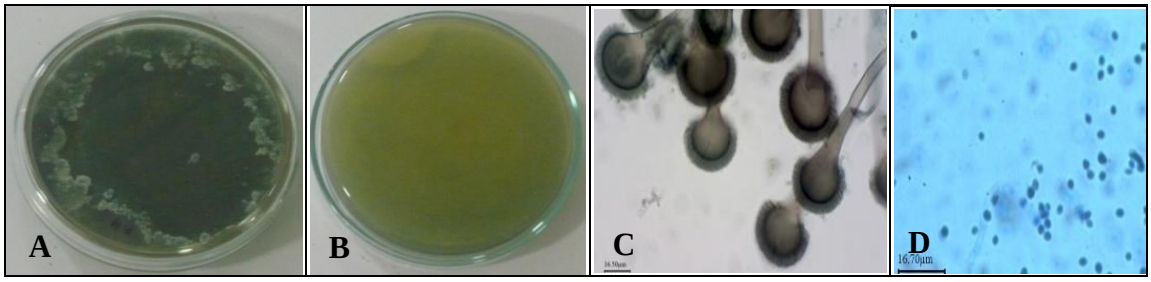
Aspergillus niger grew rapidly on Potato dextrose agar at 28˚C. Colonies were black in colour with abundant white mycelia on the media. Conidiophores were long and smooth walled, 1-2mm in length and 0.3- 0.5 mm in diameter. Conidiophores were colourless near the vesicle region. Conidia were globose, 0.5-1µm in diameter and black in colour (Plate-9.8).

Aspergillus ochraceus

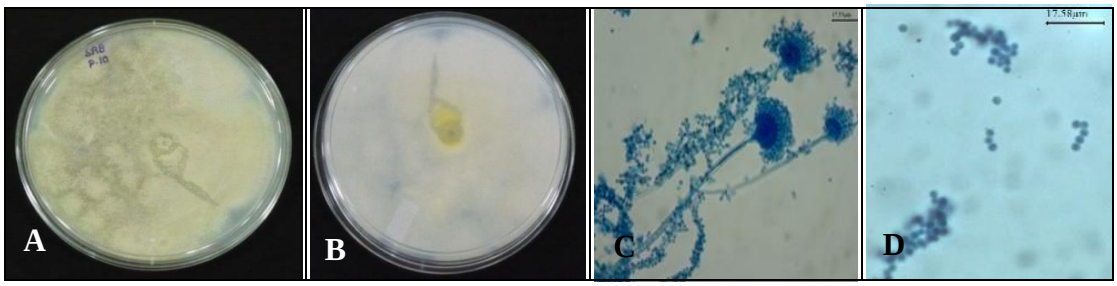
Aspergillus ochraceus showed rapid growth on Potato dextrose agar at 28˚C. Colonies were less dense and orange brown in colour with light brown on reverse. Conidiophores were long, 1-3mm in length and 0.5mm in diameter. Vesicles were round in shape with bunches of conidia. Conidia were small, globose and 1-3µm in diameter (Plate-9.9).

Plate-9: Morphological characters of endophytic fungi

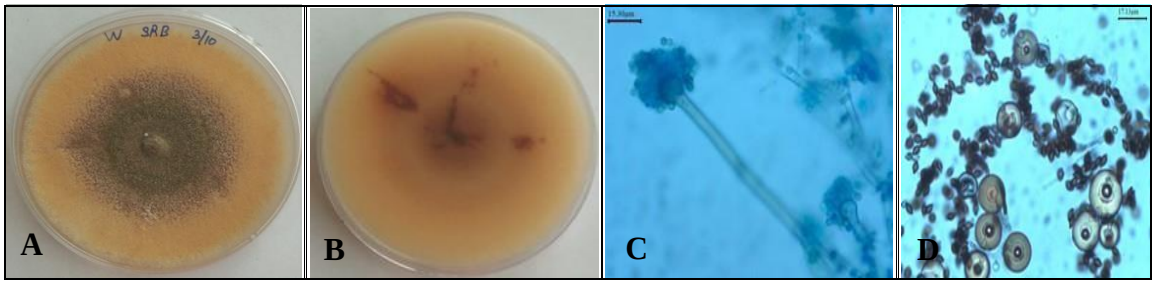
***Aspergillus fumigatus*1**



***Aspergillus fumigatus* 2**



Aspergillus nidulans



Aspergillus niger

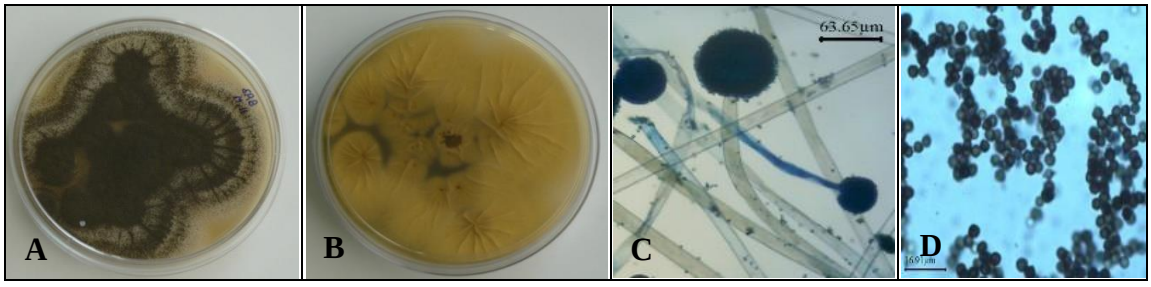


Plate-9.5, 9.6, 9.7 and 9.8 : A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

