Chapter II

INTRODUCTION

Endophytes, are usually bacterial and fungal communities that colonize the plant tissue, spend complete or part of their life cycle without causing any noticeable symptoms to the host (Petrini and Fisher, 1990). Many fossilized plant tissues like stems and leaves of land plants provided evidence for plant- microbe association, which suggested that endophyte-plant association have started along with the evolution of higher land plants (Krings et al., 2007).

Plant microbe interaction must overcome several physical and chemical barriers to establish successful association (Saikkonen et al. 1998). Their interactions can range from mutualism through commensalism to parasitism. Through commensalism and mutualism endophytes get benefits like energy, nutrients, shelter and protection from environmental stress, from the host plant. But the interactions between host plants and endophytes were poorly understood. Their interaction is affected by various factors like mode of transmission, pattern of infection, plant age, environmental conditions and genetic background (Schulz et al., 1999). Many years of research on plants also suggested that plants are mostly in symbiosis with mycorrhizal fungi or endophytic fungi (Kogel et al., 2006).

Endophytes can persist for certain period of time inside the host plants without causing symptoms. This includes pathogen during their period of latency, which is referred as the period between the time a pathogen invades and develops in the plant and the time the parasitic relations begin. When the traditional methodology is used for isolation of endophytes, only faster growing culturable fungi were obtained and it is highly suggested that some or numerous endophytes were never isolated. To overcome these problems, pathogenecity test (artificial inoculation of microbes in plants) was introduced to distinguish pathogen and endophytes (Guo et al., 2001). Those endophytes whose colonization and development never produced disease symptoms on the host are known as “true endophytes” (Sessa et al., 2018).

The artificial inoculation of test organisms in plants is essential for studies of various aspects of plant pathology including epidemiology, etiology, disease resistance, host-parasite interaction and disease control (Giri et al., 2013).

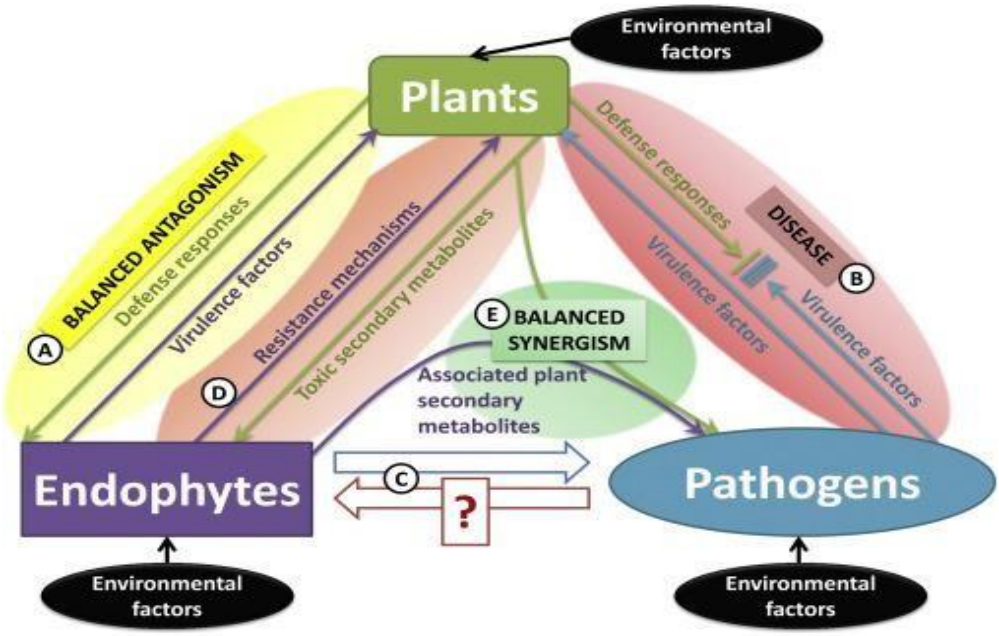
Plants act as a richest source of natural products to treat various ailments. Nearly 80% of people depend on herbal drugs for their primary healthcare. 50% of small molecule drugs used by people were based on natural products and rest is being synthetic. With the increase in demand for herbal drugs, natural products and secondary metabolites, the use of medicinal plants is growing rapidly throughout the world (Chen et al., 2016). Now-a-days some of the plant species were threatened due to over harvesting and natural anthropogenic habitat destruction.

Sometimes it is difficult to access plant bioactive compounds because of the low level of accumulation in native plant species, long growth period required for plant maturation and the difficulty in recovery of bioactive compounds. Thus, it is essential to find different approaches to produce the plant-derived biologically active compounds, in particular, those products derived from endangered or difficult-to-cultivate plant species, to meet the demand. This can be done by the applications like plant cell and tissue culture, heterologous production, total chemical synthesis, semi-synthesis or by microbially – produced or plant-extracted natural product occurring more abundantly in nature (Rai et al., 2016) or by exploiting the ability of endophytes residing in plants to produce the same or similar bioactive compounds as their hosts (Venieraki et al., 2017). The endophytic fungal diversity from asymptomatic tissues of *C.dipsaceus*, have been studied and the present study was undertaken to distinguish between true endophytes and latent pathogens.

REVIEW OF LITERATURE

Usually pathogens and mycorrhizae are the best known fungi associated with plants. Apart from these, there are numerous species of fungi inhabiting the intercellular spaces of plant tissues known as endophytes. They are present in almost all plant species. Transmission and surviving capacity of endophytes in plant tissues make them unique and show multidimensional interaction with the host plant. Therefore different symbiotic lifestyles occur in plants. Depending on the species involved, their interaction ranges from antagonism to mutualism (Saikkonen et al., 1998). Normally healthy plant parts were used for endophyte survey. Therefore, sometimes latent pathogens will be isolated if sampling is done before symptoms appear (Mostert et al., 2000). To date many research has been done to study interaction of endophytes with host plant similar to plant growth promoting microbes present in the rhizosphere.

Fig.3- Balanced antagonism between plant and endophytic fungi

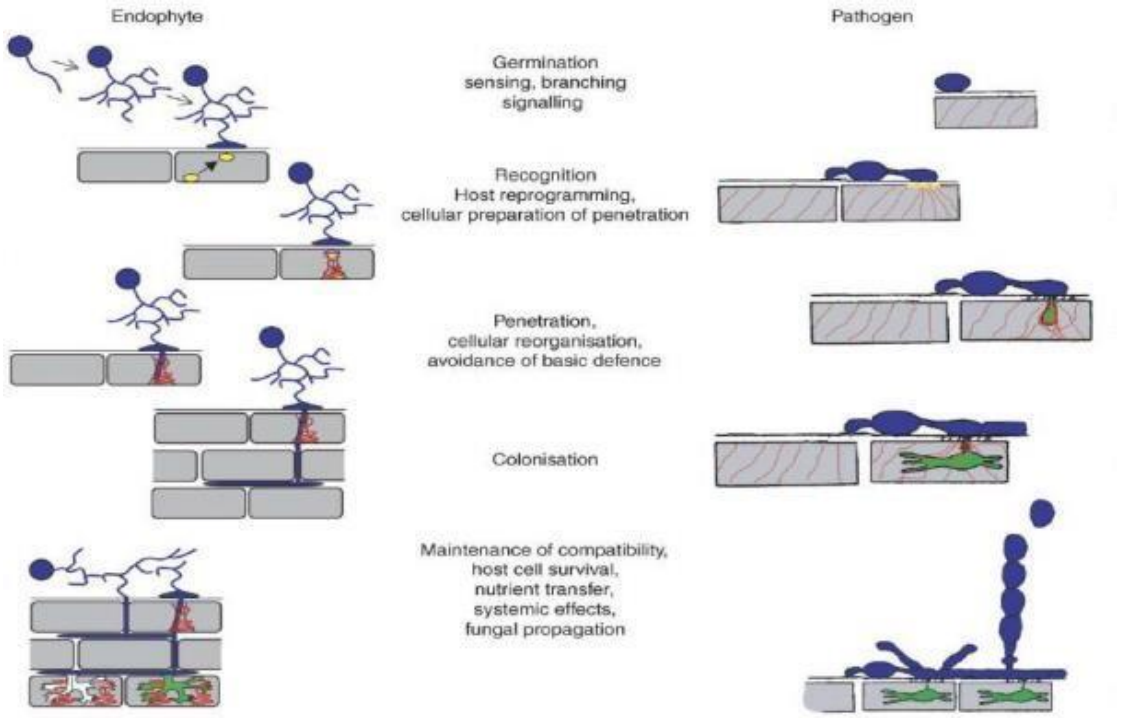


Source: Schulz and Boyle 2005

As long as endophytes show virulence and plant defense is balanced, their interaction will be asymptomatic. This type of colonization is known as balanced antagonism (Fig.3). When their interaction becomes unbalanced, it results in the formation of disease in the host plant. Their balance and imbalance status depends on the status of

the partners which are influenced by environmental factors, nutritional status and developmental stages of the partners. Endophytes and pathogens possess same structural similarities and same virulence factors. Both produce phytotoxic metabolites and exoenzymes which are necessary to infect and colonize the host (Kogel et al., 2006). Based on environmental conditions, the type of interaction between an endophyte and the plant is controlled by the genes of both partners (Moricca and Ragazzi, 2008).

Fig.- 4: **Symbiotic development of endophytes and pathogens**



Reproduction and transmission of endophytes

Reproduction and transmission process of endophytes were often used to specify their spread within and among the host plants. But they are obviously different processes. The reproductive and transmission mode of the endophytes depend on the life history of the host, growth pattern and maturity of the plant. Reproduction specifies the sexual and asexual process in endophytes. Transmission process explains mechanism by which fungal infection is distributed to host plant (Fig.4).

Through vegetative growth

The endophytic fungi grow completely inside the host plant tissues without producing any external structures or fruiting bodies on the host. This type of reproduction is completely internal and occurs through vegetative growth of hyphae by entering into the ovules of the host. Through this entry they infect seeds and transmitted to next generation and the transmission is known as vertical transmission. But the viability of hyphae is less than the viability of seeds (Clay, 1986).

Through spores

In contrast to vertical transmission, fungus may produce spores to promote horizontal transmission. But the ecological and evolutionary life history of endophytes can be understood, if we identify whether endophytes reproduce by mitotic asexual or meiotic sexual spores. Asexual reproduction of endophytes takes place through vertical transmission (seeds) and horizontal transmission (spores or hyphae), whereas sexual reproduction takes place through sexual spores and it is always horizontal. These spores were produced on leaves or inflorescences of the host plant. Spores germinate on stigma and develop through style to infect ovules (Clay K, 1990). Sexual reproduction of some fungal species produces relatively higher genotypic diversity in populations of fungal endophytes.

Mimicking of endophytes

In contrast to pathogenic fungi, endophytes develop a mutualistic relationship with the host plant. Isolated endophytes, under submerged culture condition have the ability to produce many bioactive secondary compounds. These compounds were same as those produced by the respective host plants. This condition makes the expectation that endophytic fungi can serve as an alternative source of important plant secondary metabolites. This possibility was further proved by discovery of taxol (paclitaxel) from endophyte *Taxomyces andreanae* that produces the same bioactive secondary metabolite as its host *Taxus brevifolia* in 1993 (Stierle et al.,), several studies explained that plant-derived secondary metabolites are produced by endophytes (Zhao et al., 2011). Some important examples are as follows,

***Salvia* sp. (Lamiaceae)**

Salvia species possess many important medicinal properties with high pharmacological potential, due to the presence of polyphenols or terpenoids (Wu et al., 2012). Important compounds like tanshinones, salvianolic acids, rosemarinic acid, carnosic acid, carnosol and salvinorin A were produced by different species of *Salvia* like *S. miltiorrhiza* and *S.divinorum*. These compounds were considered as potent source of anti-carcinogenic, antiatherosclerosis, antihypertensive, cardiovascular drugs and cerebrovascular drugs and as food additives (Wu et al., 2012 ; Chun-Yan et al., 2015). Endophytic fungi isolated from *Salvia* species showed the capacity to produce tanshinones and salvianolic acids under submerged culture condition. However, the yield was quite low (Li et al., 2016).

***Catharanthus roseus* (L.) G.Don (Apocynaceae)**

Catharanthus roseus is a well known medicinal plant which has the capability to produce potential anticancer compounds like Vincristine, Vindesine, Vinorelbine and Vinblastin (Kumar et al., 2014). *C.roseus* possesses wide range of endophytic fungi (Palem et al., 2015). Among them *Fusarium oxysporum, Talaromyces radicus* and *Eutypella* spp. were capable of producing Vinblastine and Vincristine (Kuriakose et al., 2016).

***Coleus forskohlii* (Willd.) Briq. (Lamiaceae)**

Coleus forskohlii is widely cultivated in Southern India. Their roots were used in Indian folk medicine to treat various human ailments (Kavitha et al., 2010). They produce important bioactive compound like forskolin. Their potential application includes anti-HIV or antitumor activities, hypertension, heart failure and body weight control (Pateraki et al., 2014). Mir et al., (2015) isolated *Rhizoctonia bataticola* an endophytic fungi from inner tissues of stem and leaves of *C. forskohlii* and was found to synthesize forskolin under submerged culture condition.

***Macleaya cordata* (Willd.) R.Br. (Papaveraceae)**

Macleaya cordata possesses an important alkaloid compound Sanguinarine (SA). It has antibacterial, antihelmintic, antitumor, antiinflamatory properties and used as feed additives (Kantas et al., 2014). Fifty five endophytic fungi were obtained from leaves of *M.cordata* and they have the capacity to produce sanguinarine (SA) (Wang et al., 2014).

***Cajanus cajan* (L.) Millsp. (Fabaceae)**

Cajanus cajan (pigeon pea) leaves exhibit various therapeutic effects on sickle cell anemia, plasmodiosis and hepatic disorders. Cajaninstilbene acid and cajanol are a major bioactive compound with pharamacological properties like anti-inflamatory, analgesic, antiplasmodial and antioxidant activity (Liang et al., 2013). Totally two hundred and forty five endophytic fungi isolated from leaves, stem and roots of pigeon pea were screened for the production of cajaninstilbene acid or cajanol. Three endophytic fungi isolated from leaves were capable of producing Cajaninstilbene acid and one strain isolated from roots stably produced cajanol (Zhao et al., 2013).

