Determination of Total Phenol and Antifungal Activities of Some Mangrove Associated Endophytic Fungi

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BY

MD. YEAMINUL HAQUE

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Approved as to Style and contents

By

Chhoa Mondal

Assistant Professor

(Supervisor)

Dr. Mst. Sabiha Sultana

Professor

(Co-Supervisor)

Prof. Dr. Sarder Safiqul Islam

(Chairman, Examination Committee

And Head, Agrotechnology Discipline)

AGROTECHNOLOGY DISCIPLINE

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KHULNA-9208

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ABSTRACT

Mangrove associated endophytic fungi are the potential sources bioactive compounds. Considering the significance of endophytic fungi, the present experiment was undertaken to determine the total phenol and antifungal activity of these endophytic fungi. The results showed that EASF-1 endophytic fungi collected from Sundarbans showed the highest Total Flavonoid Contents (TFC) (109.6 µg QE/mg of extract) and Total Polyphenol Contents (TPC) (138.4 µg GAE/mg of extract) followed by EASF-2, EASF-3. The Batul-4 and the Kewra-1 endophytic fungi showed the lowest amount of Total Flavonoid Contents (TFC) (4.113 µg QE/mg of extract) and Total Polyphenol Contents (TPC) (45.15 µg GAE/mg of extract). Among the ten endophytic fungi, EASF-3 showed the best result in case of antifungal activity and was effective against four pathogenic fungi whereas other showed moderate activity. Most effective endophytic fungi were EASF-1, EASF-2 and EASF-3 because they revealed antifungal activity against pathogenic fungus (*Bipolaris sorokiniana* of Wheat, *Fusarium oxysporum* of Tomato, *Alternaria solani* of Tomato and *Colletotrichum musae* of Banana). As they were efficient against the pathogenic fungi they were subjected to molecular identification and were identified as *Paecilomyces tenuis, Talaromyces sp.* and *Trichoderma harzianum*, respectively. These pathogens' extracts have the potentiality to use as alternative to fungicides in future.

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CHAPTER I

INTRODUCTION

Endophytes are organisms living as symptomless colony, maybe during a part of their life cycle, inside the host plants (Stone et al., 2000). Endophytes belong to diverse taxa such as bacterial, fungal, protistic, archaeal and are generally considered as mutualists.

Endophytic fungi living asymptomatically in plant tissues may present in almost all plants (Schulz et al., 1993). One species of an endophyte may be associated with many plant species, and many species of endophytes may be present in the same species. Some endophytes remain as latent in the host plant, while others may interact with other endophytes, pathogenic or non-pathogenic (Petrini, 1986 and Carroll, 1986). Endophytes have evolved mechanisms to live within the plant by defending themselves against all physical and chemical weapons of the plants. Therefore, endophytes provide two main strategies, one for obtaining novel bioactive secondary metabolites with the help of modern tools of chemistry such as selective high-resolution tandem mass spectrometry and secondly, they provide clue about mode of action of these bioactive metabolites.

Endophytic fungi are of biotechnological interest due to their potential as a source of secondary metabolites that has been over useful for novel agriculture. Antifungal activities of plant endophytic fungi have been interacted by secondary metabolites extracted from fungi (Dong et al., 2003). Endophytic fungi have been shown to produce several agriculturally important compounds those affect against fungi (Hata et al., 1998).

Metabolites produced by endophytes are being recognized as a versatile way of antimicrobial agents. Some endophytes have been known to possess superior biosynthetic capabilities, owing to their presumable gene recombination with the host, while residing and reproducing inside the healthy plant tissues (Stone et al., 2000). A high (80 %) proportion of endophytic fungi produce biologically active compounds in tests for antifungal and herbicidal activities (Strobel et al., 2004). The continued development of new antimicrobial compounds is important to overcome the

difficulties related to the treatment of infections caused by resistant pathogens in accordance with Bills et al., (2002). Thus, it can be said that endophytic fungi have emerged as an alternative source for the production of new antimicrobial agents.

Endophytes are rich source of bioactive compounds. Many endophytic fungi or its extracts have displayed broad-spectrum antimicrobial activity. It is amazing to know that endophytes can live in plants, which have capable of having metabolites for antimicrobial activity. An endophytic fungus from Mangrove plant has antifungal activity and the analysis of endophytic crude extracts revealed to have acetic acid (35.05%), valeric acid, 3-methyl-, methyl ester (21.25%), and butane-2, 3-diol (12.24%) (Huang et al., 2008). *Paecilomyces tenuis, Talaromyces sp,Trichoderma harzianum* isolated from *Excoecaria agallocha L* have antimicrobial activity against pathogenic fungi such as *Bipolaris sorokiniana* (Wheat), *Fusarium oxysporum* (Tomato), *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana). Isolating endophytic fungi from Mangrove plant might yield antimicrobials, which can fight against pathogenic fungi.

Bioactive products that can be produced by endophytes can be classified as saponins, phenols, flavonoids, steroids, tannins, alkaloids, terpenoids, and others. These compounds defend the plant against phytopathogenic fungi. For example, flavonoids are well-known substances, and their production involves a variety of processes such as cell signaling, plant growth, and reproduction (Hallman et al., 1997).

Pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life (Agrios, 2004). In addition, in some cases fungi are indirectly responsible for toxic disorders among consumers because of the production of mycotoxins.

Plant pathogens such as fungi, bacteria, nematodes and viruses cause various diseases in plants or may damages the plants (Montesinos, 2003). More importantly, fungi are the main pathogens that harm the plants. Fungal diseases cause a considerable loss of crop yields in agricultural industries worldwide. For example, fungi such

as *Fusarium* spp., growing on plants, are able to produce mycotoxins that can seriously harm consumers. Aflatoxin B1 and B2 and fumitoxins produced by *A. flavus* and *A. fumigatus* are some examples of mycotoxins (Singh et al., 1991). Antimycotics play an important two major roles in agriculture; firstly, they are used to control fungal growth on plants and fruits. Secondly, they can be used to prevent or to ease the problem of post harvest spoilage of plants and fruits (Hof, 2001). The presence and growth of this fungus in food and animal feed threatens human and animal health, respectively.