***Aspergillus terreus* 1**

*Aspergillus terreus*1 produced white yellow coloured colonies with brown shades on reverse. Colonies were velvety. Conidiophores were smooth walled, 6-7mm in length and 2-3mm in diameter. Vesicles were hemispherical to dome shaped. Sterigmata were seen submerged in vesicles. Conidia were compactly arranged and they showed uniform diameter (0.6µm) (Plate-9.10).

***Aspergillus terreus* 2**

Aspergillus terreus 2 showed fast growth on Potato dextrose agar at 28˚C. Colonies were pale brown to dark brown in colour with cottony growth. They were dark brown in colour on reverse side. Conidiophores were 3-4cm in length and 0.5-1mm in diameter. Vesicles were spherical, loose in texture, pale brown in colour. Conidia were small, smooth, globose, brown coloured with 4-5µm in diameter (Plate-9.11).

***Aspergillus terreus* 3**

Colonies were pale orange coloured in center surrounded by white mycelia. They were slow in growth on potato dextrose agar media and reached 5-6cm in diameter within 15 days. Pale orange coloured pigment were seen on reverse side. Conidiophores were long, smooth with globose to hemispherical vesicle. Conidial heads were compact and globose, 4-5µm in diameter (Plate-9.12).

***Aspergillus ustus* 1**

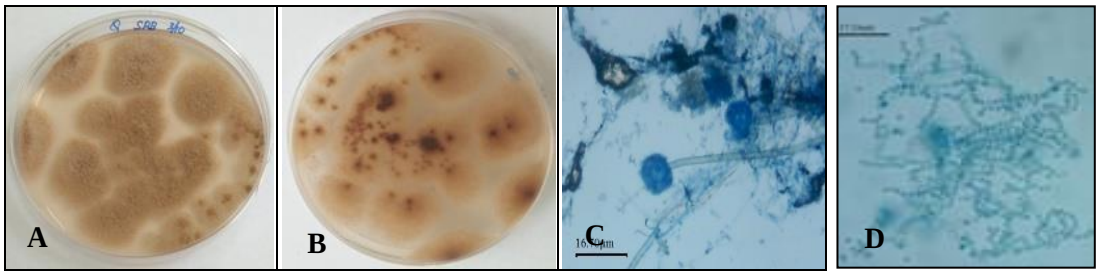
*Aspergillus ustus*1 produced grayish white colour colonies with pale grey on reverse side. Conidiophores were 3-4mm in length and 0.4mm in diameter. Vesicles were subclavate in shape. Conidia were light grey, globose, rough textured with 4-5µm in diameter (Plate-9.13).

***Aspergillus ustus* 2**

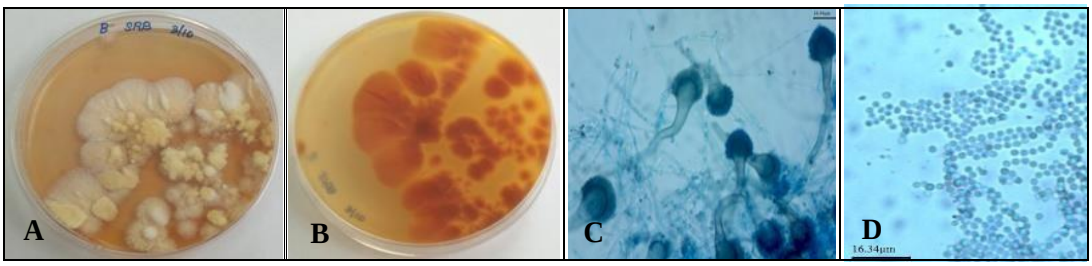
Colonies showed rapid growth on Potato dextrose agar with 3-4cm in diameter within 10 days. Morphologically they possessed white- yellow coloured mycelia which looked cottony. They produced light greenish yellow pigment on reverse. Conidiophores were, 1-2mm in length and 0.3-0.5mm in diameter. Vesicles were hemispherical in shape with submerged sterigmata. Conidia were grey in colour, small, globose in shape with 3-4mm diameter (Plate-9.14).