***Cephalotaxus hainanensis* H.L.Li (Cephalotaxaceae)**

Cephalotaxus hainanensis is native to china. Their bark and leaves were used in Chinese folk medicine, to treat cancer and acute myeloid leukemia. Homoharringtonine (HHT) an alkaloid produced from this plant help to achieve this. Two hundred and thirteen endophytic fungal strains were isolated from bark of *Cephalotaxus hainanensis*. Among them *Alternaria tenuissima* was reported to be capable of producing homoharringtonine (Hu et al., 2016).

***Cinchona* spp. (Rubiaceae)**

Until the discovery of synthetic antimalarial compounds, quinine extracted from stem and roots of *Cinchona* was used as a strong natural antimalarial compound (Kaufman and Ruveda, 2005). Twenty one endophytic fungi were isolated from *Cinchona ledgeriana.* Among them *Phomopsis*, *Diaporthe*, *Schizophyllum*, *Penicillium*, *Fomitopsis* and *Arthrinium* species produced quinine (Maehara et al., 2011 and 2013).

***Passiflora incarnata* (Passifloraceae)**

Leaves of *P.incarnata* contain compounds like alkaloids, phenols, flavonoids (chrysin) and cyanogenic compounds which possess antibacterial, anti-inflammatory, anti-diabetic, anxiolytic, hepatoprotective, anti-aging, anticonvulsant and anticancer properties. Endophytic fungi namely, *Altenaria alternata*, *Colletotrichum capsici*, and *C. taiwanense* isolated from leaves of *P. incarnata* have the ability to produce chrysin (Seetharaman et al., 2017).

***Fritillaria cirrhosa* D.Don (Liliaceae)**

Fritillaria bulbs were used in Chinese medicine for antitussive activity. They have major biological active products like peimisine, imperialine-3β-D-glucoside and peimine (Wang et al., 2011). Several endophytic fungi have been isolated from bulbs of *Fritillaria*, among which *Fusarium redolens* secrete peimisine and imperialine- 3β-D-glucoside under submerged culture condition (Pan et al., 2017).

***Huperzia serrata* (Thunb. ex Murray) Trevis (Huperziaceae)**

Huperzia serrata is a traditional medicinal plant in China, used to cure number of ailments like contusions, strains, swellings, schizophrenia, myasthenia gavis and organophosphate poisoning. Their active compound comes under the class alkaloids (Huperzine A (HupA)) which helps to treat Alzheimer's disease (Zhao et al., 2013). Different groups of endophytic fungi have been isolated from different parts of *H. serrata.* The HupA-producing endophytic fungi were identified as *Penicillium griseofulvum*, *Penicillium* sp., *Aspergillus flavus, Mycoleptodiscus terrestris, Trichoderma* sp. and *Colletotrichum gloeosporioides* strain ES026 and *Shiraia* sp. (Su et al., 2017).

***Rhodiola* spp. (Crassulaceae)**

Rhodiola rosea is a perennial herb found in arctic and mountainous regions of Asia and Europe. It is used as an important food crop and folk medicine in countries like Sweden, Russia, India and China. It possess many biological properties like antioxidant, anti-aging, anti-microwave radiation, antihypoxia and also stimulates mental and physical endurance, counteracts depression, improves sleep quality and prevents high-altitude sickness. This type of biological behavior takes place by phenolic substance like salidrosides, p-tyrosol and glycosides like rosavins (Chiang et al., 2015). Different species of *Rhodiola* like *R. crenulata*, *R. angusta* and *R. sachalinensis* were screened for endophytic fungi. One of the endophytic fungi identified as *Phialocephala fortinii* was able to produce large amounts of salidrosides and p-tyrosol (Cui et al., 2016).

***Solanum nigrum* L. (Solanaceae)**

Solanum nigrum L., possesses biological behaviours like antioxidant, hepato-protective, antiinflammatory, antipyretic, diuretic, antimicrobial and anticancer properties. This is due to flavonoid and steroidal alkaloids (Jain et al., 2011). Solamargine, is the major

steroidal alkaloids which is always mixed with other alkaloids such as solasonine and solanine. Therefore it is very difficult to isolate solamargine (Milner et al., 2011). Synthesis of these chemicals is a tedious process. But the endophytic fungus *Aspergillus flavus* isolated from stem, leaves and fruits of *S.nigrum* was able to produce solamargine (El-Hawary et al., 2016).

***Piper* species (Piperaceae)**

Piperine is a major compound found in *Piper* sp. with pharmaceutical properties like antibacterial, antifungal, hepato-protective, antipyretic, anti-inflammatory, anti-convulsant, insecticidal and antioxidant. The amount of Piperine varies within species of this family. Endophytic strains like *Periconia* and *Colletotrichum gloeosporioides* isolated from *P.longum* and *P.nigrum* were reported to produce piperine under submerged culture condition (Verma et al., 2011; Chithra et al., 2014).

***Digitalis lanata* Ehrh. (Plantaginaceae)**

Genus *Digitalis* possess glycosides which is a well known cardiotonic widely used for treatments like atrial fibrillation, atrial flutter and heart failure (Alonso et al., 2009). Total of 35 fungal endophytes were isolated from stem and leaves of *Digitalis lanata* and screened for production of glycoside digoxin. Crude extracts of endophytes revealed the presence of digoxin (Kaul et al., 2013).

***Capsicum annuum* L. (Solanaceae)**

Capsaicin, an important pungent alkaloid present in fleshy parts of fruits of *Capsicum annuum* possess pharmacological properties like cardio protective influence, anti-lithogenic effect, anti-inflamatory and analgesia, thermogenic influence and beneficial effects on gastrointestinal system. An endophytic fungi namely *Alternaria alternata* isolated from fruits of *C.annuum* has been found to secrete capsaicin (Devari et al., 2014).

***Ginkgo biloba* L. (Ginkgoaceae)**

Flavones and terpenoide lactones found in bark and leaves of *Ginkgo* tree were beneficial to human health. It has been discovered as potent antagonistic, used to treat number of renal cardiovascular, respiratory and central nervous system disorders. *Fusarium oxysporum* (Cui et al., 2012) and *Pestalotiopsis uvicola* (Qian et al., 2016) isolated from bark of *G.biloba* was capable of producing terpenoid lactones.

***Silybum marianum* (L.) Gaertn. (Asteraceae)**

Silybum marianum possess silymarin and flavolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin and silydianin) which are important bioactive extract with chemoprotective and hepatoprotective properties (Feher and Lengyel, 2012). Twenty one endophytic fungi were isolated from stems, leaves, roots, and seeds of *S. marianum.* Among them, *Aspergillus iizukae* isolated from the leaves and stems were found to produce flavolignans (El-Elimat et al., 2014).

***Vinca minor* L. and *Nerium indicum* Mill. (Apocynaceae)**

Members of Apocynaceae family possess vincamine indole alkaloids (vincamine, tabersonine and catharanthine) with beneficial properties like prevention of cerebrovascular disorders, precaution of chronic ischemic stroke and reduction of vascular dementia or memory impairment (Saurabh and Kishor, 2013). This compound is mostly accumulated in leaves and stems of *V.minor* and *N.indicum*. Endophytic strains isolated from *N.indicum* and *V.minor* were screened for vincamine alkaloids by TLC, HPLC and LC-MS analysis. Results suggested that Vincamine, Ethyl-vincamine and Tabersonine was 1.200mg/L, 1.279mg/L, 0.102mg/L respectively (Yin and Sun, 2011; Na et al., 2016).

***Rheum palmatum* L. (Polygonaceae)**

Air dried roots of *Rheum palmatum* have been used in traditional medicine to treat cathartic effect on the digestive movement of the colon and protects liver damage with the help of biologically active compounds like anthraquinones including emodin, rhein, physcion, aloe-emodin. Many pharmacological researches revealed that compound rhein can lighten pain, fever and inhibit inflammation (You et al., 2013). Of the fourteen endophytic fungal strains isolated from root and stem tissues of *Rheum palmatum*. Strain R13 was found to have the capacity to produce bioactive compound rhein and emodin.

***Forsythia suspensa* (Thunb.) Vahl. (Oleaceae)**

Forsythia suspensa possesses important chemical constituents like phillyrin, forsythiaside, oleanolic acid and ursolicacid. Among these phillyrin was reported to have some important biological activities such as antioxidant, anti-inflammatory, antihyperlipidemia and antipyretic (Qu et al., 2008). Important compounds were obtained from leaves and fruits of

F.suspensa. Zhang et al., (2012) isolated 12 fungal strains from stems, leaves and fruits of *F.suspensa* and *Colletotrichum gloeosporioides* isolated from fruit was found to produce phillyrin.

***Miquelia dentata* Bedd. (Icacinaceae),**

Camptothecine (CPT) and its analog, 10-hydroxy camptothecine (10-OH-CPT) are potent inhibitors of eukaryotic topoisomerase I and are currently used as efficient anticancer drug against small lung and refractory ovarian cancers (Kai et al., 2015). But these compounds were naturally produced by several plant species like *Camptotheca acuminate* and *Nothapodytes nimmoniana* (Shaanker et al., 2008) which is used as source of commercial CPT. Fortunately, high levels of CPT and 10-OH-CPT were also found in fruits and seeds of *Miquelia dentate* (Ramesha et al., 2013). Shweta et al., (2013) isolated 23 fungal isolates from fruits of *M.dentate* and found that all isolates have the capability to produce CPT in varying quantities. Among them *A. alternata*, *Phomopsis* sp. and *Fomitopsis* sp., were identified as CPT-producers with the highest yield.

***Cucumis* sp. (Cucurbitaceae)**

Cucurbitaceae family includes some medicinal and nutritionally important crops like cucumber and melon. Among them *Cucumis* sp., were widely used as traditional herbal medicine, with antiinflammatory, antitumor, hepatoprotective, cardiovascular and for immunoregulatory activities (Shweta et al., 2003). This is achieved by various biological compounds like Cucurbitacin, triterpenes, sterols and alkaloids. Cucurbitacins are high triterpenoids which gives bitter taste to cucurbits (Alghasham, 2013). Cucurbitacins and their derived compounds were largely isolated from *Cucumis* sp. Cucurbitacins have the capacity to reduce proliferation of cancer cells, head and neck squamous cell carcinoma, pancreatic cancer, hepatocellular carcinoma etc., (Guo et al., 2014). These compounds can be obtained either from natural sources or prepared. But the detection of cucurbitacins in endophytes was still not explored which would help to understand the interaction between *Cucumis* sp., and endophytes.

Many examples were available to explain that endophytic fungi have the capacity to produce secondary metabolites like their host plant. But sometimes this expectation is unfulfilled, because continuous subculturing of endophytes makes them lose the production

of secondary metabolites,though the reason for such loss is not studied. It could be due to silencing of genes in axenic cultures. Some studies suggest that genetic recombination of endophytes with host could lead to the inclusion of secondary metabolite producing genes of host into endophytes. But the mechanism behind this process is still unknown (Tan and Zou, 2001; Sachin et al., 2013).

Role of endophytes on host plants

Endophytes affect plants in various ways. But their potential function is not yet clear. Most of the research on endophytes suggests that they are beneficial to their host by promoting plant growth through various mechanisms. They can promote growth of the host plant by enhancing plant resistance to biotic and abiotic stresses. Apart from that, they are also capable of nitrogen (N) fixation, solubilization of phosphate, enhance uptake of phosphorus (P), production of siderophores, ACC deaminase, and plant hormones such as auxin, abscisins, ethylene, gibberellins, and indole acetic acid (IAA), which are important for plant growth development and regulation (Malinowski and Belesky, 2000) .

Role of endophyte in plant growth and nutrient uptake

The mechanism behind nutrient uptake by plants colonized by endophytes is still in debate. The extramatrical mycelium extending from the host roots increase the surface area and therefore increase host's access to soil nutrients. They can promote plant growth either directly (Biological nitrogen fixation, phosphate solubilization, Siderophores synthesis and Phytohormone (IAA) production) or indirectly. Endophyte like *Aspergillus* species had the highest phosphatase activity which increase phosphate solubilization and nutrient uptake resulting in increased biomass of plants. Similar to arbuscular mycorrhizae, *Aspergillus* and *Penicillium* secrete organic acids which increase the availability of inorganic phosphate. They can also increase plant yield by producing phosphatase enzyme. Some important agronomical grasses like rye (*Lolium* sp.) and fescue (*Festuca* sp.) were colonized by fungal endophytes which benefit host plants by increasing tolerance to extreme temperature, nutrient deficiency and drought. They also produce alkaloids that protect hosts against various diseases and insect pathogens (Barrow and Osuna, 2002).

Role of Endophytes in Production of Phytohormones

Endophytes enhance plant growth and biomass by producing plant growth hormones without disturbing nutrient uptake and metabolic processes. Rommert et al. (2002) reported that endophyte culture extracts has the capability to enhance plant growth under *in vitro* as well as *in vivo* conditions with the help of soluble agents in the culture extracts.

Role of Endophytes in Hosts Tolerance to Stress

Endophytes help the host plants to tolerate and survive in drought, salts and extreme temperatures. Redman et al., (2002) studied the surviving capacity of *Dichanthelium lanuginosum* at high soil temperature (57˚C). Presence of endophytic fungus *Curvularia* sp., helped to increase the plant fitness at high temperature and under water stress condition than the endophyte free plant.

***In vitro* assessment of true endophytes**

In vitro assessment of true endophytes can be done using Koch's postulates theory. According to that, pathogenic fungal organism must cause disease when inoculated into a healthy and susceptible laboratory model. To find true endophytes, pathogenicity concept was used, where isolated organisms were reinoculated into host plant to distinguish between latent pathogens and endophytes.

Photita et al., (2001) isolated 61 endophytic fungal taxa from *Musa acuminata* and compared with previous studies on pathogenic fungi from tropical hosts and concluded that endophytes isolated were established as pathogens of banana and supported the hypothesis that some endophytes are latent pathogens.