Farmers use chemical fungicides in plant agriculture to control fungal diseases, for example, *Colletotrichum musae* of banana. However, many fungicides are toxic to humans and they can cause environmental contamination or may result in fungicide residues on food products. On the other hand, biological control using microorganisms to repress plant disease, offers an alternative, environmentally friendly strategy for controlling phytopathogens (Chang et al., 2006). The screening of endophytic fungi is also another alternative that may produce chemical fungicides that are relatively non-toxic and cost-effective.

With the consideration of above facts the present study has been conducted to fulfill the following objectives:

Objectives

- To determine total phenolic compound present in the endophytic fungi.
- To appraise the antifungal activity of endophytic fungi against some fungal phytopathogens.
- To carryout molecular identification of endophytic fungi according to their performance.

CHAPTER II

REVIEW OF LITERATURE

2.1. Extraction of Secondary Metabolites from Endophytic Fungi

Choi et al. (2005) reported that a great deal of information on the role of endophytic microorganisms in nature has been collected. The capability of colonizing internal host tissues has made endophytes valuable for agriculture as a tool to improve crop performance. In this review, we addressed the major topics concerning the control of insects-pests by endophytic microorganisms. Several examples of insect control are described, notably those involving the interactions between fungi and grazing grasses from temperate countries.The mechanisms by which endophytic fungi control insect attacks are listed and include toxin production as well as the influence of these compounds on plant and livestock and how their production may be affected by genetic and environmental conditions. The importance of endophytic entomo-pathogenic fungi for insect control is also addressed. As the literature has shown, there is a lack of information on endophytes from tropical hosts, which are more severely affected by pests and diseases. Having this in mind, we have included an updated and extensive literature in this review, concerning new findings from tropical plants, including the characterization of endophytic fungi and bacteria microbiota from several Amazon trees, citrus and medicinal plants among others.

Ananda and Sridhar (2002) conducted that mangrove plant species are a valuable source of useful metabolites, their endophytes have gained more importance. Randomly sampled surface-sterilized whole root segments of four mangrove plant species, Acanthus ilicifolius, Avicennia officinalis, Rhizophora mucronata, and Sonneratia caseolaris from the mangroves of Udyavara (Karnataka) on the west coast of India, were characterized for fungal communities by direct plating, damp chamber, and bubbling chamber incubation methods. The richness of endophytic fungal species from whole root segments after direct plating and damp chamber incubation was greatest for R. mucronata than for other plants. Incubation of whole root segments in bubbling chambers yielded conidia of two freshwater hyphomycetes. The present

study showed that fungi in mangrove roots are composed of a consortium of soil, marine, and freshwater fungi.

Tejesvi et al. (2007) reported that the Endophytic fungi have proven their usefulness for drug discovery, as suggested by the structural complexity and chemical diversity of their secondary metabolites. The diversity and biological activities of endophytic fungi from the Terminalia species have been reported. Therefore, we set out to discuss the influence of seasons, locations, and even the plant species on the diversity of endophytic fungi, as well as their biological activities and secondary metabolites isolated from potent strains. Secondary metabolites were reported, among these, metabolites are the well-known anticancer drugs, a group that includes taxol, antioxidant compounds, isopestacin, and pestacin. This summary of data illustrates the considerable diversity and biological potential of fungal endophytes of the Terminalia species and gives insight into important findings while paving the way for future investigations.

Lane et al. (2003) conducted that all plants in natural ecosystems appear to be symbiotic with fungal endophytes. This highly diverse group of fungi can have profound impacts on plant communities through increasing fitness by conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption, or decreasing fitness by altering resource allocation. Despite more than 100 yr of research resulting in thousands of journal articles, the ecological significance of these fungi remains poorly characterized. Historically, two endophytic groups (clavicipitaceous (C) and nonclavicipitaceous (NC)) have been discriminated based on phylogeny and life history traits.

Cannon and Simmons (2002) showed that NC-endophytes represent three distinct functional groups based on host colonization and transmission, in planta biodiversity and fitness benefits conferred to hosts. Using this framework, we contrast the life histories, interactions with hosts and potential roles in plant ecophysiology of C- and NC-endophytes, and highlight several key questions for future work in endophyte biology. The implications for estimation of fungal diversity in tropical systems are explored.

Azevedo et al. (2000) found out endophytes are microorganisms that exist within the tissues of living plants. Generally the relationship between the plant and its endophytes is symbiotic whereby the endophytes colonise the internal tissues of the plant without any adverse effects on the host. In the past two decades, there has been growing interest in endophytes and their origins, their biodiversity, endophyte-host interactions, their role in ecology and the characterisation of their secondary metabolites. However, the sheer diversity of plant-endophyte relationships means that only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology. While there is some information available about the medicinal properties of Acacia, there is no information about the endophytic microorganisms of these plants. With increased need for new bioactive compounds with medical, industrial or biotechnological applications, we have investigated the bioactive properties of fungal endophytes of Acacia species.

Weber et al. (2004) reported that endophytic fungi were isolated from fermentations of a Phomopsis species in the course of a screening of endophytic fungi from the medicinal plant Erythrina crista-galli. For this Argentinean leguminosa antiinflammatory and neuroleptic activities have been described. The compound exhibits antifungal, antibacterial and weak cytotoxic acticity. The antiinflammatory activity was tested in different reporter gene assays (TNF-alpha, STAT1/STAT2 and NF-kappaB) and in an ear edema model in mice. In the reporter gene assays 1 exhibited no activity, whereas 1 showed interesting antiinflammatory activity in the mouse ear assay. The compound is a polyketide lactone and its structure was elucidated by spectroscopic methods.

Sturz et al. (2004) conducted that endophytes lives, reproduce and forms mycelia that grow between the cells of a plant, more in the leaf sheaths and reproductive structures. When seed production starts, endophyte grows upward in the host plant. After seed formation, endophyte infects outer layers of the seed and endophytes transfers from plants in a seed production field to seed when such seeds germinates and grows, endophytic microbes starts infecting the other host. Endophytic fungi may express different symbiotic lifestyles in response to the host genotypes and environmental factors. Lifestyle expression of endophytes is a post colonization phenomenon which involves biochemical and genetic communications between endophytic microbes and

host. Basically grass species, have been entirely studied in relation to their endophytic biology.

Tran et al. (2010) reported that the growth stimulation by the endophytes may also by nitrogen fixation, phosphate solubilization, production of siderophore and also supply of essential vitamins to plants phytohormones, production of volatile substances like 2-3 butanediol and aceotin for plant growth. Endophyte produces adenine ribosides that found affect nematodes propagation.