Plate-9: Morphological characters of endophytic fungi

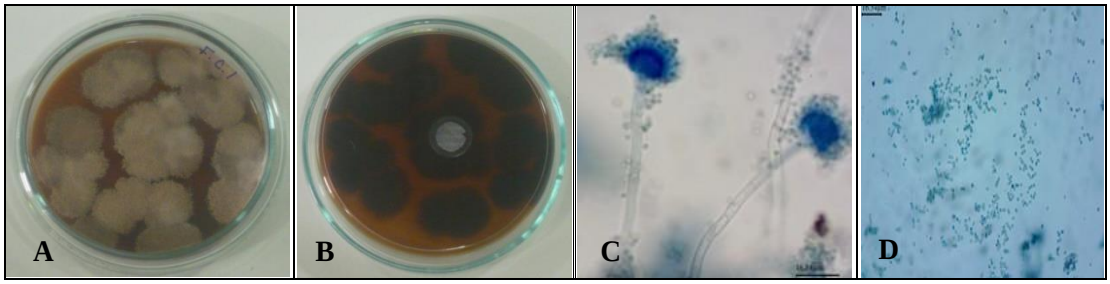
Aspergillus ochraceus



***Aspergillus terreus*1**



***Aspergillus terreus* 2**



***Aspergillus terreus* 3**

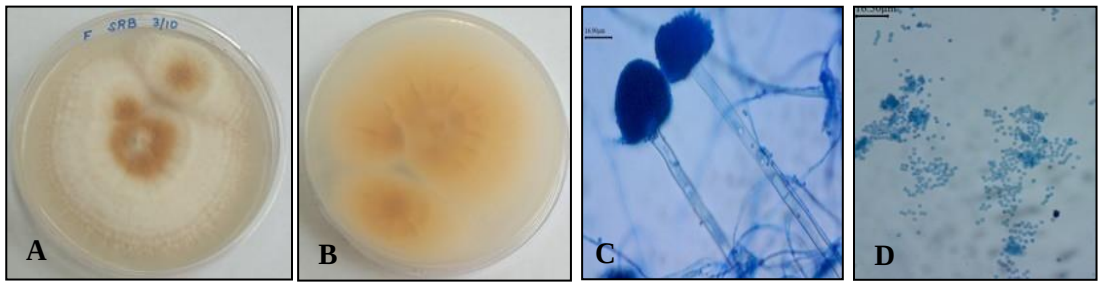
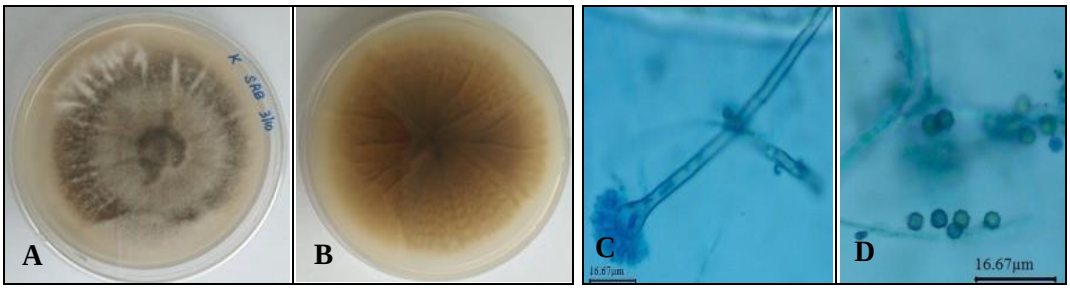


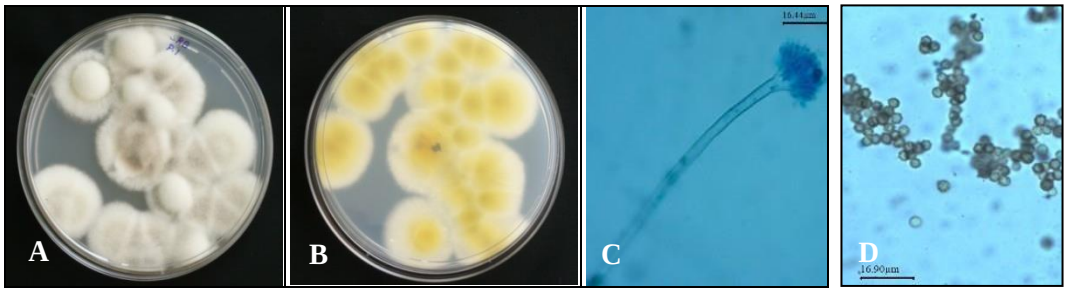
Plate :9.9, 9.10, 9.11 and 9.12 : A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

Plate-9: Morphological characters of endophytic fungi

***Aspergillus ustus*1**



9.14 *Aspergillus ustus* 2



***Aspergillus ustus* 3**

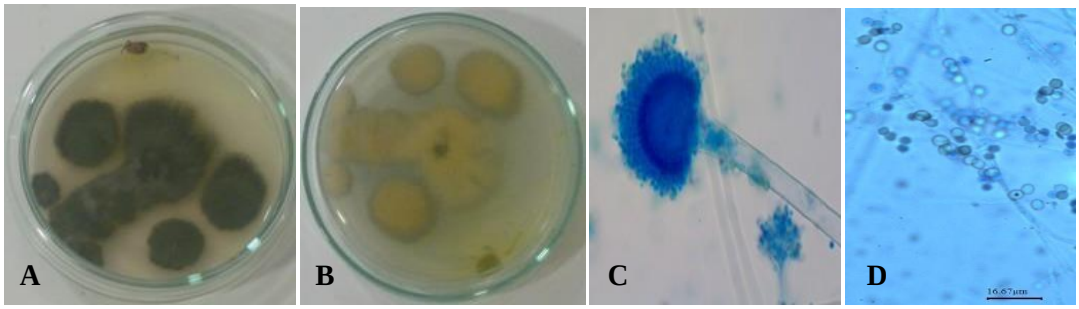


Plate:9.13, 9.14 and 9.15: A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

***Aspergillus ustus* 3**

*Aspergillus ustus*3 produced greenish colour colonies with pale grey on reverse side. Conidiophores were 3-6mm in length and 0.3mm in diameter. Vesicles were subclavate in shape. Conidia were dark green, globose, rough texture with 4-5µm in diameter (Plate-9.15).