Photita et al., (2004) isolated endophytic fungi from wild banana (*Musa acuminata*) and identified as *Cladosporium musae, Colletotrichum gloeosporioides, Cordana musae, Deightoniella torulosa, Guignardia cocoicola, Periconiella musae* and *Pestalotiopsis* sp. Isolated endophytes were reinoculated using wounding method on healthy banana leaves under *in vitro* conditions to test their pathogenicity. Among them *Deightoniella torulosa* was able to cause leaf spot disease which indicated that they may be latent pathogen.

Soumya et al., (2018) isolated *Acremonium bolchii, Acremonium strictum, Aspergillus flavus, A. fumigatus, A.glaucus, A.japonicus, Chaetomium globosum, Curvularia lunata, Eurotium repens, Oidium* sp., *Penicillium digitatum, P.restrictum* and one Sterile

mycelia as endophytic fungi from *Eichhornia crassipes*. To find true endophytes, isolated endophytic fungi were reinoculated into leaves of *E.crassipes* by wounding method. Among them, *A. flavus*, *A. fumigatus*, *A. glaucus*, *Curvularia lunata*, *Eurotium repens*, *Oidium* sp., *Chaetomium globosum* and sterile mycelia showed disease symptoms on leaves which is otherwise denoted as latent pathogens.

Romero et al., (2001) isolated *Alternaria zinniae*, *A. helianthi*, *A. alternate*, *Cylindrocarpon* sp., *Curvularia brachyspora*, *Fusarium* sp., *Nigrospora oryzae*, *Penicillium funiculosum* and *Periconia* sp. as endophytes from *Parthenium hysterophorus*. Seier and Romero (1997) isolated pathogenic fungi from leaves of *Parthenium hysterophorus* and found that *Cylindrocarpon* sp., *Penicillium funiculosum*, *Periconia* sp, *A. helianthi* and *A. alternate* had the ability to act as latent pathogens.

Sessa et al., (2018) explored fungal diversity from healthy twigs of apple, pear, peach and blueberry trees and aimed to discriminate between endophytic fungi and latent pathogens. About 843 isolates were obtained from bark tissues of all tree species, of which *Diaporthe brasiliensis*, *D. foeniculina*, *D.inconspicua*, *D. terebinthifolii*, *Diaporthe* sp.1, *Cytospora*-like isolates and *Pestalotiopsis* spp. did not produce any disease symptoms on inoculated shoots and considered as true endophytes. In the meantime isolates of *Diaporthe* namely *D. oxe*, *Diaporthe* sp.2, *D. infecunda* and *D. serafiniae*, *Botryosphaeria dothidea*, *Neofusicoccum parvum* and *N. austral* were found to be latent pathogens because they produced sunken cankers and necrosis on inoculated shoots. Results suggested that healthy shoots can host more number of endophytes as well as disease causing fungi.

Gorzynska et al., (2019) isolated 12 different endophytic fungi from *Carex secalina*, and found no visible symptoms on the leaves. But the isolated organisms were reported as pathogenic fungi in different species. Among them, *Colletotrichum* species has the ability to cause anthracnose disease which is recently reported as eighth most important group of plant pathogenic fungi in the world. Therefore, authors considered that isolated endophytes have the chance to act as latent pathogens.

Interaction between endophytes and plant pathogenic fungi

Endophytic fungi have the capability to produce antibiotic substances under submerged fermentation condition which is used to inhibit several plant pathogenic fungi.

Such type of compounds under *in planta* condition could constitute defense mechanism against plant pathogens. It is also achieved by direct inoculation of endophytic fungi in host plant (Kim et al., 2007). For example, in wheat, endophytes *Chaetomium* and *Phoma* were reinoculated as well as their culture filtrate was applied. As a result they reduced the foliar disease caused by *Puccinia* and *Pyrenophora* spp (Istifadah and McGee, 2006).

Arnold et al., (2003) reported that six different species of endophytes were isolated from *Theobroma cacao* L and reinoculated into endophyte free seedling of the host, which significantly reduced the severity of leaf disease caused by *Phytophthora* sp., when compared to uninoculated plants. Endophytes help plants to survive against pathogens which could be the result of direct competition between endophytes or they may produce zone of inhibition against the entry of pathogenic fungi.

Inoculation of endophytes isolated from another host plant sometimes alters the plant biochemistry and protect the plant against pathogens. Waller et al., (2005) inoculated *Piriformospora indica* into monocotyledons barley and dicotyledon like *Arabidopsis* and found that, it not only increased yield but also provided salt stress tolerance.

Multiple species of fungus may penetrate and infect plants. But only small units act as pathogens and produce diseases. Such type of plant disease cycle is shared by pathogens or endophytes. Any fungus after entering into plant may act either as endophyte or pathogens but their infection process is different. It depends on characteristics of fungus and host plant.

Inoculation methods for fungi on plants

Tefera and Vidal (2008) compared the effect of different inoculation methods for *Beauveria bassiana* on Sorghum, like soaking of seeds in culture, through soil and spraying on leaves and growing plants in sterilized and unsterilized soils. They tested the endophytic colonization of sorghum by the entomopathogenic fungus *Beauveria bassiana*. They recorded the results 20 days after the application of fungus. Seed inoculation does not show colonization in stem and leaves under unsterilized soil condition. Leaf inoculation method was considered as best to introduce *Beauveria bassiana* into sorghum either through sterile or nonsterile soil.

Giri et al., (2013) used five different types of artificial inoculation methods like spraying, infiltration, wounding, spore suspension drop and spore suspension drop along with agarose method for studying various aspects of plant pathology. He studied the

pathogenesis of *Alternaria brassicae* on *Brassica juncea*. (Indian mustard) and suggested that spore suspension drop along with agarose method was most useful to fix inoculum on target site and it will help to handle the inoculated plants easily.

Wijesooriya and Deshappriya (2016) isolated 27 endophytic fungal isolates from Kuruluthuda rice variety and tested those isolates on plant growth and yield, using spore suspension and plate method under green house and field condition. All endophyte inoculated plants showed considerable difference in plant growth, number of tillers and yield when compared with non inoculated plants.

MATERIALS AND METHODS

Cultures of 22 endophytic fungi isolated from *Cucumis dipsaceus* were maintained at 25±2˚C on potato dextrose agar. The isolates were inoculated into the host under different conditions to assess whether the organisms are true endophytes or latent pathogens.

Assessment of True endophytes through pathogenecity test

Preparation of inoculum (Tefera and Pringle, 2004)

Conidial suspension was prepared from 3 week old sporulating endophytic cultures. It was prepared by scraping mycelia and spores from actively growing endophytic fungal cultures. Scraped mycelia and spores were collected in 50ml of sterile distilled water and filter the suspension through four layers of cheese cloth to remove most of the mycelia. Filtered spore suspension was centrifuged at 2000xg for 5 minutes and resuspended in deionized water. Centrifugation was repeated in order to obtain clear spore suspension. After final wash, supernatant was discarded and spores were resuspended in distilled water containing 0.05% tween-20. Spores in the suspension were counted using haemocytometer and the concentration was adjusted to 10^4 spores ml^{-1}. To check the viability of conidia, germination test was carried out using PDA media and cultures were incubated at 25±2˚C for 4 days.

Seed source

Mature and dried fruits of *Cucumis dipsaceus* were collected from foothills of Maruthamalai, Coimbatore. Seeds were surface sterilized using 2 to 4% sodium hypochlorite solution followed by 75% ethanol for 5 minutes. Then the seeds were rinsed three times with sterile distilled water. Treated seeds were placed on sterile filter paper to dry for 30 minutes (Tefera and Vidal, 2009).

Preparation of Soil

Required quantity of soil and sand were mixed in equal proportion and divided into two parts. One part was treated as non sterile and another part was sterilized. Separate experiments were conducted using sterile and non sterile soils. Within each experiment, a completely randomized block design with six replicates per treatment (inoculation method) was used.

Experimental Structure

No. of Species : 1 (*Cucumis dipsaceus*)

No. of growth medium : 2 (Sterile and Non-sterile soil)

No. of treatments : 22 (isolates)

No. of Inoculation methods : 3 (Soil, Leaves and seed)

No. of replicates/ treatment : 6

Inoculation of endophytes into the host (Wijesooriya and Deshappriya, 2016)

Isolated endophytes were inoculated into *Cucumis dipsaceus* through three different ways namely soil drenching, seed and leaf spraying on plants grown using different media like sterilized (autoclaved at 121˚C for 15 minutes) and unsterilized soils.

For seed inoculation, 50 g of seeds were soaked for 16 hours in spore suspension or with distilled water for non-inoculated controls. Seeds were placed in 100ml beakers and 25ml of standard volume of inoculum was stirred to form a homogenous mixture. Seeds were then grown on sterile and non-sterile soil conditions.

Leaf inoculation was done by spraying 3ml of conidial suspension, on the leaves of seven days old seedlings, using a hand sprayer. The spray was directed to the leaves but might incidentally drift to the stems. To avoid conidial runoff to the soil, the top of each pot was covered with aluminum foil. The control plants were inoculated with sterile distilled water. For soil inoculation, a 3ml conidial suspension was applied around the root zone of each seedling. The control plants were inoculated with 3ml sterile distilled water. Seedlings in all treatments were watered daily.

Observations

The plants were maintained under green house condition and watered regularly. The effect of endophytic fungus on plant growth was determined by measuring parameters like plant height, fresh and dry weight of shoot and root and leaf number.

Plant height

Plant height was measured from surface of the soil to the tip of the stem using a scale and measurements were expressed in centimeter.

Leaf number

Fully opened leaves of each seedling were counted.

Fresh weight

The seedlings were uprooted and the whole plant was weighed and the fresh weight was expressed in grams /seedling.

Dry weight

The uprooted plants were dried at 70˚C for 48 hours and dry weight was recorded when the constant weight obtained and expressed in grams /seedling.

Statistical analysis

The growth parameters were analyzed using ANOVA and the means were separated by Duncan Multiple Range Test (DMRT) to determine the effects due to treatments. The data were analyzed using SPSS (2.0) software.

Assessment of Plant-endophyte interaction using biochemical method

HPLC analysis of Cucurbitacin B (Sturm and Stuppner, 2000)

Preparation of standard solution (Chanda et al., 2019)

A standard stock solution of Cucurbitacin B was prepared by dissolving 1 mg of cucurbitacin B in 1 mL Ethyl acetate. Further dilution was carried out to prepare calibration samples in the concentration range of 1–100 µg/mL. The standard solution, was filtered through 0.45 µL syringe filter prior to injection.

Preparation of plant sample

Young leaves of *C.dipsaceus* were harvested and extracted in petroleum ether and the residue was obtained using filtration. The residue was extracted with ethyl acetate and concentrated using reduced pressure. The dried extracts were redissolved in small quantity of ethyl acetate.

Analysis of Cucurbitacin B production by endophytic fungi (Liu and liu, 2018)

Preparation of Czapek's Dox Broth (g/L)

Sucrose	- 30
Sodium nitrite	- 2.0
Dipotassium hydrogen phosphate	- 1.0
Magnesium sulphate	- 0.5
Potassium chloride	- 0.5
Ferrous sulphate	- 0.01
Distilled water	- 1000ml

All the above chemicals were suspended in 1000ml of distilled water and pH was adjusted to 7.3. CDB was sterilized at 121˚C for 20 minutes. Production of culture filtrate was tested for the production of cucurbitacin B by growing them in Erlenmeyer flask (1L) containing 500ml of Czapeks dox broth. After incubation at room temperature for 15 days fermentation products (mycelia and culture filtrate) were separated using muslin cloth. The culture filtrate was extracted thrice with equal volume of ethyl acetate (1:1) and the organic phase was collected and concentrated to obtain crude extract. The crude extract was redissolved in small quantity of ethyl acetate and used for further process.

Analytical conditions

The presence of Cucurbitacin B in *C.dipsaceus* was determined using HPLC (Shimadzu SP 20A). Separation was carried out on C18 column and optimal separation was obtained with the mobile phases water (A) and water-acetonitrile (20:80 v/v)(B). The temperature of the column was kept at 40 °C and the injection volume was 20 μL. The total run time was set for 10 min. The flow rate of mobile phase was set at 1.0 mL/min and the maximum absorption was recorded at 230 nm. Standard, plant extract and each sample were chromatographed twice and the data were reported.

Molecular identification of endophytic fungi

Endophytic fungi that produced less disease symptoms on *C.dipsaceus* and those organisms that enhanced the growth of the plants, were selected for DNA sequencing. The cultures were subjected to study the phylogeny by molecular technique. The identity

of the organisms was confirmed by the molecular studies. Selected endophytic fungal cultures were inoculated into CDB and incubated for 15 days. Cultures were filtered to obtain the fungal biomass. Fresh fungal mycelium was used for genomic DNA isolation.

Isolation of genomic DNA

Preparation of extraction buffer

EDTA - 10 mM (pH 8.0)

Tris HCl - 0.1M (pH 8.0)

NaCl - 2.5 M

CTAB - 3.5%

Proteinase K - 150µl (20mg/ml)

200mg of fresh fungal mycelia was transferred to 1.5ml of sterilized Eppendrof tube and 800µl of extraction buffer was added with mixed sterilized 0.5-1mm glass beads. The mixture was vortexed for 5 minutes using homogenizer. The samples were placed in water bath at 65˚C for 30 minutes and then centrifuged at 10000rpm for 10 minutes at room temperature. To the collected supernatant equal volume of chloroform: isoamylalcohol (24:1) was mixed and centrifuged again at 10000rpm. Supernatant was collected and equal volume of ice cold isopropanol was added. Samples were incubated at -20˚C for 1-2 hours. The samples were then centrifuged at 13000rpm for 15minutes to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800µl of 70% ethanol and air dried. Dried DNA pellets were dissolved in 200µl of TE buffer (10mM Tris-HCl, pH-8.0, 1mM EDTA). 5µl of RNAse A (20mg/ml) was added to DNA sample, mixed and incubate at 37˚C for 1 hour. DNA was air dried and stored in TE buffer for further PCR amplification (Mishra et al., 2014).