Jeewon et al. (2004) have explained the ability of endophytic fungal production of phytotoxic metabolites and exoenzymes, which are used for cell wall penetration and colonization of the host plant. After the cell wall penetration by endophytes, cellular reprogramming may takes place to accommodate new organism in the host and also to maintain cell integrity for a long lasting interaction. Remarkably, when endophyte enters into the host, the host defense mechanisms may get activated. Therefore the endophyte infected plants are more vigorous, drought resistant and toxic to herbivores, nematodes and pathogens than uninfected plants.

Butler (2004) conducted that the endophytic fungi have the ability to produce phyto hormones which help to promote plant growth and regulation. have suggested many endophytic fungi to have the ability for nitrogen fixation, phosphate solubilization, siderophore and ACC deaminase production. Endophytic fungal colonization in root region can increase the surface area and thereby increase the host access to soil nutrients. Endophytes with phosphate solubilization capacity assist the host plant for easy mobilization of phosphate which otherwise will be unavailable to the plant.

Zhang et al. (2006) has reported ability of endophytic fungal metabolites to helpplant growth and adaptation to the stress conditions. Endophytes live inside the plant tissue and are generally plant beneficial in nature. However, the stability of plant and endophytes interaction may be affected by stress conditions.

Lane et al. (2003) have pointed out that under certain stress conditions pathogenic endophytes may likely to lose their virulence and can get transformed into non pathogenic endophytic organisms. In the case of pathogenic Colletotrichum magna

mutation has been reported to result in the loss of virulence factor and transformation into endophytic life.

Silva et al. (2007) have reported a similar effect with Piriformospora indica by studying the ability of the fungal culture filtrate to increase the growth of maize plant. Endophytic fungi may help the host plants from environmental stress conditions such as drought, salts, and high temperatures. In the herbal plant Dichanthelium lanuginosum, which lives in areas where soil temperatures can reach 57°C, the presence of endophytic fungi *Curvularia sp.* is considered to protect the plant from stress condition better than endophyte free plants. At the same time Piriformospora indica, have been reported for the ability to increase antifungal activity and salt stress tolerance in barley. Such beneficial effect of endophytic fungi on the defense status of the plant and survival under stress conditions can increase the plant growth also.

Strobel and Daisy (2003) conducted that endophytic fungi are capable of increasing the photosynthetic capacity of host plant also. Epichloe sp. isolated from Achnatherum inebrians was found to increase the chlorophyll content and photosynthetic rate of the host plant. The fungal endophyte Epichloe typhina from Dactylis glomerata also reported for the same effect Endophytic fungi play an important role in control of environmental pollution.

Faeth and Fagan (2002) have reported application of endophytes in phytoremediation. The ability of endophytic fungi to break down complex compounds can have important applications. An endophytic fungi isolated from Nicotiana tabaccum has found to increase biomass production under different level of cadmium stress. Endophytic fungi have also been studied for their ability to degrade synthetic polymer polyester polyurethane. The endophyte infected plants were found to have more shoot and root biomass due to phyto remediation.

Rudgers et al. (2004) conducted that fungal endophytes are extremely common and highly diverse microorganisms that live within plant tissues, but usually remain asymptomatic. Endophytes traditionally have been considered plant mutualists, mainly by reducing herbivory via production of mycotoxins, such as alkaloids. However, the vast majority of endophytes, especially horizontally-transmitted ones

commonly found in woody plants, apparently have little or no effect on herbivores. For the systemic, vertically-transmitted endophytes of grasses, mutualistic interactions via increased resistance to herbivores and pathogens are more common, as predicted by evolutionary theory. However, even in these obligate symbioses, endophytes are often neutral or even pathogenic to the host grass, depending on endophyte and plant genotype and environmental conditions.

Hyde and Lee (1995) found out Endophytic fungi are the store house of naturally occurring bioactive compound. Plants have been exploited for human benefits for over thousands of years and have been the storage house of compounds for medicine. Endophytic fungi are a kind of fungi that colonize and reside in living and internal tissues of plants without causing any disease to the plant and reproduce by vegetative and sexual methods. Endophytes constitute a large diversity of microbial adaptations that have developed in special and hidden environments. Because of their huge diversity and particular habituation, they can provide a good area for research in the field of making new medicines and novel drug like molecules. Many microorganisms have developed resistance against the current pathogen, so it is necessary to search for new fungi. In order to provide aid and comfort in all aspects of agriculture and it is very important to make fungicide which has safe bioactive compounds which are not dangerous for environment.

Strobel et al. (2002) showed that all plant species holds endophytic fungi belong to the various classes, including Dothideomycetes, Eurotiomycetes, Sordariomycetes, Pezizomycetes and Leotiom-ycetes. The classic traditional identification of fungi method was historically developed based on the morphological characters with the phenotypic access. Morphological characterization method involves types of sporulation, the spore structure, colony, hyphal morphology, color of the hyphae and pigmentations.

Suryanarayanan and Murali (2006) isolated 373 fungal endophytes belonging to 15 genera from five medicinal plants collected in Himachal Pradesh, India. Some of them are *Aspergillus, Penicillium, Alternaria, Curvularia, Haplosporium, Phoma, Nigrospora, Colletotrichum, Cladosporium, Stemphylium, Fusarium, Geotrichum, Phomopsis, Trichoderma* to mention in few. The study revealed that, the highest

colonization rate was observed in Adhathoda vasica (93.05 %), followed by *Ocimum sanctum* (91.66 %), Viola odorata (85 %), Cannabis sativa (82.81 % and lowest in Withania somnifera (61.11 %).

Waller et al. (2005) isolated 1051 fungal endophytes inhabiting Cephalotaxus hainanensis Li collected from Hainan, China. About 21 genera of endophytic fungi were identified its morphological characteristics and molecular ITS sequences. The dominant species was P. quercella and Colletotrichum boninense with relative frequency of 42.06 % and 36.84 % respectively. Out of 1051 endophytic fungi, 21 strains exhibited strong antimicrobial activity where 17 strains (80.95%) showed activity against at least one test bacterial pathogens and 14 strains (66.67%) against at least one test fungal pathogens. Neonectria macroconidialis exhibited significant inhibition against Streptococcus agalactiae (28 mm) followed by S. aureus (20 mm), *Rhizoctonia solani* (20 mm), *E. coli* (20 mm) and *B. subtilis* (14 mm). *Verticillium bulbillosum* exhibited strong activity against *F. oxysporum* (22 mm) and *Strep agalactiae* (32 mm). These fungal endophytes were prolific source for medicine development.