***Chaetomium* sp.**

Chaetomium sp. showed rapid growth at 28˚C on Potato dextrose agar media. Colonies were pale black in colour, hyphae were septate, globose and hair like appendages on the surface. Margins of the colonies were not evenly arranged. It does not possess any aerial mycelium. This fungus produce abundant ascocarps that are ovate in shape. Lateral hairs were present around ascocarps which are scanty, dark brown in colour and straight. Asci possessed clavate shaped ascospores, that were brown in colour and globose to subglobose in shape (Plate-9.16).

Chaetomium globosum

Colonies showed rapid growth at 28˚C on Potato dextrose agar media with pale pink coloured mycelia producing pink colour pigment on reverse. It possessed only aerial mycelium with swollen edges (Plate-9.17).

***Chaetomium* sp.**

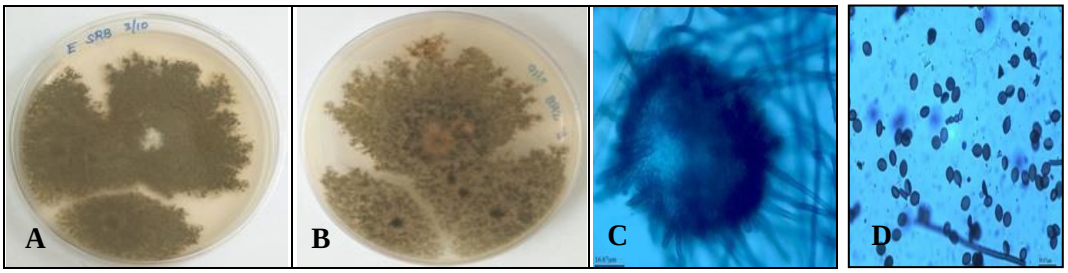


Plate: 9.16: A- Front side of the colony; B- Reverse side of the colony; C-Ascocarps; D-Ascospores

9.17 *Chaetomium globosum*

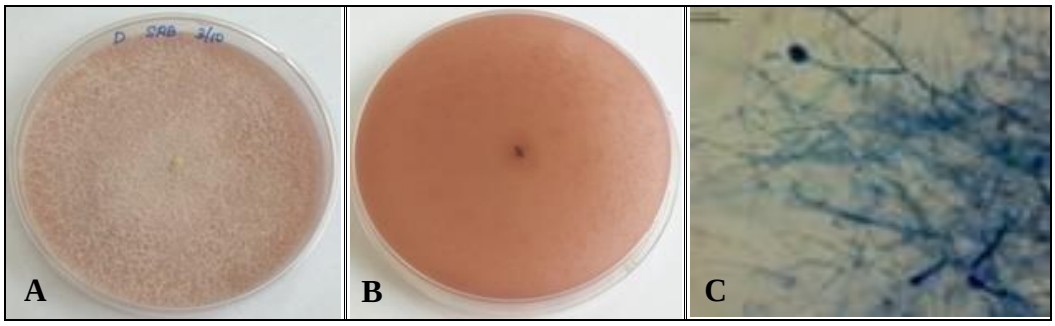


Plate: 9.17: A-Front side of the colony; B- Reverse side of the colony; C-Sterile mycelia

Melanospora zamiae

Melanospora zamiae produced offwhite colonies on Potato dextrose agar media without any aerial mycelium. Colonies became 5cm in diameter after 12 days. Reverse side of the colonies were pale white. It possessed ascocarps that are subglobose in shape. Numerous ascospores were present, that are ellipsoidal in shape (Plate-9.18).

Nodulisporium gregarium

Colonies produced by *N.gregarium* were white- ash coloured with dark blue colour on the reverse side. They have the capability to produce pigments. They were simple and unbranched forms. Length of the conidiophores cannot be distinguished. They form conidiogenous cells. Conidiogenous cells refer to repeated branching of condiophores (Plate-9.19).

Penicillium javanicum

Colonies showed fast growth on Potato dextrose agar media and possessed bright yellow mycelia that looks textured surrounded by white mycelia with 3-4cm in diameter. Conidiophores biverticillate, 1-2mm in length and 0.2mm in diameter. Conidia were grayish green in colour surrounded by green or white margins. Exudates were clear yellow droplets seen at the center of the colony. They have the ability to produce brown coloured soluble pigment (Plate-9.20).