Agarose gel electrophoresis

Preparation of 1X TBE buffer

10.8 g Tris and 5.5 g Boric acid were dissolved in 900 mL distilled water and 4 mL 0.5 M EDTA (pH 8.0) was added. Volume was adjusted to 1 L and stored at room temperature.

Quality of isolated DNA was assessed using 0.8% (w/v) agarose gel. The gels were placed in electrophoresis tank containing 1X TBE buffer and DNA samples were loaded along with 5X Bromo phenol blue and the gel was run in 50V until the dye reaches the bottom of the gel. Quality of DNA was visualized using gel documentation system.

PCR amplification

PCR amplification was carried out using specific primers ITS1 and ITS4 which possess the following sequence: ITS1(5′-TCCGTAGGTGAACCTGCG G-3′) and ITS4 (5′-TCC TCC GCTTAT TGATAT GC-3′). PCR amplification was carried out using 20µl of reaction mixture containing 1µl of genomic DNA, 2µl of each primer, 0.5µl *Taq* DNA polymerase, 2µl of 10X *Taq* DNA polymerase buffer, 2µl of 10mM dNTPs and 12.5µl of Millipore water. The reaction was performed using PCR with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45sec, annealing at 55.5°C for 60sec and extension at 72°C for 60sec with a final extension at 72°C for 10 min. The amplified product was analyzed using 2% (w/v) agarose gel, purified and was further used for DNA sequencing.

Sequencing protocol for fungi

Single-pass sequencing was performed on each template using 18S rRNA ITS (fungi) universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to capillary electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). Sequence data was aligned and analysed for identifying the sample.

Sequence analysis

A comparison of the 18S rRNA gene sequences with the sequence available in the GenBank of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search tool (BLAST) was done to obtain the best homogenous sequences. Sequence from different endophytic fungi were aligned and compared with sequences retrieved from NCBI using multiple sequence alignment software (Clustal W). The aligned data was used for further phylogenetic analysis using Neighbor joining method and all the sequence were deposited in NCBI gene bank.

EXPERIMENTAL RESULTS

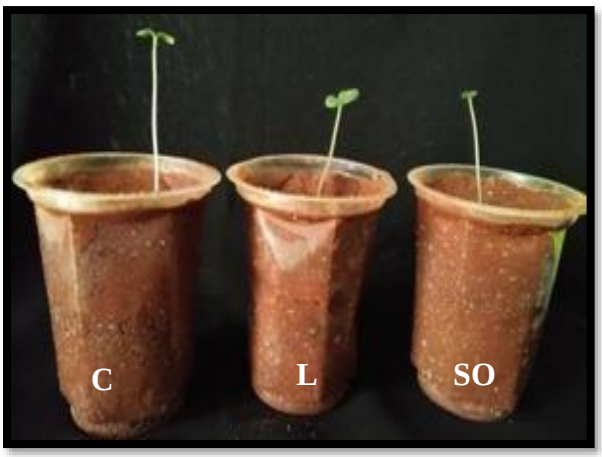
Concepts of true endophytes or latent pathogens were developed based on the relationship of endophytes with the host plant. The relationship varies based on the environmental condition of the host plant, age of the plant, soil condition etc., Therefore, to distinguish between true endophytes and latent pathogens following studies were undertaken.

Assessment of the status of fungi through pathogenecity test

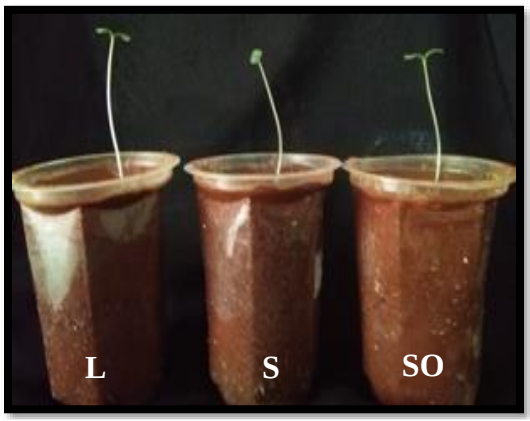
The effect of all twenty two endophytic fungi on growth of *C.dipsaceus* was determined under *in vivo* condition. Dry fruits of C.dipsaceus were collected for this study (Plate-10). Various parameters with reference to plant growth after inoculation with endophytes were recorded as the criteria to find out the best artificial inoculation method for pathogenecity studies. Effects of endophytic fungus on growth of plants were shown in Plate- 11.

Plate 10: Dry fruit of *C.dipsaceus* showing seeds

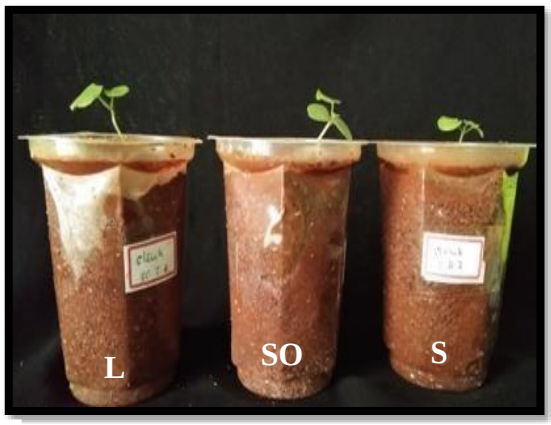
Plate- 11: Influence of endophytic fungi on the growth of *C. dipsaceus*



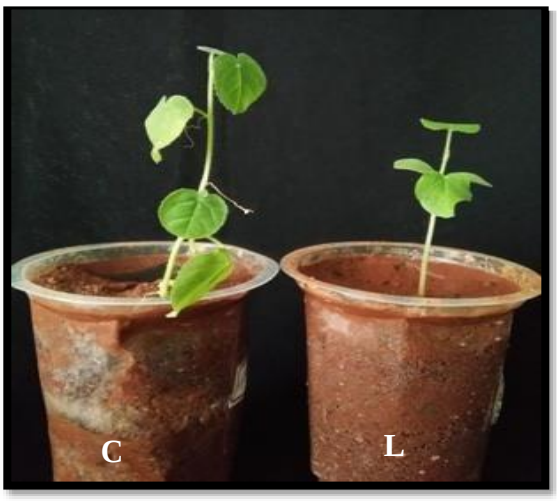
Plant growth after 7 days inoculated with *A.terreus*



Plant growth after 7 days (*T.radicus*)



Plant growth after 13 days (*C. globosum*)



Plant growth after 22 days (*T.radicus*)

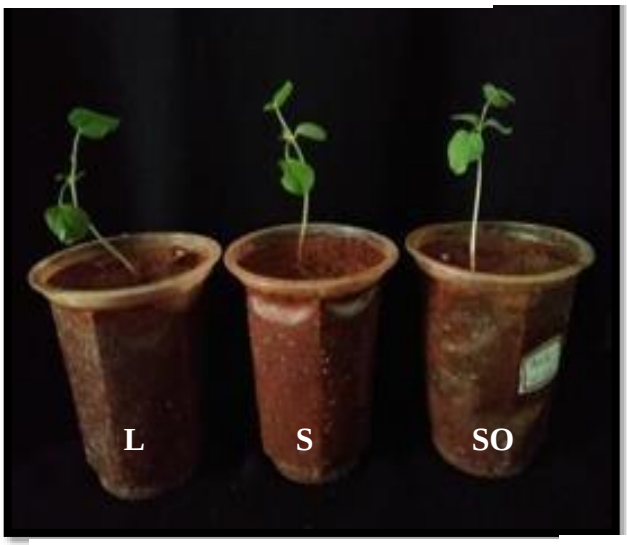
Plant growth after 22 days (*A.terreus*)

C-Control**; L-** leaf inoculation**; S-**Seed inoculation**; So-** Soil inoculation

Plate 11 (Contd.) : Influence of endophytic fungi on plant growth

Plant growth after 22 days (*C.globosum*)

Control plants under different soil conditions



Plant growth after 28 days (*C.globosum*)

Plant growth after 30 days

Seedlings with disease symptoms

C-Control**; L-** leaf inoculation**; S-**Seed inoculation**; So-** Soil inoculation;
US- Unsterilized soil; **ST-** Sterilized soil

By using different substrate (Sterile and non-sterile soil) and inoculation methods all the 22 fungal endophytes were inoculated separately in host plant. Plant growth parameters were recorded from 7th day of germination after inoculation with endophytic fungal isolates. When microbially (isolated endophytes) treated seeds were sown in different substrates, their germination percentage was low. Only three isolates namely *T.radicus*, *C.globosum* and *A.terreus* promoted seed germination. In contrast, when non treated seeds were sown, they resulted in quick germination and growth of seedlings. Among them, 17 isolates namely *Aspergillus aculeatus*, *A.flavus* 1, *A.flavus* 2, *A.fumigatus* 1, *A.fumigatus* 2, *A.nidulans*, *A.niger*, *A.ochraceus*, *A.terreus* 1, *A.terreus* 2, *A.ustus* 1, *A.ustus* 2, *A.ustus* 3, *Chaetomium* sp., *M.zamiae*, *N.gregarium* and *P.lilacinum* didnot promote any plant growth. Their pathogenecity on host plants was high. Seedling height, leaf number, fresh and dry weight of shoot, root varied significantly among the treatments. Five endophyte strains were found to be effective growth promoters. But their induction of growth in plants was less compared to control (Table 7-11).

But, when isolated endophytes were inoculated through soil and leaves some plants showed stunted growth, withering of leaves and lesions developed on plants. Among the 22 isolates tested only 5 endophytic fungus namely *Aspergillus* sp., *A.terreus*, *C.globosum*, *P.javanicum* and *T.radicus* induced growth of host plants.

Aspergillus sp. significantly induced growth of the plant when grown in sterilized soil. Unsterilized soil also promoted plant growth but compared to sterilized soil it showed only limited growth in plants. Among the inoculation methods, inoculation through leaves showed significant growth next to control plants in terms of seedling height (5.38±1.12cm), leaf number (5.17±1.25), fresh weight of shoot (0.20±0.01g), root (0.08±0.01g) and dry weight of shoot (0.10±0.06g), root (0.02±0.01g) (Table-7).

T.radicus promoted significant growth in host plant when grown in sterilized soil. Leaf inoculation method gave significant seedling height (6.03±0.08cm), leaf number (5.50±0.43), fresh weight of shoot (0.78±0.03g), root (0.23±0.02g) and dry weight of shoot (0.37±0.02g), root (0.09±0.01g) (Table-8). Compared to control, artificial inoculation of microbes to the host showed only slow growth.

Table 7: Growth response of *Cucumis dipsaceus* inoculated with endophytic fungi *Aspergillus* sp.

Substrates	Treatments[$]	Seedling height (cm Plant^{-1})	Leaf Number (plant^{-1})	Fresh weight (g)		Dry weight (g)	
				Shoot	Root	Shoot	Root
Unsterilized soil	T1	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c
	T2	0.92±0.05c	0.83±0.01c	0.10±0.01c	0.02±0.01c	0.05±0.01c	0.10±0.01b
	T3	**5.28±0.42b**	**4.83±0.31b**	**0.50±0.05b**	**0.22±0.01b**	**0.25±0.02b**	**0.11±0.01b**
	T4	12.55±0.39a	7.00±0.26a	0.88±0.03a	0.53±0.03a	0.43±0.02a	0.25±0.02a
Sterilized soil	T1	1.55±0.99c	3.0±1.37b	0.30±0.03a	0.24±0.03b	0.16±0.01b	0.12±0.02ab
	T2	2.87±1.97bc	2.17±1.38b	0.72±0.04a	0.22±0.12b	0.15±0.03b	0.24±0.09a
	T3	**5.38±1.12b**	**5.17±1.25ab**	**0.20±0.01a**	**0.08±0.01b**	**0.10±0.06b**	**0.02±0.01b**
	T4	12.13±0.40a	6.67±0.42a	0.82±0.04a	0.49±0.03a	0.39±0.03a	0.22±0.02a
Substrate (S) (1,40)		1.941ns	2.955**	1.412ns	3.164ns	0.357ns	5.329*
Treatment (T) (3,40)		77.619***	14.435***	6.870**	33.384***	46.174***	13.771***
S × T (3,40)		6.596**	4.668**	2.315ns	1.855ns	4.299*	5.460**

[$]T1- Seed; T2- Soil, T3- Leaves, T4-Control

[#]Mean ± S.E

Mean in a column followed by a same superscript(s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test

***Significant at P< 0.001 and ** significant at P< 0.01, ns- non significant

Table 8: Growth response of *Cucumis dipsaceus* inoculated with endophytic fungi *Talaromyces radicus*

Substrates	Treatments[$]	Seedling height (cm Plant^{-1})	Leaf Number (plant^{-1})	Fresh weight (g)		Dry weight (g)	
				Shoot	Root	Shoot	Root
Unsterilized soil	T1	2.08±1.33b	1.50±0.96c	0.22±0.14b	0.07±0.01b	0.07±0.01b	0.03±0.01b
	T2	4.08±1.30b	3.0±0.97c	0.42±0.13b	0.12±0.04b	0.19±0.05b	**0.07±0.02b**
	T3	**4.28±1.36b**	**4.17±1.42ab**	**0.52±0.17ab**	**0.13±0.04b**	**0.24±0.08b**	0.06±0.01b
	T4	12.55±0.39a	7.0±0.26a	0.88±0.03a	0.53±0.03a	0.43±0.02a	0.25±0.02a
Sterilized soil	T1	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c
	T2	5.68±0.12b	5.33±0.49b	0.42±0.02b	0.18±0.03b	0.21±0.01b	**0.12±0.01b**
	T3	**6.03±0.08b**	**5.50±0.43b**	**0.78±0.03a**	**0.23±0.02b**	**0.37±0.02a**	0.09±0.01b
	T4	12.13±0.40a	6.67±0.42a	0.82±0.04a	0.49±0.03a	0.39±0.03a	0.22±0.2a
Substrate (S) (1,40)		0.128ns	0.739ns	0.009ns	0.369ns	0.546ns	0.288ns
Treatment (T) (3,40)		57.160***	13.942***	20.286***	82.165***	27.870***	50.510***
S × T (3,40)		8.307***	11.158***	5.466**	7.693***	9.814***	7.968***

[$]T1- Seed; T2- Soil, T3- Leaves, T4-Control

[#]Mean ± S.E

Mean in a column followed by a same superscript(s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test

***Significant at P< 0.001 and ** significant at P< 0.01, ns- non significant

Artificial inoculation of *P.javanicum* into the host plant using unsterilized soil as substrate induced plant growth. Inoculation through leaves showed significant seedling height (5.03±1.01cm), leaf number (4.83±1.08), fresh weight of shoot (0.63±0.13g), root (0.17±0.03g) and dry weight of shoot (0.28±0.06g) and root (0.08±0.01g) (Table-9).