Schulz et al. (2002) conducted that Endophyte-associateded plants often grow faster than non-associated ones. This may be due to the endophyte's production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances. For instance, the growth-promoting phytohormone indole-3-acetic acid was reisolated from cultures of a root-associated endophytic bacterium *Azospirillum brasilense* SP 7 the tall fescue fungal endophyte Acremonium coenophialum *Aureobasidium pullulans* and *Epicoccum purpurascens*. Endophytes also promote plant growth by a number of other mechanisms such as phosphate solubilization activity the production of a siderophore by supplying biologically fixed nitrogen and by supplying essential vitamins to plants. Other beneficial effects of endophytes on plant growth include osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism.

Stone et al. (2004) had been seen that endophytic association with plants may lead to increase in production of chemical toxins after damage to the plant has occurred.

Along with the production of novel chemicals, many endophytes have been shown to have a natural capacity for xenobiotic degradation. The ability of some endophytes to show resistance to heavy metals/antimicrobials and degrade organic compounds is due to their exposure to various compounds in the plant/soil. This natural ability to degrade these chemical compounds is being explored to improving phytoremediation. Endophytic fungi cause symptomless infections in healthy tissues of plants. This cryptic guild of fungi is regarded as a benchmark for estimating fungal biodiversity. We studied endophyte distribution, diversity and host recurrence in 24 tree hosts (belonging to 17 plant families) of two dry tropical forests of the Nilgiri Biosphere Reserve.

Pandey et al. (2003) conducted thatof 81 endophyte taxa were isolated from 3600 tissue segments. Fifty-six species were isolated from more than one host. We discerned two groups of fungi in both forests, one group consisting of the ubiquitous forms that dominated the endophyte assemblage of many hosts and the second represented by the less frequent forms. Host density influenced the composition and distribution of endophytes in one of the forests. The existence of ubiquitous forms reduced the diversity of the endophytes in the plant communities. Our results suggest that dry tropical forests are not hyperdiverse with reference to endophytes and that the generalists among endophytes be identified before extrapolating data to calculate global fungal diversity.

Schardl et al. (2004) inoculated pea plants with a Pseudomonas endophyte capable of degrading the organo chlorine herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D).When inoculated plants were exposed to 2,4-D, they showed no accumulation of the herbicide into their tissues and experienced little or no signs of phytotoxicity, where as uninoculated plants showed significant accumulation of 2,4-D and displayed signs of toxicity including a reduction in biomass, leaf abscission and callus development on their roots.

Murali et al. (2006) reported an endophytic strain significantly increased shoot and root growth of rice in growth chamber experiments, and also under field conditions Significant increases in rice grain yield and N-content were observed. Grain yield was observed to increase by 46%, grain N-content by 53%, straw yield was increased by

15% and straw N-content by 39%, compared to uninoculated plants. Inoculation with Acetobacter diazotrophicus wild-type strain resulted in a significant increase in the height of N-limited sugarcane plants compared with un-inoculated plants. Inoculation of diazotrophic endophytes enhanced plant growth, where as their co-inoculation with some other types of bacteria acted synergistically to give effects greater than single-strain inoculants.

Redman et al. (2001) reported from plants found in various environment which includes tropic, temperate, aquatic, oceans, xerophytic, deserts, antarctic, geothermal soils, rainforests, mangrove swamps and also coastal forests. Endophytes found to improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens. Endophytic fungi are able to protect their host plant from drought conditions and also found to increase heat tolerance so it is said that endophytes can acts as biological trigger to turn on the stress response more quickly. Endophytes control the activation of host stress response systems by acting as biological triggers. Enhanced drought tolerance is recognized through studies of endophyte infected species. Endophyte infected grasses respond to poor soil nutrition by an increased growth rate.

Malinowski and Belesky (2000) conducted that fungi may express different symbiotic lifes tyles in respond to host genotypes and environmental factors. Both pathogenic and nonpathogenic fungi isolated from asymptomatic plant tissues, suggestive of that both pathogenic and nonpathogenic microbes express lifestyles or remain dormant in expectation of plant senescence and life style expression is a post colonization phenomenon communicating between the symbiont and host. It is recognized that mutalistic fungi collectively may confer several host fitness benefits, such as growth improvement or tolerance to drought, disease, herbivory, insect and temperature to plant.

Rosenblueth and Martinez (2006) showed that endophytes interact with host mutualistically. Endophytic fungi found to produce alkaloids in the plants. Some of the agronomic species infected with endophytes showed resistance to toxic effects on vertebrate and invertebrate herbivores. Endophytic bacteria found within plant tissue as biotrophic symbionts and either obligate or facultative. Endophytic bacteria can

colonize thousands of different plant species, while some of them are restricted to selected plant families. Bacterial endophyte produces large number of phytohormones, such as auxins, cytokinins and the gibberellins. Examination indicates that interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environmental conditions.

Fisher et al. (1993) conducted that the endophytic fungal assemblages of leaves, xylem and bark of Eucalyptus nitens trees taken from their natural growth range in Australia and from introduced areas in England have been studied to detect site and climate related differences in the endophyte communities. Sixty-four fungal taxa have been isolated, but only twenty acount for a relative importance of more than 5% in any of the tissues examined. The Australian and British samples are clearly separated according to their geographic origin. The endophytes responsible for the separation are mainly *Cytospora eucalypticola*, very abundant in the sample units from Australia and otherwise present only in twigs from Britain that underwent drying, and Botryosphaeria dothidea, completely absent from the Australian samples. Australian and British isolates of *Cytospora eucalypticola* belong probably to different strains. Possible explanations for the site-related differences between Australian and British samples are presented and briefly discussed.

Giménez et al. (2007) conducted that Endophytes (fungal and bacterial organisms) have attracted great interest over the past few years because their presence benefits the host plant (development and defence) and they are a source of secondary metabolites of potential interest and thus play an important role in the regulation of plant communities and their herbivores. There are an increasing number of reports on their identification and on the production of secondary metabolites. Their role in plant-pathogen and plant-insect interactions is receiving increasing attention because of their potential use in pest control, however, little is known about their physiology and the regulation processes of the plant-endophyte interaction.