Talaromyces radicus

Colonies were greyish green in colour with active growth at 28˚C on Potato dextrose agar media. Surface of the colonies were rough with pale yellow colour on reverse side. It possessed simple and branched conidiophores with two to three phialides. Spores were spherical shaped and arranged in form of chains. Structure of *T.radicus* resembles like *Penicillium* (Plate-9.21).

Purpureocillium lilacinum

Colonies on Potato dextrose agar media attained 4-5cm diameter of growth within 12 days. Mycelia grew radially with branches of conidia in center by producing light yellow diffusible pigment. Conidiophores were 4-5mm in length with hemispherical vesicle. Conidia were columnar in shape (Plate-9.22).

Melanospora zamiae

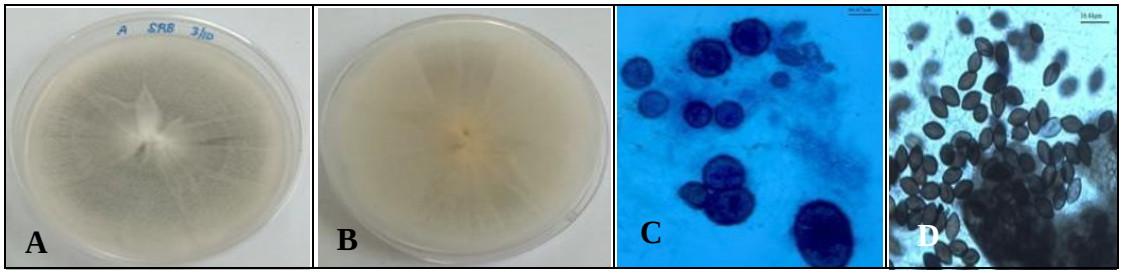


Plate: 9.18: A-Front side of the colony; B- Reverse side of the colony; C- Ascocarps; D- Ascospores

Nodulisporium gregarium

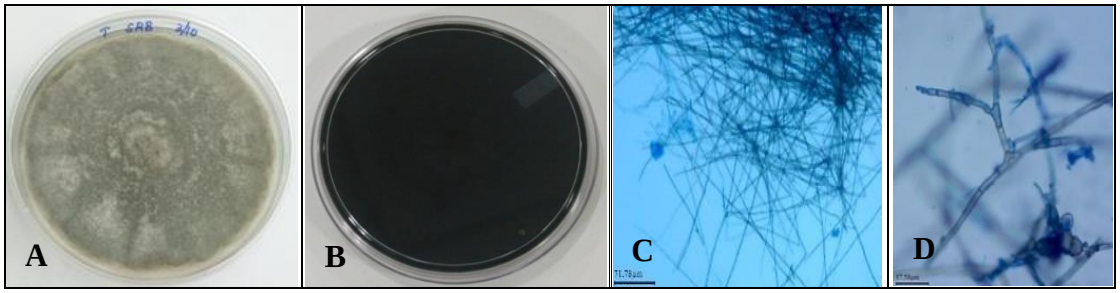


Plate: 9.19 : A-Front side of the colony; B- Reverse side of the colony; C-Conidiogenous cells

Penicillium javanicum

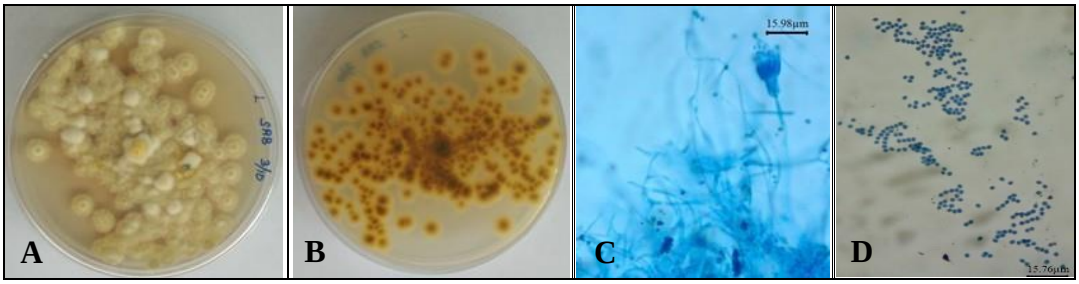
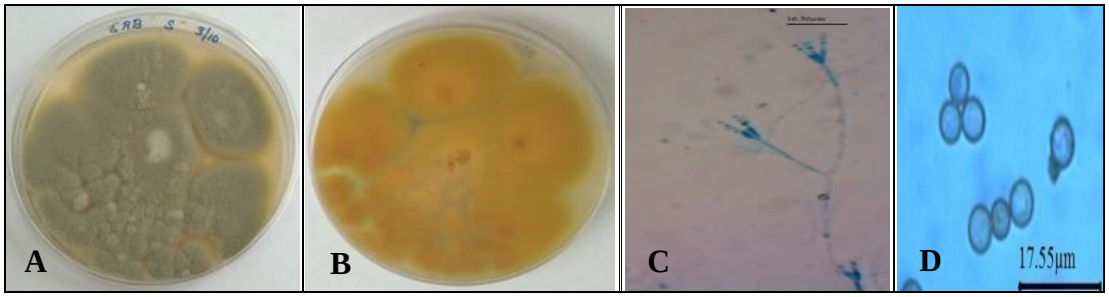