C.globosum induced plant growth when the plants grown in unsterilized soil condition. *C.globosum* also showed significant seedling height (5.95±0.25cm), leaf number (4.67±1.50), fresh weight of shoot (0.56±0.11g), root (0.26±0.06g) and dry weight of shoot (0.04±0.01g), root (0.02±0.01g) (Table-10) when inoculated through leaves.

A.terreus sp. significantly induced plant growth in sterilized soil. Among the inoculation methods, inoculation through leaves showed significant seedling height (3.93±1.27cm), leaf number (3.67±1.65), fresh weight of shoot (0.45±0.13g), root (0.40±0.16g) and dry weight of shoot (0.07±0.03g), root (0.02±0.01g) (Table-11).

Table-9: Growth response of *Cucumis dipsaceus* inoculated with endophytic fungi *Penicillium javanicum*

Substrates	Treatments[$]	Seedling height (cm Plant^{-1})	Leaf Number (plant^{-1})	Fresh weight (g)		Dry weight (g)	
				Shoot	Root	Shoot	Root
Unsterilized soil	T1	0.00 ±0.00c	0.00 ±0.00b	0.00±0.00b	0.00 ±0.00c	0.00 ±0.00c	0.00 ±0.00c
	T2	2.25±1.42c	1.83±0.01b	0.23±0.15b	0.06±0.01c	0.10±0.06c	0.03±0.01bc
	T3	**5.03±1.01b**	**4.83±1.08a**	**0.63±0.13a**	**0.17±0.03b**	**0.28±0.06b**	**0.08±0.01b**
	T4	12.55±0.39a	7.0±0.26a	0.88±0.03a	0.53±0.03a	0.43±0.02a	0.25±0.02a
Sterilized soil	T1	0.00 ±0.00c	0.00 ±0.00c	0.00 ±0.00c	0.00 ±0.00c	0.00 ±0.00b	0.00 ±0.00c
	T2	3.23±1.25b	4.17±1.35ab	0.47±0.15ab	0.18±0.04b	0.24±0.08a	0.09±0.01b
	T3	**3.80±1.21b**	**3.33±1.50b**	**0.42±0.19b**	**0.09±0.01bc**	**0.21±0.06a**	**0.04±0.01bc**
	T4	12.13±0.04a	6.67±0.42a	0.82±0.04a	0.49±0.03a	0.39±0.03a	0.22±0.02a
Substrate (S) (1,40)		0.064ns	0.037ns	0.008ns	0.018ns	0.038ns	0.004ns
Treatment (T) (3,40)		64.312***	18.677***	20.604***	81.617***	20.116***	55.274***
S × T (3,40)		1.099ns	1.510ns	1.419ns	3.062*	1.479ns	2.357ns

[$]T1- Seed; T2- Soil, T3- Leaves, T4-Control

[#]Mean ± S.E

Mean in a column followed by a same superscript(s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test

***Significant at P< 0.001 and ** significant at P< 0.01, ns- non significant

Table-10: Growth response of *Cucumis dipsaceus* inoculated with endophytic fungi *Chaetomium globosum*

Substrates	Treatments[$]	Seedling height (cm Plant^{-1})	Leaf Number (plant^{-1})	Fresh weight (g)		Dry weight (g)	
				Shoot	Root	Shoot	Root
Unsterilized soil	T1	0.90±0.05c	0.83±0.05b	0.12±0.10c	0.04±0.01c	0.26±0.05b	0.11±0.02b
	T2	5.10±1.03b	4.67±1.02a	0.45±0.14b	0.12±0.04c	0.19±0.06b	0.06±0.02bc
	T3	**5.95±0.25b**	**4.67±1.50a**	**0.56±0.11b**	**0.26±0.06b**	**0.04±0.01c**	**0.02±0.01c**
	T4	12.55±0.39a	7.00±0.26a	0.88±0.03a	0.53±0.03a	0.43±0.02a	0.25±0.02a
Sterilized soil	T1	2.12±1.30bc	2.17±1.30b	0.36±0.16b	0.31±0.14a	0.16±0.07b	0.15±0.02a
	T2	0.00±0.00c	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00c	0.00±0.00b
	T3	**3.13±1.42b**	**2.67±1.20b**	**0.23±0.1bc**	**0.06±0.02b**	**0.10±0.05bc**	**0.03±0.01b**
	T4	12.13±0.40a	6.67±0.42a	0.82±0.04a	0.49±0.03a	0.39±0.02a	0.22±0.02a
Substrate (S) (1,40)		3.457ns	4.244*	3.564ns	0.221ns	3.746ns	0.214ns
Treatment (T) (3,40)		53.163***	11.610***	15.825***	27.751***	21.122***	23.058***
S × T (3,40)		2.566ns	3.461*	2.366ns	0.779ns	2.501ns	1.013ns

[$]T1- Seed; T2- Soil, T3- Leaves, T4-Control

[#]Mean ± S.E

Mean in a column followed by a same superscript(s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test

***Significant at P< 0.001 and ** significant at P< 0.01, ns- non significant

Table-11: Growth response of *Cucumis dipsaceus* inoculated with endophytic fungi *Aspergillus terreus*

Substrates	Treatments[$]	Seedling height (cm Plant^{-1})	Leaf Number (plant^{-1})	Fresh weight (g)		Dry weight (g)	
				Shoot	Root	Shoot	Root
Unsterilized soil	T1	1.83±1.16b	0.83±0.03b	0.10±0.04b	0.05±0.01b	0.10±0.03b	0.04±0.01b
	T2	**2.02±1.29b**	**1.83±1.17b**	**0.17±0.10b**	**0.09±0.05b**	**0.09±0.02b**	**0.05±0.01b**
	T3	0.98±0.03b	2.17±1.42b	0.22±0.14b	0.10±0.02b	0.01±0.00b	0.02±0.01b
	T4	12.55±0.39a	7.00±0.26a	0.88±0.03a	0.53±0.03a	0.43±0.02a	0.25±0.01a
Sterilized soil	T1	1.02±0.3b	3.67±1.20ab	0.12±0.01b	0.08±0.01b	0.20±0.06b	0.18±0.02ab
	T2	3.27±1.40b	1.17±0.05b	0.34±0.01b	0.15±0.03b	0.05±0.01c	0.04±0.01c
	T3	**3.93±1.27b**	**3.67±1.65ab**	**0.45±0.13b**	**0.40±0.16a**	**0.07±0.03bc**	**0.07±0.01bc**
	T4	12.13±0.04a	6.67±0.42a	0.82±0.04a	0.49±0.03a	0.39±0.03a	0.22±0.2a
Substrate (S) (1,40)		0.968ns	1.129ns	1.240ns	3.338ns	0.435ns	2.449ns
Treatment (T) (3,40)		46.192***	9.184***	15.817***	16.514***	28.801***	15.129***
S × T (3,40)		1.104ns	1.091ns	1.070ns	2.715ns	1.249ns	2.486ns

[$]T1- Seed; T2- Soil, T3- Leaves, T4-Control

[#]Mean ± S.E

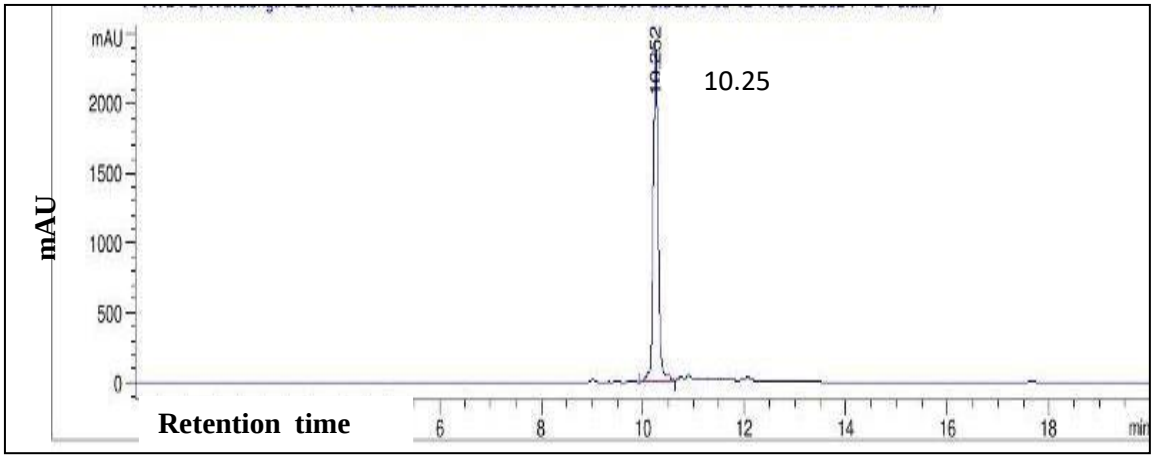
Mean in a column followed by a same superscript(s) are not significantly (P> 0.05) different according to Duncan's Multiple Range Test

***Significant at P< 0.001 and ** significant at P< 0.01, ns- non significant

Assessment of Cucurbitacin B through HPLC

Endophytes have the capacity to produce secondary metabolites similar to their host plant. To prove this statement and find true endophytes associated with *C.dipsaceus* HPLC analysis was carried out. Cucurbitacin B an important compound from various *Cucumis* sp., have been taken for analysis. Detection of Cucurbitacin B was done in host plant (*C.dipsaceus*) and their associated endophytes (*Aspergillus* sp., *A.terreus, C.globosum, P.javanicum* and *T.radicus*).

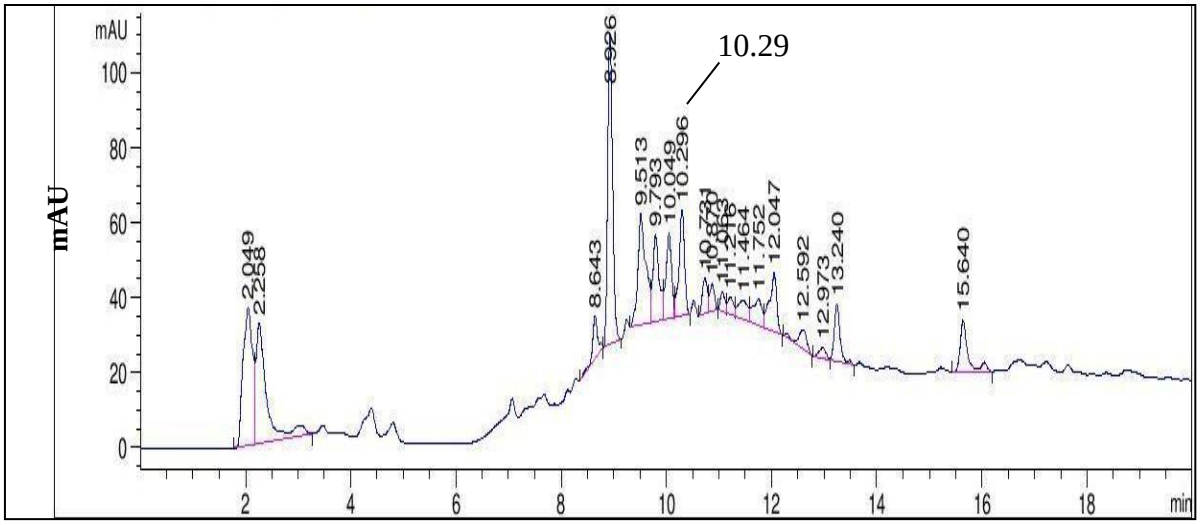
Fig. 5: HPLC chromatograph depicting the peaks of Cucurbitacin B- standard



Standard solution of Cucurbitacin B, leaf extract of *C.dipsaceus* and culture filtrate of isolates was injected separately and peaks were recorded under the optimized chromatographic conditions. The results of the study showed the presence of Cucurbitacin B in leaf extracts and culture filtrate of isolates by comparing with chromatograph of standard.

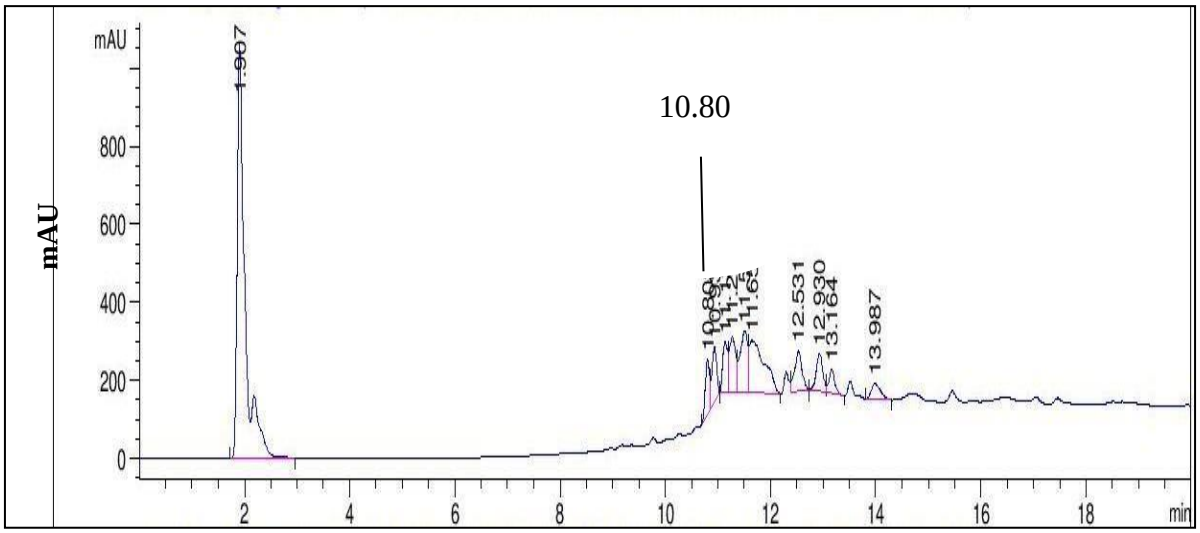
HPLC of standard Cucurbitacin B showed that compound eluted at a retention time of 10.252 minutes (Fig.5). Upon observation of crude extracts of plants (Fig. 6) and fungal endophytes (Fig 7-11) it was found that they possess cucurbitacin B determined by comparing their retention time with that of standard compounds. The compounds present in little quantities were depicted by small peaks at different retention times. One of the isolate *A.terreus* exhibited a sharp and major peak at the retention time of 10.920 which corresponds to cucurbitacin B thereby proving to be a potential source for the isolation of this compound. Different retention times at which cucurbitacin B eluted from plant and endophytic fungal extracts were summarized in Table-12.