Jalgaonwala et al. (2011) reported that this review describes information on the role of endophytic microorganisms and some of naturally occurring bioactive compounds obtained from endophytic fungi isolated from various host plants. In the recent past years, a great deal of information on the role of endophytes in nature has been

collected. The main topics addressed are isolation of endophytes, host endophyte relationship, fungal endophyte, their diversity, physiological role of endophyte, biological activities and chemistry.

Lodge et al. (1996) found out Endophytic fungi inhabit living plant tissues without causing disease symptoms. Although abundant, the extent of their contribution to fungal biodiversity remains unclear. Since endophytic fungi are poorly known, especially in the tropics, current estimates of fungal species are probably conservative. Here we tested strategies for sampling endophytic fungi in tropical plants. We compared the number of fungi isolated from 400 mm2 leaf pieces that were divided into increasingly small fragments. Leaf pieces were surface-sterilized, cut into fragments and plated on culture media. For a given area, cutting leaf pieces into smaller fragments significantly increased the number of fungal morphospecies recovered. There was a strong linear relationship between size of fragments and number of fungi isolated. By extrapolation, an estimated 16 ± 3 fungi could be recovered from a 2 × 2 cm leaf piece, using infinitely small fragments. This represents a large part of the fungal diversity estimated to exist in leaf endophytes in a population. We conclude that reducing the size and increasing the number of leaf fragments will increase the number of fungal species isolated. This strategy will help to estimate real values of endophytic fungal diversity.

Maria and Sridhar (2003) showed that twenty-five endophytic fungi com prising three ascomycetes, 20 m itosporic fungi and two sterile fungi were recovered from two halophytes (A canthus ilicifolius and Acrostichum aureum) of a west coast m angrove h a b ita t in India. Overall colonisation of tissue segm ents by endophytes ranged betw een 74.5 % (*Acanthus ilicifolius*) and 77.5 % (*Acrostichum aureum*). A nalysis using the Jaccard's similarity coefficient revealed 16-25 % similarity in endophyte assem blage among different tissues, and 24.5 % between the two hosts. Out of four tissues screened, species richness and diversity were high in stems of *Acanthus ilicifolius* and roots of *Acrostichum aureum*. The most dominant endophyte was *Colletotrichum sp.* in prop roots of *Acanthus ilicifolius*, and *Yeast sp.* 1 in rhizom es of *Acrostichum aureum* . Am ong the dominant endophytes (colonisation frequency > 5 %), A crem onium and Yeast sp. 1 were comm on to both hosts. Acanthus ilicifolius showed dominance of a single species, (*Colletotrichum sp*), while in Acrostichum

aureum m ultiple species dominance was seen (*Acrem onium sp.*, P *énicillium sp.* and *Yeast sp.* 1). Only one typical m arine m itosporic fungus (*Cumulospora marina*) was recovered from the roots of a canthus ilicifolius.

Narayan et al. (2007) conducted that evaluation of some endophytes have been carried for their possible antimicrobial activity from various parts of medicinal plants belonging to Jalgaon Maharashtra (India). A total of seventy eight bacterial endophytes and one hundred fourty two fungal endophytes were isolated from the aerial and underground parts of selected medicinal plants. Fifteen positive endophytic bacterial isolates and fourteen positive endophytic fungal isolates possess antifungal and antibacterial activity respectively. Bacterial isolates KB4 from roots of roots of P.glabra, NB6 from stem of E.globulus and HB3 from rhizomes of *C.longa* have strong antifungal activity. Endophytic fungi AFR1, AFR4, AFR7 from roots of *A.vera* possess strong antibacterial activity against *S. typhi* in dual culture assay.

Schulz et al. (1999) showed that fifty-three endophytes were isolated from whole stem and xylem tissues taken from a mixed stand of Pinus sylvestris and Fagus sylvatica. Rhinocladiella atrovirens appeared confined to the xylem of Fagus, *Coniothyrium sp*, pini to the xylem of Pinus. Coryneum betulinum, *Cryptocline sp.* 1 and 2, *Cylindrocarpon album* and *Melanconium atrum* occurred in whole stems of Fagus only, Coniochaeta tetraspora and Sporormiella australis in whole stems of Pinus. Five species were non-host specific and occurred in all four morphological units. A cluster analysis showed that Pinus tissues can be separated from Fagus tissues on the basis of their endophyte populations, and a K-means cluster analysis revealed that eleven of the fungi isolated were mainly responsible for this separation. A discriminant analysis performed with these fungi demonstrated good discrimination between the four tissue sample groups.

Photita et al. (2004) conducted that fungi isolated as endophytes from wild banana (*Musa acuminata*) were tested in order to ascertain whether they are capable of causing disease symptoms in healthy banana leaves. The endophytes *Cladosporium musae, Colletotrichum gloeosporioides, Cordana musae, Deightoniella torulosa, Guignardia cocoicola, Periconiella musae* and *Pestalotiopsis sp.* were inoculated on banana leaves in vitro to test their pathogenicity. Only *Deightoniella torulosa* was

able to cause leaf spots on banana leaves in vitro. This result confirms earlier reports that fungal pathogens may be latent in their host long before the outbreak of disease symptom.

Raviraja (2005) reported that eighteen species of endophytic fungi were isolated from bark, stem and leaf segments of five medicinal plant species growing within the Kudremukh range in the Western Ghats of India. The dominant endophytic fungal species isolated from these plant species were *Curvularia clavata, C. lunata, C. pallescens* and *Fusarium oxysporum*. The highest species richness as well as frequency of colonization of endophytic fungi was found in the leaf segments, rather than the stem and bark segments, of the host plant species. The greatest number of endophytic fungal species were found within *Callicarpa tomentosa* (11 species), whereas Lobelia nicotinifolia harbored the lowest number of fungal endophytes (5 species). This study provides evidence that fungal endophytes are host and tissue specific.

Syed et al. (2009) found out the carbon nutrition of leaf endophytes and free-living members of the genus Chaetomium were compared to better understand the interaction between endophytes and their host, and the potential for endophytes to have complex life cycles. Broadly similar potential utilisation of several carbon sources was observed among endophytes and free-living isolates of Chaetomium. All fungi developed measurable biomass on glucose, cellobiose, carboxymethyl cellulose and xylan, and crystalline cellulose was slowly cleared from agar. Pectin was poorly utilised by these isolates. Sequence analysis of the fungi indicates that diverse taxa of Chaetomium may colonise wheat leaves, and that fungi with similar sequences are found in other habitats. Endophytic isolates of Chaetomium may complete part of their life cycle in other habitats.