Plate: 9.20: A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

Talaromyces radicus



Purpureocillium lilacinum

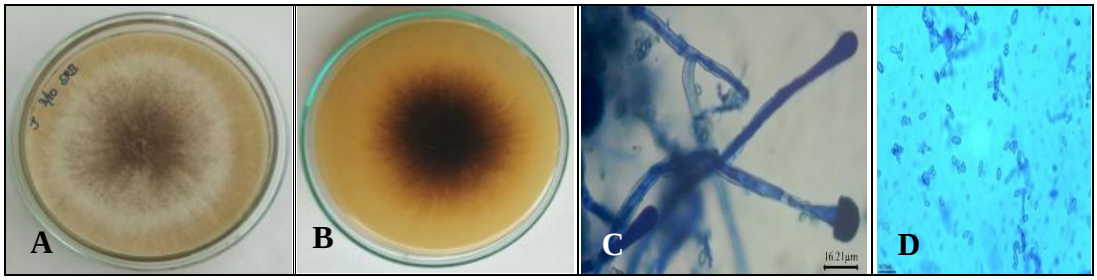


Plate:9.21 and 9.22: A- Front side of the colony; B- Reverse side of the colony; C- Conidiophores; D-Conidia.

DISCUSSION

Plants are considered as an excellent reservoir of endophytic microbes. They establish in the internal tissues of host plants without causing any visible symptoms. Studies on endophytes have been carried out mainly for two reasons (1) it act as an important ecological group which possess many unknown fungal species (Petrini, 1991) and (2) as a source of novel bioactive secondary metabolites (Stierle et al., 1993; Suryanarayanan et al., 1998). They have been recorded from plants such as herbs, shrubs, tree species and vines from unique places of ecological adaptations which help to contribute high diversity of endophytes. Hyde and Soytong, (2008) expected tropical forests as leading producers of endophytes as they possess high diversity of plants. But Kodandapani et al., (2008) stated that dry tropical forests of South India were accumulated with a lot of litter and experience long dry periods along with periodic ground fires. Spores present in litters of fire prone forest were heat tolerant and some of these fungi later enter into plants as endophytes (Suryanarayanan et al., 2011).

Some environmental factors such as temperature, rainfall and atmospheric humidity affect the host plant and decide the diversity and colonization frequency of endophytes. There are numerous species of endophytic fungi found in the inner spaces of plant. These endophytes interact positively with the environment and have potential towards plant improvement and disease control (Nair and Padmavathy, 2014).

Therefore, efforts have been taken to choose the plant with some ethanomedicinal values. As a result, the plants were sampled from foothills of Marudhamalai situated in Coimbatore district, Western Ghats of South India. As the tropical regions were richest source of plant diversity with good climatic condition almost all the plant species examined from this region harbor more endophytes.

Most important treatment before isolation of endophyte is surface sterilization of the plant parts. It is essential for isolation of pure endophytes that ensures the reduction of the contaminants, depending on the morphological characters (Softness or hardness) of plant tissues. In the current study, surface sterilization technique was optimized to obtain maximum fungal endophytes from *C.dipsaceus*. To the best of our knowledge, this is the first comprehensive report concerning the endophytic fungi from *C.dipsaceus*.

The population density of endophytic fungi is less compared to epiphytes. Hence to avoid contamination, plant samples were thoroughly surface sterilized before inoculating them on agar media. In this study, simple sterilization protocol was done for isolation of endophytic fungi from plant tissues of *C.dipsaceus*. Surface sterilization was carried out using 70% ethanol but it was not effective to eliminate epiphytes. Use of sodium hypochlorite at high concentration alone cannot support effective sterilization because they are mild sterilizing agent. Therefore, in the present study, two sterilizing agents in different concentrations with varying time of exposure were tested for sterilization of explants for isolation of endophytic fungi. In case of *C.dipsaceus* high survival percentage of explants was obtained with a combination of sodium hypochlorite and ethanol. After sterilization, explants showed a significant reduction in fungal contamination but the sterilizing agents did not give suitable results if the time and concentration were increased.

Our results corroborated with the results of Karunai selvi and Balagengatharathilagam, (2014). They obtained 80% survival capacity of explants in *Lagenaria sinceraria* on the usage of 70% ethanol for 2min and 1% NaOCl for 5min. Similarly, Glassner et al., (2015) obtained highest survival percentage by treating the plant material with same concentration of ethanol and NaOCl as mentioned above but the exposure time of tissues in ethanol (5min) was high. In contrast, Yan et al., (2011) and Nayak et al., (2016) obtained 85-90% survival percentage of *Cucumis sativus* and *C.maderaspatnus* by using high concentration (4-5%) of NaOCl by lowering the tissue exposure time.