Fig.6: HPLC chromatograph depicting the peaks of *Cucumis dipsaceus*



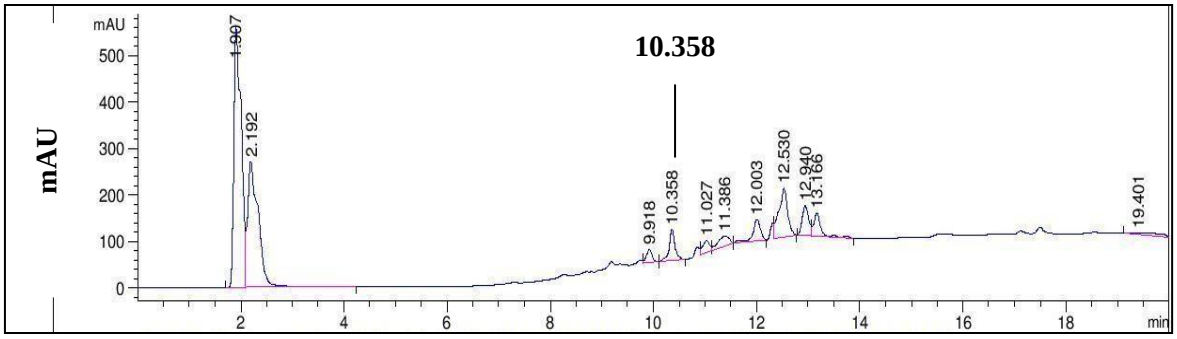
Retention time

Fig.7 : HPLC chromatograph depicting the peaks of *Aspergillus* sp.,



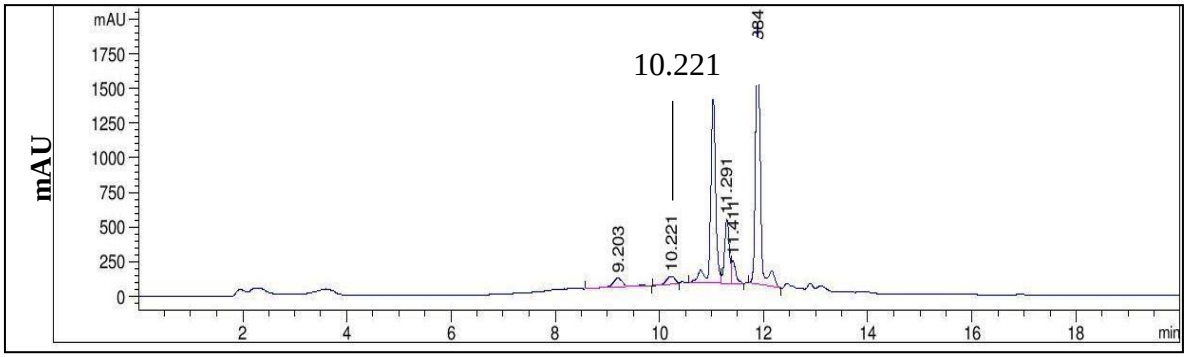
Retention time

Fig.8: HPLC chromatograph depicting the peaks of *Talaromyces radicus*



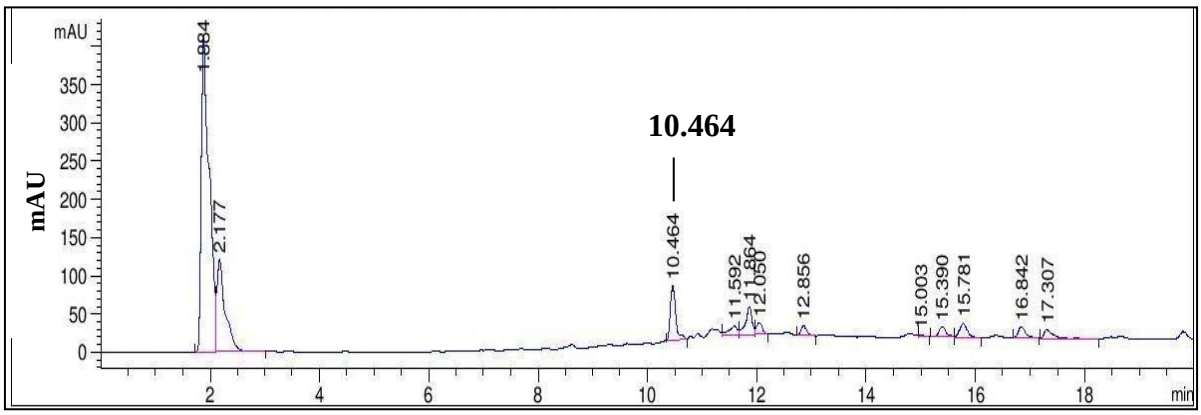
Retention time

Fig.9: HPLC chromatograph depicting the peaks of *Penicillium javanicum*



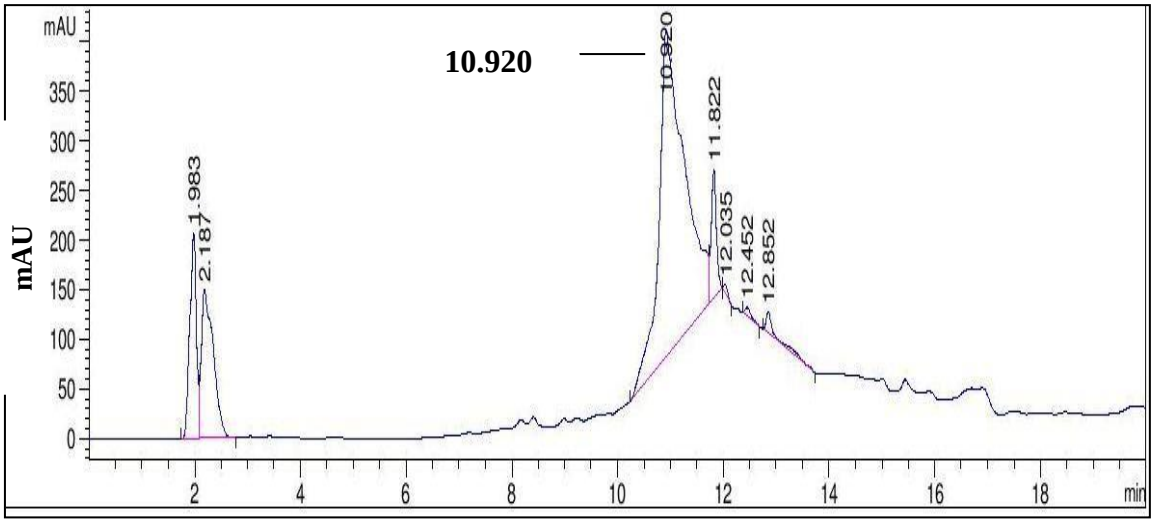
Retention time

Fig.10: HPLC chromatograph depicting the peaks of *Chaetomium globosum*



Retention time

Fig.11: HPLC chromatograph depicting the peaks of *Aspergillus terreus*



Retention time

Table-12: Retention time of Cucurbitacin B of standard, extracts of *C.dipsaceus* and the endophytes

S.No.	Source	Retention time (minutes)
1.	Standard	10.252
2.	*C.dipsaceus*	10.296
3.	*Aspergillus* sp.,	10.802
4.	*A.terreus*	10.920
5.	*T.radicus*	10.358
6.	*C.globosum*	10.464
7.	*P.javanicum*	10.221

Molecular characterization of true endophytes

Based on the assessment using plant studies and HPLC technique five isolates namely *Aspergillus* sp., *A.terreus, C.globosum, P.javanicum* and *T.radicus* was found to have the capacity to act as true endophytes of *C.dipsaceus*. The cultures were subjected to molecular characterization and the results were shown below.

Identification of a Fungal culture using Small Sub Units (SSU: *18S rRNA*) based Molecular Technique

A. *Aspergillus* sp.

Fig.12: Consensus Sequence (851bp) of *Aspergillus* sp.

GCGCAATCCAGCTCCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCT
CGTAGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGT
CCGGCTGGACCTTTCCTTCTGGGGAATCCCATGGCCTTCACTGGCTGTGGGTG
GAACCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGC
TCGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTG
GTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATT
CAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAA
GCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGA
AGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGAT
CGGGCGGCGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTT
TTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGAC
GGAAGGGCACCACAAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG
GAAACTCACCAGGTCCAGACAAAATAAGGATTGACAGATTGAGAGCTCTTTC
TTGATCTTTTGGATGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGT
CTGCTTAATTGCGATAACGAACGAGACCTCGGCCCTTAAATAGCCCGGTCCG
CGTCCGCGGGCCGCTGGCTTCTTAGGGGGACTATCGGCTCAGCCGATGGAAG
TGCGCGGCAATAACA

Fig.13: Phylogenetic tree of *Aspergillus* sp.

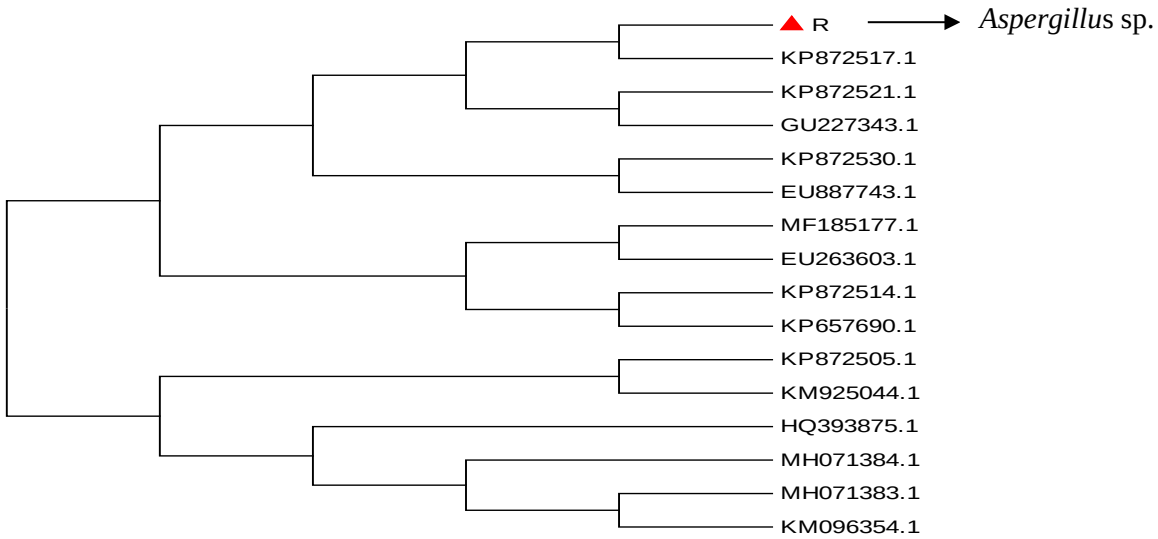


Table- 13: Sequence Alignments View of *Aspergillus* sp.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
MF185177.1	*Aspergillus* sp. strain DX4H	1550	1550	99%	0.0	99%
KP872530.1	*Aspergillus* sp. Y30-2	1550	1550	99%	0.0	99%
KP872521.1	*Aspergillus* sp. Y19-2	1550	1550	99%	0.0	99%
KP872517.1	***Aspergillus* sp. Y14-1**	1550	1550	99%	0.0	99%
KP872514.1	*Aspergillus* sp. Y37-1	1550	1550	99%	0.0	99%
KP872505.1	*Aspergillus* sp. Y38-1	1550	1550	99%	0.0	99%
KM925044.1	*Aspergillus* sp. LFJ1403	1550	1550	99%	0.0	99%
HQ393875.1	*Aspergillus versicolor* strain PSFNRO-2	1550	1550	99%	0.0	99%
GU227343.1	*Aspergillus versicolor* strain HDJZ-ZWM-16	1550	1550	99%	0.0	99%
EU887743.1	Ascomycota sp. Ex1	1550	1550	99%	0.0	99%
EU263603.1	*Aspergillus versicolor* strain DAN13	1550	1550	99%	0.0	99%
MH071384.1	*Aspergillus* sp. strain 3Y	154	154	99%	0.0	99%
MH071383.1	*Aspergillus* sp. strain 3G	154	154	99%	0.0	99%
KP657690.1	*Aspergillus* sp. P4-7	154	154	99%	0.0	99%
KM096354.1	*Aspergillus versicolor* strain MF557	154	154	99%	0.0	99%