Tanaka et al. (2005) showed that the biosynthesis of secondary metabolites by filamentous fungi their biological role is often less clear. The assumption is these pathways have adaptive value to the organism but often the evidence to support this role is lacking. We provide the first genetic evidence that the fungal produced secondary metabolite, peramine, protects a host plant from insect herbivory. Nine additional genes, which show striking conservation of microsynteny with *Fusarium*

graminearum and other fungal genomes, were identified on the perA-containing cosmid. Associations between *perennial ryegrass* and an *E. festucae* mutant deleted for perA lack detectable levels of peramine. A wild-type copy of perA complemented the deletion mutant, confirming that perA is a NRPS required for peramine biosynthesis. In a choice bioassay, plant material containing the perA mutant was as susceptible to Argentine stem weevil (ASW) (Listronotus bonariensis) feeding damage as endophyte-free plants confirming that peramine is the *E. festucae* metabolite responsible for ASW feeding deterrent activity.

Zhao et al. (2010) found out plant endophytic fungi are an important and novel resource of natural bioactive compounds with their potential applications in agriculture, medicine and food industry. In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi. During the long period of co-evolution, a friendly relationship was formed between each endophyte and its host plant. Some endophytes have the ability to produce the same or similar bioactive compounds as those originated from their host plants. This chapter mainly reviewed the research progress on the endophytic fungi for producing plant-derived bioactive compounds such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin etc. The relations between the endophytic fungi and their host plants, some available strategies for efficiently promoting production of these bioactive compounds, as well as their potential applications in the future are also discussed. It is beneficial for us to better understand and take advantage of plant endophytic fungi.

Faeth and Fagan (2002) conducted that secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism but carry out protective functions in the human body. For instance, secondary metabolites can protect the body from free radicals, modulate the immune system and kill pathogenic microbes. Plant secondary metabolites also contribute to the plant defense against herbivory.

Tejesvi et al. (2008) showed that secondary metabolites are heterogeneous group of natural compounds that may assist in survival and basic functions of the plants, such

as symbiosis, metal transport, competition, differentiation and so on. They are also widely used for pharmaceutical, medical, or agricultural purposes including natural antibiotics which are capable of inhibiting microbial growth. Secondary metabolites have a scientifically proven effect on health but many of these effects are still unknown and their effects are currently being intensively investigated and researched. Endophytes, produce a wide variety of novel bioactive secondary metabolites with unique structure which are synthesized by various metabolic pathways. Because of this, endophytic fungi as a promising source of secondary metabolites with high pharmaceutical significance for the development of drugs. The wide range of applications for endophytic metabolites involved its use in production of agrochemicals, antibiotics, immune suppressants, anti parasitic, antioxidants, and anticancer agents.

Gunatilaka et al. (2006) conducted that endophytic fungi are key resources for exploiting bioactive metabolites. The role of secondary metabolites produced by endophytes in host such as signaling, defense and regulation of the symbiosis was suggested the potential of discovering plant specific novel metabolite synthesis from endophytic fungi to be exploited as alternative source for bioactive metabolites. Endophytic fungi thus represent an untapped reservoir of unique chemical structures that have been involved in host plant protection and communication.

Bailey et al. (2006) reported that boosted by the general aim of exploiting the biotechnological potential of the microbial component of biodiversity, research on the secondary metabolite production of endophytic fungi has remarkably increased in the past few decades. Novel compounds and bioactivities have resulted from this work, which has stimulated a more thorough consideration of various natural ecosystems as conductive contexts for the discovery of new drugs. Thriving at the frontier between land and sea, mangrove forests represent one of the most valuable areas in this respect. The present paper offers a review of the research on the characterization and biological activities of secondary metabolites from manglicolous strains of species belonging to the genus Talaromyces. Aspects concerning the opportunity for a more reliable identification of this biological material in the light of recent taxonomic revisions are also discussed.

Newman et al. (2003) reported that secondary metabolites from fungal endophytes have an enormous functional diversity. Even though, the functional role of such biologically active metabolites in respect to the benefits and disfavor to the host plant is not yet clearly known, microbial endophytes are well known to biosynthesize several bioactive metabolites with potential applications as antioxidant, antimicrobial, anticancer, insecticidal drug leads, immunosuppressive and antidiabetics.

Huang et al. (2008) showed that Endophytic fungi from Mangrove plants are good source of functional metabolites. Endophyte infection found to alters pattern of gene expression in the host plant. Interaction between endophyte and plant is mainly controlled by the genes of both organism and host plant modulated by the environment. Endophytes fromangiosperms as well as gymnosperms have been studied for presence of novel secondary metabolites. Primary metabolites are common in all living cells and are involved in the formation of biomass and generation of energy, in contrary secondary metabolites are produced by one or few species only.

Silva et al. (2007) showed that secondary metabolites are low molecular weight compound, they are not required for growth in pure culture and are produced as an adaptation for the specific function in nature. Bioprospecting is most frequently used phrase to describe the collection and screening of the biological material for commercial purposes. The natural products produced by endophytes have vast range of bioactivities, representing a vast reservoir offering an enormous potential for exploitation in medicinal and industrial uses. Crude extracts from culture broth of endophytes found to show antibacterial, antifungal, antiviral, anti-inflammatory and antitumor activities.

Strobel et al. (2001) conducted that microorganisms are likely to harbor metabolic pathways that lead to the production of novel secondary metabolites. Endophytes from plant origin are investigated for their secondary metabolite production. Endophytic microbes are nonpathogenic in nature, production of secondary metabolites enable them to survive in the plant interstitial space. Many of the important secondary metabolites have been extracted and characterized from endophytic microbes. This includes alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols. In addition natural product also often

serves as lead structures whose activity can be enhanced by exploitation through synthetic chemistry.