In Cucurbitaceae, only few plant species of *Cucumis* have been screened for the evaluation of endophytic fungi. In the present investigation a total of 22 isolates were obtained from different parts of plant (leaf and stem). Mycelia sterilia was also reported from this study. The plants were found to be colonized with different endophytic fungi. Dominance of endophytes not only depended on plant species but also on their location (Huang *et al.*, 2008). Previously many researchers (Naik et al., 2008; Naik et al., 2014; Nalini et al., 2014) reported the diversity of endophytic fungi from various host plants of southern Western Ghats. Each of them isolated more than 3000 isolates from different parts of the plants. Richness of endophytic fungi in plants of Western Ghats may be due to ecological adaptation of host and favorable climatic condition for endophytes growth and sporulation.

Maximum number of endophytes can be recovered by following certain procedures they are (i) Isolation method- if the methods are inefficient, similar fungal species will be obtained from each site, (ii) seasons play important role in recovery of endophytes. Sample collection after rainy period helps to obtain more endophytes because this season favours the fungal growth by providing high humidity and considerable temperature, (iii) major effect on diversity of endophytes is due to their geographical location. Therefore, sample collection from various sites of same region help to recover more endophytes (Yadav et al., 2016). But in the present study, only 22 isolates have been recovered; this might be due to collection of plants from single site or by the usage of single isolation method. Only one isolation method was adopted, this is to eliminate as much epiphytes as possible. Some species showed different morphology, this might be due to usage of different media for isolation process.

In the present investigation, colonization frequency was higher in leaves than in stem. These results corroborates with the findings of Verma et al., (2007) (*Azadirachta indica*) who reported that colonization frequency was greater in leaves than bark. Gond et al., (2011) reported highest colonization frequency of leaves in *Nyctanthes arbor-tristis*. Reddy et al., (2014) and Su and Niu, (2018) also reported highest colonization frequency in leaves and roots than in stem and fruits of some Cucurbetaceous plants. This may be due to the large surface area of leaves exposed to the outer environment and the presence of stomata providing passage to the entry of fungal mycelia. Sometimes endophytes in leaf may also act as saprophytes in certain cases to enhance the litter degradation when leaf senescence occurs or plants die.

Bezerra et al., (2015) reported highest colonization frequency in stem of *Bauhinia forficate*. Sometimes colonization frequency increased with the increase in altitude. Similar reports were given by Rana et al., (2017) on observing the colonization frequency of endophytic fungi in *Rhododendron campanulatum.* The overall colonization frequency was 17.22% in this study. Highest colonization frequency was found in leaf (7.22%) followed by the stem (5.09%) and root (4.90%) samples.

The isolates from *C.dipsaceus* were identified as belonging to seven genus namely, *Aspergillus, Chaetomium, Melanospora, Nodulisporium*, *Talaromyces*, *Penicillium* and *Purpureocillium.*

Aspergillus was found to be the most dominant endophyte in *C.dipsaceus*. Various species of *Aspergillus* like *A.aculeatus*, *A.flavus*, *A.fumigatus*, *A. nidulans*, *A. niger*, *A.ochraceus*, *A.terreus* and *A.ustus*, were reported. Previously *Aspergillus* species was reported as common endophyte of *Cucumis sativus* (Yan et al., 2011) and in some Cucurbitaceae plants like Cucumber, Bitter gourd, Ridge gourd, Snake gourd, Pumpkin and Ash gourd (Reddy et al., 2014). Our study suggested that *Aspergillus* was not organ specific. They were isolated from both leaf and stem tissues.

Santos et al., (2003) obtained *A.aculeatus*, *A.flavus* and *A.niger* as endophytes from root cortex, root xylem, stem cortex, leaves and fruits of *Melia azedarach* also, Gautam et al., (2013) recorded *Aspergillus* as the most frequently occurring genera with three species *A.niger*, *A.flavus* and *A.nidulans* from leaves, root and petiole of *Cannabis sativa*. Sandhu et al., (2014) isolated *A.niger* and *A.fumigatus* as endophytes from leaves, stem and root of *Ricinus communis*, on PDA media and SDA media Venkatachalam et al., (2015) obtained *Aspergillus* sp., *A.niger* and *A.terreus*. *Aspergillus ustus* have been less studied as endophytes. Facey et al., (2016) isolated *A.ustus* from a mangrove plant *Avicennia germinans.* Goutam et al., (2016) also recorded *A.terreus* as endophytes from leaf, stem and root tissues of *Achyranthus aspera*. Aruna et al., (2017) reported the *Aspergillus* sp., like *A.ochraceus*, *A.niger*, *A.flavus* and *A.nidulans* from root, stem, leaves, flowers and fruits of *Calotropis gigantea*.

Aspergillus can be saprophytic, pathogen and even beneficial to plants, animals and humans. Some *Aspergillus* sp. (Palencia et al., 2010) has been known to cause disease in plants but it has been isolated as endophytes from many host plants (Paulussen et al., 2017). This may be due to latent pathogenecity of *Aspergillus* inside the host plants.