Talaromyces radicus

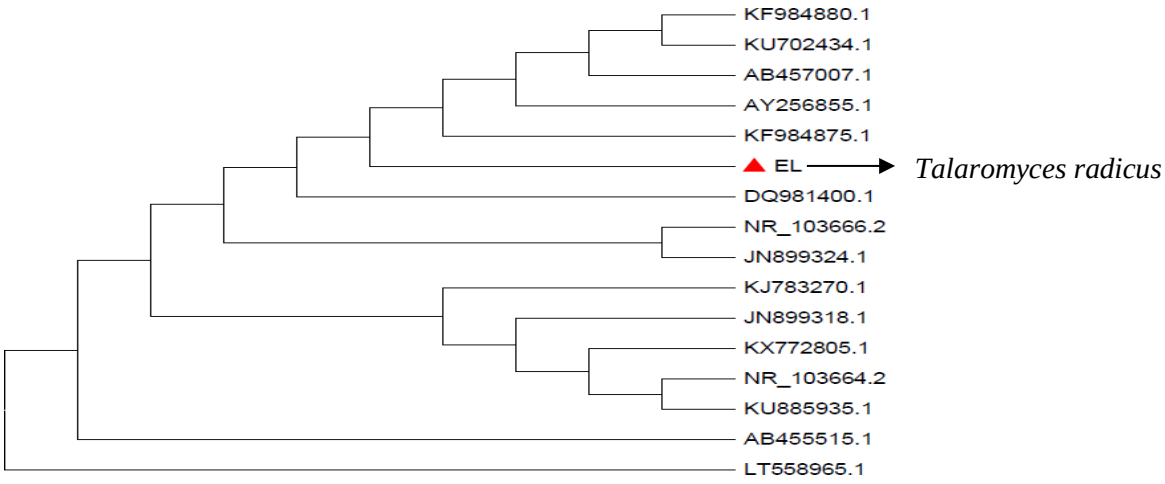
Fig.14: Consensus Sequence (604 bp) of *T.radicus*

CTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTTCGAATGA
GCCCAACCTCCCACCCGTGTCTACCGTTACCGCGTTGCTTCGGCGGGCCCACT
GGGGCCTCGCCCCGGTCGCCGGGGGGCTTCTGCCCCCGGGCCCGCGCCCGCC
GAAGCGCCCTGGAACCCTGTCTGAACAGTGAGTCTGAGTGTGATATTGAATC
ATTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCG
TCATTTCTGCCCTCCAGCACGGCTGGGTGTTGGGCGCTGTCCCCCCGGGGACA
CGCCCCAAAGGCAGTGGCGGCGCCGCGTCGGGTCCTCGAGCGTATGGGGCTC
TGTCACCCGCTCGGGAGGGACTCGGTCGGCGCTGGTCTTCCCCCAGGCGGCC
CTTTCGGGCTCGTCTCCTTCCGGTTGACCTCGGATCAGGTAGGGCTACCCGCT
GAACTTAAGCATATCAATAAGCGGAGGAA

Table-14: Sequence alignment view of *Talaromyces radicus*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB457007.1	*Talaromyces radicus* genes for, strain: FKI-3765-2	1096	1096	98%	0.0	100%
KF984875.1	***Talaromyces radicus*** strain DTO181D5	1077	1077	96%	0.0	100%
NR_103666.2	*Talaromyces radicus* CBS 100489	1075	1075	99%	0.0	99%
JN899324.1	*Talaromyces radicus* strain CBS 100489	1075	1075	99%	0.0	99%
KF984880.1	*Talaromyces radicus* strain DTO181D4	1070	1070	96%	0.0	99%
KU702434.1	*Talaromyces* sp. isolate TLT73	1057	1057	95%	0.0	99%
AY256855.1	*Talaromyces radicus* isolate FRR 3761	1048	1048	93%	0.0	100%
AB455515.1	*Penicillium* sp. FKI-3389	1037	1037	100%	0.0	98%
DQ981400.1	*Talaromyces radicus* strain AZ-5	1037	1037	95%	0.0	99%
NR_103664.2	*Talaromyces islandicus* CBS 338.48	1033	1033	99%	0.0	98%
JN899318.1	*Talaromyces islandicus* strain CBS 338.48	1033	1033	99%	0.0	98%
KX772805.1	*Talaromyces islandicus* strain ANF36	1029	1029	99%	0.0	98%
LT558965.1	*Talaromyces* sp. strain DI16-143	1029	1029	99%	0.0	98%
KU885935.1	*Talaromyces islandicus* strain EN-501	1024	1024	100%	0.0	97%
KJ783270.1	*Talaromyces islandicus* strain CICC 4034	1024	1024	99%	0.0	97%

Fig.15: Phylogenetic tree of *Talaromyces radicus*



Chaetomium globosum

Fig.16 :Consensus Sequence (896bp) of *C.globosum*

TTTCGTGTGTGGGGCCCCGCAGACGCGGTAAATCCAGCTCCAATAGCGTATA
TTAAAGTTGTTGAGGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTAGCCGGC
CGGTCCGCCTCACCGCGTGCACTGGCTCGGCTGGGCCTTTCCTTCTGGAGAAC
CGCATGCCCTTCACTGGGTGTGCCGGGGAACCAGGACTTTTACCGTGAAAAA
ATTAGATCGCTTAAAGAAGGCCTATGCTCGAATACATTAGCATGGAATAATA
GAATAGGACGTGTGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTA
ATAGGGACAGTCGGGGGCATCAGTATTCAATTGTCAGAGGTGAAATTCTTGG
ATTTATTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAA
TCAGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTA
ACCATAAACTATGCCGATTAGGGATCGGACGGCGTTATTACTTGACCCGTTCG
GCACCTTACGATAAATCAAAATGTTTGGGCTCCTGGGGGAGTATGGTCGCAA
GGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTG
CGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACGATGAG
GATTGACAGATTGAGAGCTCTTTCTTGATTTCGTGGGTGGTGGTGCATGGCCG
TTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCT
TAACCTGCTAAATAGCCCGCATGCTTGGCAGTGCGCCGCGTCTTAGAGGGAC
TATCGGCTCAGCCGATGGAAGTTTGAGCAATAACAGTCTAGGCCCCCCCCCC
AAAAAAT

Fig.17:Phylogenetic tree of *Chaetomium globosum*

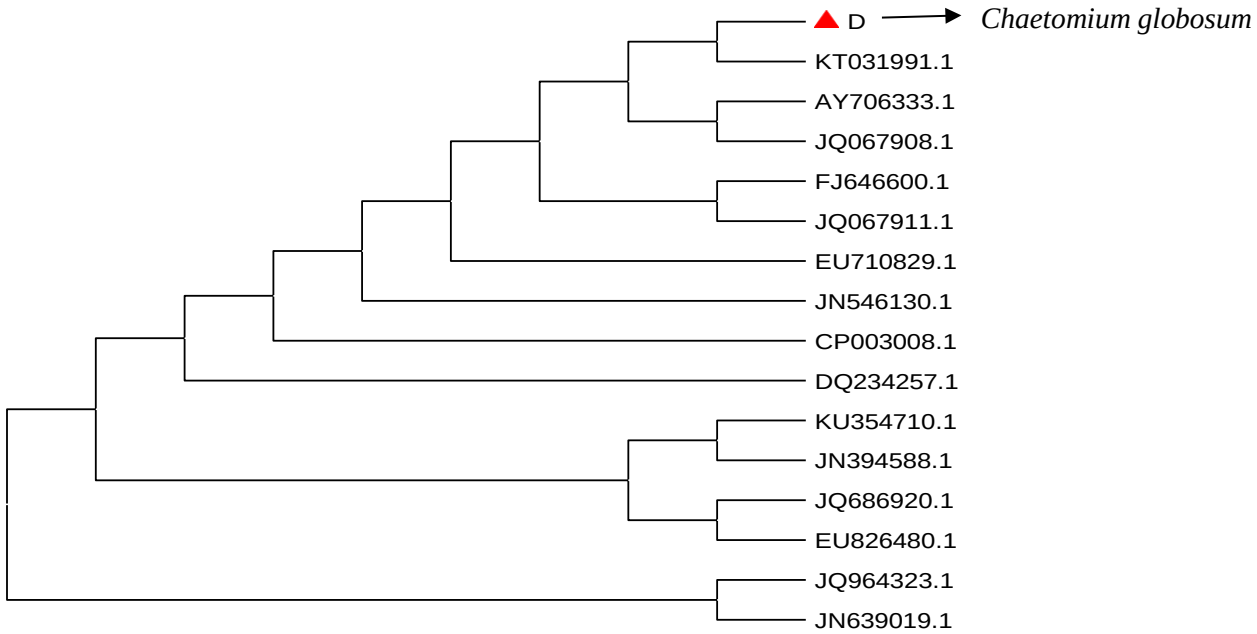


Table-15: Sequence Alignments View of *C.globosum*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KT031991.1	***Chaetomium globosum* strain VV09**	1524	1524	96%	0.0	99%
JN546130.1	*Chaetomium globosum*	1502	1502	96%	0.0	98%
AY706333.1	*Humicola fuscoatra* var. *fuscoatra* strain DAOM 35882	1502	1502	96%	0.0	98%
CP003008.1	*Myceliophthora thermophila* ATCC 42464	1496	1496	96%	0.0	98%
FJ646600.1	*Chaetomium* sp. CQ31	1496	1496	96%	0.0	98%
JQ067911.1	*Melanocarpus albomyces* strain ATCC 16460	1493	1493	96%	0.0	98%
KU354710.1	Fungal sp. strain EL001118	1491	1491	96%	0.0	98%
JQ964323.1	*Chaetomium globosum* strain WFWML4	1491	1491	96%	0.0	98%
JQ686920.1	*Chaetomium globosum* isolate W7	1491	1491	96%	0.0	98%
JQ067908.1	*Thielavia australiensis* strain ATCC 28236	1491	1491	96%	0.0	98%
JN639019.1	*Chaetomium globosum* strain NK-104	1491	1491	96%	0.0	98%
JN394588.1	*Chaetomium globosum* strain NK-103	1491	1491	96%	0.0	98%
EU826480.1	*Chaetomium* sp. CPCC 480539	1491	1491	96%	0.0	98%
EU710829.1	*Chaetomium* sp. 15002	1491	1491	96%	0.0	98%
DQ234257.1	*Chaetomium globosum*	1491	1491	96%	0.0	98%

Aspergillus terreus

Fig. 18: Consensus Sequence (869 bp) of *A.terreus*

ATACGCTATTGGAAGCTGGGAAATTACCGCGCCTGCTGGCACCCAGAATTTA
AAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGT
CCGCCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTTTTGGGGAATCCC
ATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAAAAAATTA
GAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGAAT
AGGACTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGG
GATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTG
CTGAAGACTAACTACTGCGAAAGCTTCGCCAAGGATGTTTTCATTAATCAGG
GAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCA
TAAACTATGCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGC
ACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAGCCTGCG
GCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGA
TTGACAGATTGAGAGCTCTTTCTTGATCTTTTGGATGGTGGTGCATGGCCGTT
CTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTCG
GCCCTTAAATAGCCCGGTCCGCATTTGCGGGCCGCGGGCTTCTTAGGGGGAC
CATCAGCTCTTCCGGGCCCCCGCCCCACGCCTA

Fig.19: Phylogenetic tree of *A.terreus*

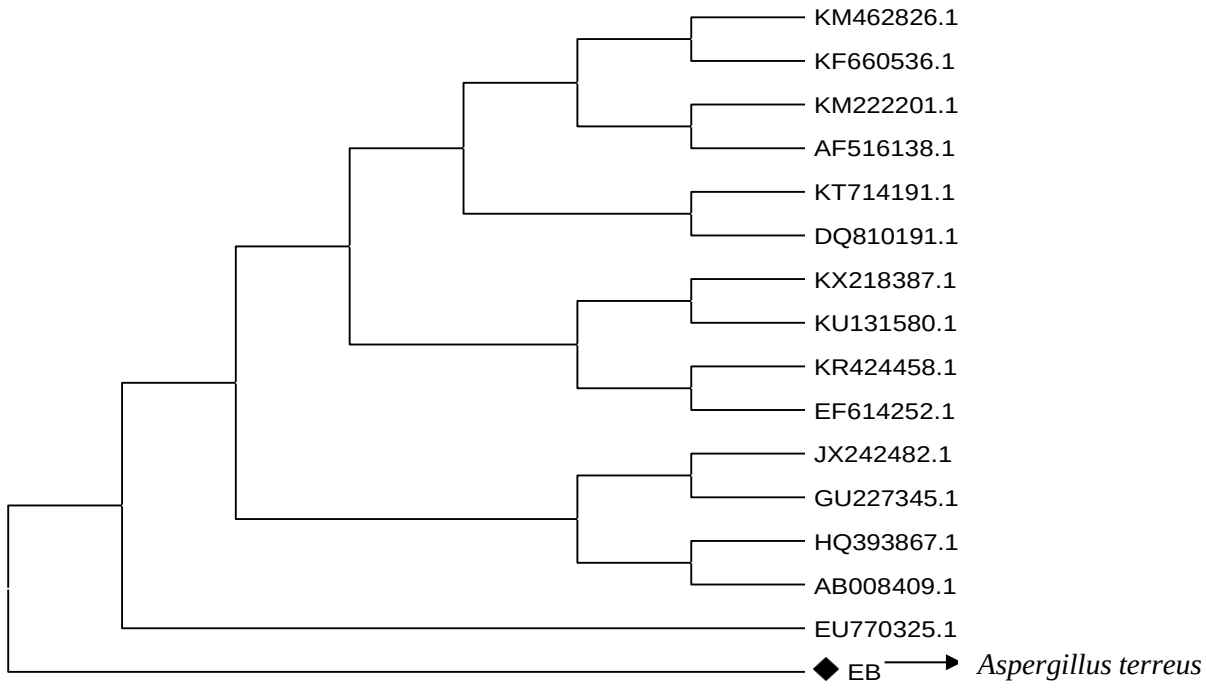


Table-16 : Sequence Alignments View of *A.terreus*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KX218387.1	*Aspergillus neoflavipes*, strain AJR1	1432	1432	91%	0.0	99%
KU131580.1	*Aspergillus terreus*, strain ASPII	1432	1432	91%	0.0	99%
KT714191.1	Fungal sp. isolate nussu_AT	1432	1432	91%	0.0	99%
KR424458.1	***Aspergillus terreus*, strain GA-B2**	1432	1432	91%	0.0	99%
KM462826.1	*Aspergillus terreus*, isolate ATE1	1432	1432	91%	0.0	99%
KM222201.1	*Aspergillus terreus*, strain 1H3-S0-P1-1	1432	1432	91%	0.0	99%
KF660536.1	*Aspergillus terreus*, strain AN4	1432	1432	91%	0.0	99%
JX242482.1	*Aspergillus terreus*, strain SMF-H10	1432	1432	91%	0.0	99%
HQ393867.1	*Aspergillus terreus*, strain PSFCRG2-1	1432	1432	91%	0.0	99%
GU227345.1	*Aspergillus terreus*, strain HDJZ-ZWM-18	1432	1432	91%	0.0	99%
EF614252.1	*Aspergillus* sp. Ar-4jing-1	1432	1432	91%	0.0	99%
EU770325.1	*Aspergillus* sp. ZL-2008	1432	1432	91%	0.0	99%
DQ810191.1	*Penicillium* sp. TP0307	1432	1432	91%	0.0	99%
AF516138.1	*Aspergillus terreus*	1432	1432	91%	0.0	99%
AB008409.1	*Aspergillus terreus*	1432	1432	91%	0.0	99%