Arnold (2007) conducted that endophytes are able to increases host fitness and competitive abilitity, by increasing nutrientional uptake, resistance to seed predators, seed germination success, tolerance to heavy metals, high salinity and good growth rate through biochemical pathways such as phytohormone indole 3 acetic acid (IAA) from fungal endophytes *Acremonium coenophialum, Aureobasidium pullulans, Epicoccum purpurascens* and *Collectotrichum Sp.* along with IAA, cytokinins were also produced by an endophytic Hypoxylon serpens. Plant provides spatial arrangement, shelter, nutrient and distribution to the next generation of microbes. Plant may provide vital compounds for the completion of the life cycle of the endophytes. Current research suggested that endophyte and plant genotypic combinations together with environmental conditions are important source of variation in endophyte host interactions. Many factors such as season, age, environment and location these may contributes and influence the biology of endophyte.

Arnold and Herre (2003) reported that endophytic microbes associated with traditionally used medicinal plants particularly of the tropics could be a rich source of functional metabolites. In this regard plant host association could also be exploited in enhancing the production of useful metabolites by the host. Genomic projects are being performed on some endophytic bacteria such as *Azoarcus sp., Herbaspirillum sp., Gluconacetobacter diazotrophicus* and *Klebsiella sp.* which will certainly be of great help for further understanding their molecular interaction with plants and may be used as well to study gene expression during endophytic life. In future such applications may consider as useful to agriculture and related fields.

2.2. Antioxidant Determination from Secondary Metabolites of Endophytic Fungi

Malinowski and Belesky (2000) conducted that natural antioxidants coumpounds are commonly found in medicinal plants, fruits, and vegetable. However, it is reported that metabolites producing endophytes can be a possible cause of novel naturaly produced antioxidants. Polysaccharides produced from plants and microorganisms which have been extensively studied and considered as natural antioxidant. Antioxidant metabolites are often obtained by endophytic fungi. Isopestacin and Pestacin were isolated from plant Terminalia morobensis and Pestalotiopsis microspore.

Bacon et al. (1977) reported that antioxidant compound in our food has a vital role as a health-protecting factor. Therefore, it is considered useful nutraceuticals on account of many health benefits. The most important function of antioxidant is trapping the free radical particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are involved in the pathogenesis of several chronical and degenerative diseases such as cancer, inflammation, neurodegenerative diseases, cardiovascular diseases and aging-related disorders. ROS and RNS can act as secondary messenger in normal physiological functions of the organism and participate in various regulatory redoxmechanism. However, problems arise with overproduction of these species which can overwhelm protective enzymes leading to destructive and lethal cellular effects.

Young et al. (2012) conducted that antioxidant works by preventing or slowing the oxidation of other molecules by free radical. Oxidation is a process of chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can generate and produce free radicals, which will initiate chain reactions that cause damage to the cells. These radicals will react with biological molecules for example DNA, proteins and phospholipids and eventuallydamage and destroy the structure of tissues and other membranes.

Tran et al. (2010) found out conversely antioxidants will terminate these chain reactions by removing and eliminating free radical intermediates, and inhibit other

oxidation reactions by being oxidized themselves. Recently, the use of natural antioxidants has been promoted and become important because of concerns regarding the safety of synthetic ones. Hence, many studies have been conducted to investigate the antioxidant activity of plant extracts. Phenolic compounds are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. There are more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Phenolics are synthesized primarily from products of the shikimic acid pathway.

Berg and Hallmann (2006) found out a common feature of phenolic compounds is the presence of at least one hydroxyl-substituted aromatic ring system. Phenolic compounds include flavonoids, phenolic acid, tannins and the less common stilbenes and lignans. Most phenolic compounds belong to the flavonoids (anthocyanin, chalcons, flavones, quercetin, kaempferol). Flavonoids are most commonly known for their antioxidant activity in vitro. Reports from in vitro studies have shown that natural phenols have antimicrobial.

Porteous et al. (2006) showed that phenolic compounds present essential functions such as contribution to the color of plants and in the reproduction and growth of plants. They act as defense mechanisms against pathogens, parasites, and predators. Plants synthesize phenolic compounds as a response to ecological and physiological conditions and mainly when they are attacked by pathogen, and insects or exposed to UV radiation and wounding.

Wang et al. (2004) reported that many cool-season grasses harbor fungal endophytes in the genus Neotyphodium, which enhance host fitness, butsimple phenols or polyphenols some also produce metabolites--such as ergovaline--believed to cause livestock toxicoses. In Claviceps species the first step in ergot alkaloid biosynthesis is thought to be dimethylallyltryptophan (DMAT) synthase, encoded by dmaW, previously cloned from Claviceps fusiformis. Here we report the cloning and characterization of dmaW from Neotyphodium sp. isolate Lp1, an endophyte of perennial ryegrass (*Lolium perenne*). The gene was then disrupted, and the mutant failed to produce any detectable ergovaline or simpler ergot and clavine alkaloids. The disruption was complemented with the *C. fusiformis* gene, which restored

ergovaline production. Thus, the biosynthetic role of DMAT synthase was confirmed, and a mutant was generated for future studies of the ecological and agricultural importance of ergot alkaloids in endophytes of grasses.

Zhang et al. (2004) conducted that the basic and common scaffold of all phenolic compounds is the aromatic ring bearing one or more hydroxyl groups. The classification of the plant phenolic compounds is based on the number of phenol units in the molecule (simple phenols or polyphenols). Based on this classification, they comprise simple phenols, coumarins, lignins, lignans, condensed and hydrolysable tannins, phenolic acids and flavonoids. Arising from a common intermediate, phenylalanine, or a close precursor, shikimic acid, more than 8,000 phenolic compounds have been discovered so far. Based upon their structure, phenolics have beendivided into two main categories: flavonoids and non-flavonoids.

Chang et al. (2002) conducted that flavonoids belong to a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in all vascular plants, where they can be in various organs: roots, stems, woods, leaves, flowers and fruits. They are represented by a group of more than 4,000 compounds, they are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection.

Guo et al. (2008) showed that the chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of chemical polymerization. Their activities are structure dependent and they help in combating oxidative stress and act as growth regulators. Flavonoids are derivatives of the FLAVONE or 2-PHENYL CHROMONE ring bearing free phenol functions, ethers or glycosides. They are complex polyphenols whose structure consists of two aromatic rings (rings A and B) and an oxygenated heterocycle.

Mc et al. (2001) found out the aim of the present study was to investigate total phenols and flavonoids content of 53 traditionally used medicinal plants of western region of India. The plants/plant parts were extracted by cold percolation method in acetone and methanol. Qualitative phytochemical analysis was done for various phytoconstituents like alkaloids, tannins, cardiac glycosides, steroids and saponins.