Yan et al., (2011) and Reddy et al., (2014) recovered *Chaetomium* sp. from *Cucumis sativus*, other Cucurbetaceous plants which corroborates our studies. *Chaetomium* sp. also occurred as endophytes in various other plants such as *Oryza sativa* (Naik et al., 2009), *Morinda pubescence* (Jena and Tayung, 2013), some medicinal plants of Western ghats (Nalini et al., 2014), *Ocimum sanctum* (Chowdhary and Kaushik, 2015), *Glycine max* (Fernandes et al., 2015), *Justicia adhatoda* (Fatima et al., 2016), *Adenium obesum* (Meenatchi et al., 2016) and *Eugenia jambolana* (Yadav et al., 2016) also found to possess *Chaetomium* sp. as endophytes.

Chaetomium globosum was reported as endophyte in plants like *Ginkgo biloba* (Zhang et al., 2013); *Adiantum capillus-veneris* (Selim et al., 2014); *Amaranthus viridis* (Piyasena et al., 2015); *Salvia miltiorrhiza* (Zhai et al., 2018) and *Litsea cubeba* (Wu et al., 2019). *Chaetomium* sp. also act as a bio control agent against various plant pathogenic fungi (Raguchander et al., 2014; Hung et al., 2015; Assad et al., 2017; Amatuzzi et al., 2018). Therefore, presence of *Chaetomium* sp. in various host plants help the plants resist against various plant diseases and also has the capability to act as plant growth stimulators (Atugala and Deshappriya, 2015).

There were no previous reports on *Melanospora. zamiae* as endophyte in *Cucumis* sp. Zida et al., (2014) reported *M.zamiae* as endophyte of Sorghum plant. They were isolated from leaves using PDA media. Sun et al., (2017) isolated *M.zamiae* from trunk of *Aquilaria crassna*. Only few reports were available on *M. zamiae* as endophytes.

No reports were available for *Nodulisporium gregarium* as endophyte of *Cucumis* sp. or any Cucurbitaceae members. Isabella et al., (2012) recorded *N.gregarium* from leaves of three mangrove plants (*Avicennia schaueriana, Laguncularia racemosa* and *Rhizophora mangle*) of Northeast Brazil. They were also reported as endophytes in plants like *Coffea arabica* (Oliveira et al., 2014) and some medicinal plants of Western Ghats regions of Goa (D'Souza and Hiremath, 2015).

There were no previous reports available for *Talaromyces radicus* as endophyte of *Cucumis* sp. or any Cucurbitaceae members. *Talaromyces radicus* as endophyte was reported by Palem et al., (2015) and Dhayanithy et al., (2019) from all parts of *Catharanthus roseus*. Similar results were obtained in the present study.

According to our knowledge, there is no previous report on *Purpureocillium lilacinum* as endophyte from *Cucumis* sp. They have been reported as endophyte from *Gossypium hirsutum* (Ek-Ramos et al., 2013), *Sophora tonkinensis* (Yao et al., 2016) and *Oroxylum indicum* (Das and Narzary, 2017). Many reports were available on *P.lilacinum* as entomopathogen and as biocontrol agent. Lopez et al., (2014) recorded their entomopathogenic activity against cotton aphids. Lan et al., (2017) evaluated biocontrol

potential *P.lilacinum* against *Verticillium dahliae* in Eggplant. Therefore, this fungus when reside inside the host plant as endophyte, can protect the host plant from various plant diseases with the help of some biological properties they possess.

Penicillium javanicum showed excellent growth in Potato Dextrose Agar. But there is no report on *P.javanicum* as endophyte from any *Cucumis* species or Cucurbitaceous plants. Many *Penicillium* sp. have been reported as endophytic fungi from various plant species like *Coffea arabica* (Vega et al., 2006); *Centella asiatica* (Devi et al., 2014); *Salvadora persica* and *Salvadora oleoides* (Korejo et al., 2014); *Senecio flavus* (Elkhayat and Goda, 2017); *Tabebuia argentea* (Murugan et al., 2017); *Artemisia* sp. (Cosoveanu and Cabrera, 2018) and *Terminalia* sp.(Toghueo and Boyom, 2019).

CONCLUSION

Endophytic fungi are one of the most important diverse groups of organisms that make association with plants. But only few plants have been studied for endophyte diversity. Plants may harbor different fungi as endophytes which has various biological activities. In the present study, *Cucumis dipsaceus* was analyzed for endophytic community. Twenty two endophytic fungi were isolated from surface sterilized leaf and stem samples. Colonization of endophytic fungi was recorded more in leaves than in stem. Isolated endophytes were identified as *Aspergillus* sp., *Aspergillus aculeatus*, *Aspergillus flavus* 1, *Aspergillus flavus* 2, *Aspergillus fumigatus* 1, *Aspergillus fumigatus* 2, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus terreus* 1, *Aspergillus terreus* 2, *Aspergillus terreus* 3, *Aspergillus ustus* 1, *Aspergillus ustus* 2, *Aspergillus ustus* 3, *Chaetomium* sp., *Chaetomium globosum*, *Melanospora zamiae*, *Nodulisporium gregarium*, *Penicillium javanicum*, *Talaromyces radicus* and *Purpureocillium lilacinum*. *Aspergillus* sp. was more dominant. All the isolated organisms were well known for its biological activity and medicinal properties. So the endophytic fungi isolated from *Cucumis dipsaceus* can be expected to have strong interaction with host plant and have the ability to produce promising bioactive metabolites.