Penicillium javanicum

Fig.20:Consensus Sequence (808 bp) of *P.javanicum*

TAATTCCAGCTCCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGT
AGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCC
GGCTGGACCTTTCCTTCTGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGA
ACCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTC
GAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGT
TTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCA
GCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGC
ATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAG
ACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCG
GACGGGATTCTATGATGACCCGTTCGGCACCTTACGAGAAATCAAAGTTTTTG
GGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAA
GGGCACCACAAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAA
CTCACCAGGTCCAGACAAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGA
TCTTTTGGATGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGC
TTAATTGCGATAACGAACGAGACCTCGGCCCTTAAATAGCCCGGTCCGCATT
GCGGGCCGCTGGCGCAATTTGCC

Fig.21: Phylogenetic tree of *P.javanicum*

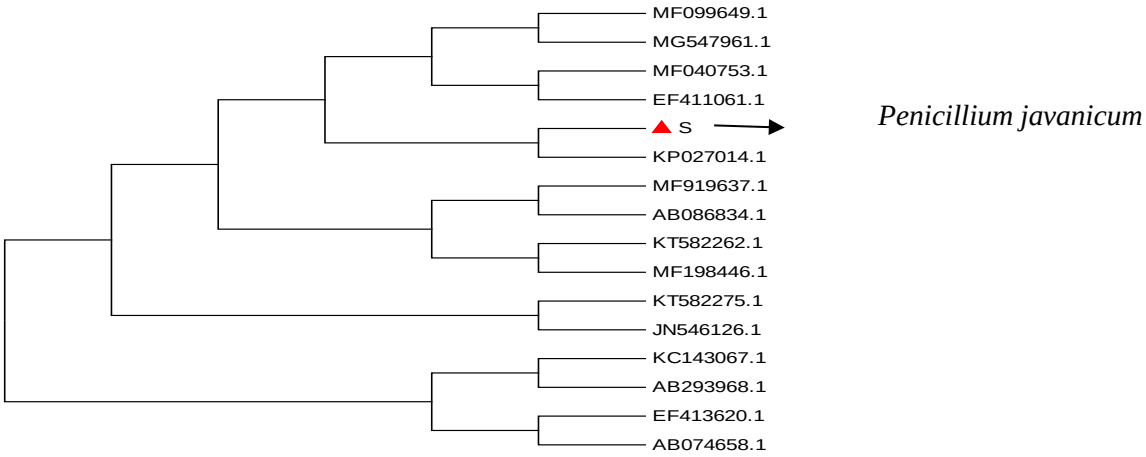


Table-17 : Sequence Alignments View of *P.javanicum*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
MF919637.1	*Penicillium javanicum* culture BCC<THA>:84314	1467	1467	98%	0.0	99%
MF099649.1	*Penicillium* sp. isolate R57	1463	1463	98%	0.0	99%
MF040753.1	*Penicillium* sp. strain R57	1463	1463	98%	0.0	99%
KT582275.1	Fungal sp. ZJ64	1463	1463	98%	0.0	99%
KT582262.1	Fungal sp. ZJ15	1463	1463	98%	0.0	99%
KP027014.1	***Penicillium javanicum*** isolate EMBOSS_009	1463	1463	98%	0.0	99%
KC143067.1	*Penicillium* sp. 6-16M	1463	1463	98%	0.0	99%
JN546126.1	*Eupenicillium javanicum*	1463	1463	98%	0.0	99%
AB293968.1	*Penicillium janthinellum*, strain: F-13	1463	1463	98%	0.0	99%
EF413620.1	*Eupenicillium javanicum* isolate AFTOL-ID 429	1463	1463	98%	0.0	99%
EF411061.1	*Penicillium limosum*	1463	1463	98%	0.0	99%
AB074658.1	Uncultured ascomycete, clone:APf3_76	1463	1463	98%	0.0	99%
AB086834.1	*Penicillium herquei*	1463	1463	98%	0.0	99%
MF198446.1	*Penicillium cyaneum* strain XMU01	1461	1461	98%	0.0	99%
MG547961.1	*Penicillium* sp. isolate LSZ-2	1458	1458	98%	0.0	99%

DISCUSSION

Plant endophyte association can act as an excellent model for studying evolution of microbial symbiosis. Endophytes have been broadly defined to include pathogens during their latency or quiescent stage until they produce visible symptoms. But for phytopathological studies, it is desirable to differentiate between latent pathogens and true endophytes. Many evidences were available for endophytic fungi occurring in lifecycle of some members of cucurbitaceae and they have been discussed by several authors. An endophyte cannot be considered to cause disease but accidently some of the genera and species which have the ability to cause disease were also regularly isolated as endophytes. Earlier, research on plant multiplication studies has targeted only latent pathogens whereas true endophytes were discarded because they could not produce any sanitary risk to plants. Besides, true endophytes act as efficient colonizers of plant which can outcompete with pathogens and reduce disease severity (Arnold et al., 2003).

The purpose of this study was to establish if any fungi regularly isolated as endophytes from *Cucumis* sp., have the ability to act as pathogen (Latent pathogens). Some of the common genera included under pathogens were isolated as endophytes they were, *Aspergillus* sp., *Fusarium*, *Alternaria* sp., etc. Some pathogens possess latent phase and become complete pathogen when the host is stressed. Microbes present in host plant may show either endophytic phase or pathogenic phase. This change in mode were influenced by change in host susceptibility caused by excessive humidity, poor nutrient supply etc., at the same time, these factors determine the duration of a particular mode.

Some of the isolates identified as endophytes in this study were also isolated as phytopathogen from some other plant species. *Aspergillus niger* isolated as endophyte was shown to be capable of causing black rot of onions, crown rot of peanuts, fruit rot of grapes (Sharma, 2012) etc., *Aspergillus aculeatus* was shown to cause bunch rot of grapes (Jarvis and Traquair, 1984), *A. flavus*, *A.fumigatus*, *A.nidulans* were able to infect bean leaves and corn kernels (Raymond et al., 2000), *A.ochraceus* cause disease in figs (Doster et al., 1996). Effect of these strain were more prevalent if plants grown under specific hot and humid growth conditions.

Other endophytic fungi like *A.ustus*, *M.zamiae*, *N.gregarium*, *Paecilomyces* sps., *P.liliacinum* did not show any effect on plant growth, in the present study. Our preliminary pathogenicity test on these isolates were able to cause disease symptoms like inhibiting the plant growth, yellowing of leaves, senescence of leaves at earlier stage of growth etc., indicating that they may be latent pathogens and able to infect host plant severely under stress or later during the plant growth (Yang et al., 2018). There may be some reasons for non colonization of endophytes on host plant. They are, sustainable effect by plant growth medium (sterilized and unsterilized soil) on inoculated organisms or by different methods of inoculation used. While taking plant growth medium, autoclaving of soil resulted in eliminating microbes present in them which otherwise make the inoculated organisms to compete or antagonized with soil microbes (Tefera and Vidal, 2009). Under unsterilized soil condition, biotic and abiotic factors may influence the colonization of endophytes and promote plant growth (Kessler et al., 2003).

Under unsterilized soil condition, isolates like *A. flavus*, *A.ustus*, *A.niger* and *M.zamiae* does not shown any endophytic colonization in plants when inoculated through seed and leaf. The reason behind the non colonization of endophytes was not clear and requires further investigations. But isolates like *A.terreus*, *T.radicus* and *C.globosum* showed slow growth and disease symptoms under unsterilized soil condition, this is might be due to low colonization and this may occurred by the stronger influence of biotic factors on inoculated isolates than the abiotic factors.

Lingg and Donaldson (1981) and Pereira et al., (1993) explained the fungistatic effects of soil and soil antagonism. According to them, unsterilized soil condition, biotic antagonisms suppress the germination of conidia of *Penicillium urticae* or prevented the isolates from entering into host. For example, some common soil fungus like *Penicillium* sps., and *Aspergillus* sp., produced water soluble inhibitors (Shields et al., 1981) or metabolites that acted (Majchrowicz et al., 1990) against inoculated isolates.

Aspergillus terreus, *Chaetomium globosum*, *Penicillium javanicum* and *Talaromyces radicus* promoted slow and stable growth on host compared to control plants. This is because, before the inoculation of endophytes, some potential fungal species which act as biocontrol agent with high production of alkaloids, terpenes and other resources may

exist on host plants. But after inoculation, sometimes due to competition between microbes, endophytes can suppress the growth or activity of potential fungal species. Mechanism of growth enhancement was not carried out in the present study. However some previous studies suggested that, they can promote plant growth in various ways, such as production of siderophores, supplying biological nitrogen fixation and by secreting plant growth hormones (Herre et al., 2007).

Our results contradicted with results of Waqas et al., (2012) who reported a significant increase in shoot length and plant biomass in Cucumber seedlings when inoculated with *Penicillium*. Halo et al., (2018) reported *A.terreus* as best isolate to defend against *Pythium aphanidermatum* which caused damping off disease in cucumber. The results from their study suggested that *A.terreus* increased the survival rate of cucumber seedlings from 38-39% when they were used as biocontrol agents. No disease symptoms were observed on cucumber seedlings.

Our results coincided with the results of Hung et al., (2015) and Tomilova and Shternshis (2006). They studied the *in vitro* and *in vivo* effects of crude extracts of *Chaetomium* sp., as biological control agents in controlling *Phytophthora nicotianae* on *Citrus maxima*. Under *in vitro* condition, application of spores resulted in increasing plant height and act as strong antifungal agent.

The inoculated endophytes- plant interaction promotes plant growth and development. Endophytic fungus act as potential partners which improves plants usage of soil resources (nutrients and water) and stress tolerance. In turn plants provide carbohydrates to fungus which provide stable interaction between the partners (Zeilinger et al., 2016).

Assessment of true endophytes using HPLC

Similar to our studies, Kaul et al., (2013) detected Digoxin from leaves of *Digitalis lantana* and n-hexane extracts of five endophytes isolated from the host plant. The results suggested that both host plant and endophytic fungi have the capacity to produce digoxin. Kumar et al., (2013) performed the HPLC analysis of crude extract of the fungal endophyte *Fusarium oxysporum* inhabiting *Catharanthus roseus* and isolated vinblastin and vincristine followed by subsequent characterization using NMR.

Rhein is an excellent anthroquinone found in *Rheum palmatum* L. Therefore, You et al., (2013) reported that endophytic fungus isolated from *R.palmatum* produced rhein.

Likewise, Wang et al., (2014) reported the isolation of secondary metabolites from chloroform crude extracts of endophytic fungi *Fusarium proliferatum* inhabiting *Macleaya cordata*. HPLC analysis showed that both host plant and endophyte possess the sanginarine compound. Hu et al., (2016) reported the presence of Homoharringtonine (HHT) in chloroform crude extracts of endophytic fungus *Alternaria tenuissima* isolated from *Cephalotaxus hainanensis*.

Methanolic extract of endophytic fungus *Phoma glomerata* inhabiting *Salvia miltiorrhiza* showed the presence of salvianolic acid (Li et al., 2016). Bilobalide an active compound in medicinal products were detected both in host plant *Ginkago biloba* and their respective endophyte *Pestalotiopsis uvicola* (Qiran et al., 2016).

The present study confirms the presence of Cucurbitacin B from fungal endophytes of *C.dipsaceus*. This is probably first report of Cucurbitacin B from the endophytes. They are almost present in many genus of cucurbits like *Trichosanthes*, *Cucurbita*, *Cucumis* and *Citrullus*. They are also found to be present in many other families like Scrophulariaceae, Cruciferae, Datiscaceae, Primulaceae, Rubiaceae etc. They contribute to different pharmacological actions (Dinam et al., 1997). The bitter taste of plant species like cucumber has been attributed to the presence of cucurbitacins. They were used for the most effective therapies like Anti- artherosclerotic activity (Esterbauer, 1993), antitumor activity (Liu et al., 2000) and antiinflammatory activity (Jayaprakasam et al., 2003) etc. These potential applications of cucurbitacin B add upto the isolation of this compound from alternative sources like fungal endophytes.

CONCLUSION

Among the different endophytic fungi isolated from *C.dipsaceus*, *Aspergillus* sp., *A.terreus*, *C.globosum*, *P.javanicum* and *T.radicus* were found to be true endophytes and *Aspergillus aculeatus*, *Aspergillus flavus* 1, *Aspergillus flavus* 2, *Aspergillus fumigatus* 1, *Aspergillus fumigatus* 2, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus terreus* 2, *Aspergillus terreus* 3, *Aspergillus ustus* 1, *Aspergillus ustus* 2, *Aspergillus ustus* 3, *Chaetomium* sp., *Melanospora zamiae*, *Nodulisporium gregarium* and *Purpureocillium lilacinum* were found to be latent pathogens. Isolates which are identified as true endophytes were found to produce Cucurbitacin B, a potent secondary metabolite with many pharmacological importances. Hence these fungi can be used for the production of Cucurbitacin B at industrial level.