Total phenol and flavonoid conent was quantitatively estimated. Total phenolic contents were determined by the Folin-Ciocalteus reagent method. Total phenolic content was expressed as GAE (Gallic Acid Equivalents).

Suryanarayanan and Kumaresan (2000) showed that total flavonoid content was determined by the aluminium chloride colorimetric method. Total flavonoid content was expressed as quercetin equivalents. Tannins were present in more number of plants followed by cardiac glycosides and steroids. The Mangifera indica showed highest phenolic content, maximum content being in the methanol extract followed by Strychnos nux-vomica, Origanum marjoram, The Aristolochia bracteolate showed highest flavonoid content, maximum content being in the acetone extract followed by Phyllanthus reticulates, Argemone mexicana. Many reports suggest a positive correlation between total phenolic content and antioxidant activity so it can be stated that the Mangifera indica may possess good antioxidant property because of its high phenolic content.

Seena and Sridhar (2004) conducted that phenol and flavonoids are very common metabolite products from endophytes and have shown to be toxic to insect but not to mammals. Bioactive metabolites, such as steroids, terpenoids and diterpenes also are generated by endophytes. Endophyte also produces extracellular hydrolyases to establish a resistance mechanism against plant invasion which includes some of the extracellular enzymes like cellulases, proteinase, lipases and esterases. The actions of these enzymes found to support the hypothesis of co-evolution between endophytes and their hosts. Number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganisms.

2.3. Antifungal activity of Secondary Metabolites of Endophytic Fungi

Mandyam et al., (2010) have suggested possible competition between endophytes and the pathogen during pathogen entry. Endophytes are also considered to improve overall host performance against disease by the alteration of host nutritional chemistry. This may involve production of toxic alkaloids and feeding deterrents by endophytes. Non pathogenic *Fusarium oxysporum* isolated from Lycopersicon esculentum was found to produce soluble toxic metabolites against Meloidogyne incognita and which was identified to be lethal within 24 hours exposure.

Tran et al. (2010) conducted that the bioactivity of the fungal endophytes was examined and a number of isolates exhibited antibacterial and antifungal properties. Other isolates also exhibited amylase activity and were thus able to hydrolyse starch. This study showed that fungal endophytes are readily isolated from the phyllodes of Acacia species and that these exhibit promising bioactive properties. Thus, endophytes from Australian native plants may be a useful source of novel bioactive compounds.

Kusari et al. (2009) have reported the production of hypericin from endophytic fungus which was found to have an antimicrobial activity against several bacteria and fungi. Similarly helvolic acid has been reported to be produced by *Pichia guilliermondii* isolated from the medicinal plant Paris polyphylla var. yunnanensis.

Simanjuntak et al. (2010) conducted that endophytic fungi are novel producers of bioactive natural products. The genome sequencing of endophytic fungal species has revealed its significant biosynthetic potential, due to the presence of a large number of cryptic and diverse biosynthetic pathways. Hence, artificial use of biological efforts to activate these silent pathways can have important role to discover new natural products.

Maria et al. (2005) found out the antimicrobial potential of 14 endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum* towards selected bacteria (*Bacillus subtilis, Enterococcus sp., Klebsiella pneumoniae, Pseudomonas aerugionsa, Salmonella typhi* and *Staphylococcus aureus*) and fungi (*Candida*

albicans and *Trichophyton metagrophytes*) was tested using ethyl acetate extract of fungi cultivated under solid-state fermentation (SSF). Crude ethyl acetate extracts purified by TLC of four endophytes grown under SmF showed many fluorescent fractions. Two fractions of Aspergillus sp. 3 showed high antimicrobial activity. One of the fractions of Pestalotiopsis sp. showed considerable inhibition of *Bacillus subtilis*, Staphylococcus aureus and Candida albicans. Seven endophytic fungi were assessed for the production of extracellular enzymes (*amylase, cellulase, chitinase, laccase, lipase, protease* and *tyrosinase*) by culture plate method. Cellulase and lipase activity was present in all fungi, while amylase and protease in a few. No fungi exhibited chitinase, laccase and tyrosinase activity.

Orale et al. (2009) conducted that the function of invasive fungal infections has increased significantly during cancer chemotherapy, organ transplantation, and allogeneic bone marrow transplantation. However, only a few numbers of antifungal agents are now available for the treatment of various life-threatening fungal infections. The new antifungal agents have been available in the market and the development of protection to antifungal drugs has become increasingly superficial, especially in patients with long term treatment. Microbial natural products have been an alternative natural cause for the isolation of unique molecules for various therapeutic applications. Endophytes microorganisms live in internal tissues of plant species and represent as an abundant and symbiotic source of bioactive and chemically unique compounds with potential for desecration in a wide range of agricultural, medical and industrial field. In present different metabolites obtained from fungal endophyte with ability as antifungal agents.

Taghavi et al. (2005) reported that the requirement for new antifungal agents generally, comes from the increasing resistance of pathogenic microbes towards antibiotics. Many microorganisms of agricultural concern are also known to acquired resistance to commonly used antimicrobial chemical compounds. So the interest in natural methods of pathogen control through new, eco-friendly agents is increased. The biologically active natural products from endophytes are excellent resources for medicine, agriculture and industry.

CHAPTER III

MATERIALS AND METHODS

3.1 Site of the Experiment

The *in vitro* experiment was conducted at the Plant Protection Laboratory, Agrotechnology Discipline and Pharmaceutical Chemistry Laboratory, Pharmacy Discipline, Khulna University, Khulna during the period from February 2018 to February 2019.

3.2 Samples Collection

Pure cultures of pathogenic fungi were collected from Plant Protection Laboratory, Agrotechnology Discipline. Isolates of endophytic fungi were collected from Microbiology Laboratory, Pharmacy Discipline, Khulna University, Khulna.

Table 3.1 List of fungal species along with their host

Type of Fungi	Fungal Species	Host
Pathogenic Fungi	*Bipolaris sorokiniana*	Wheat
	Fusarium oxysporum	Tomato
	Alternaria solani	Tomato
	Colletotrichum musae	Banana
Endophytic Fungi	EASF-1	Gewa
	EASF-2	Gewa
	EASF-3	Gewa
	Batul-2	Batul
	Batul-3	Batul
	Batul-4	Batul
	Kewra-1	Keora
	Kewra-3	Keora
	Dakur-5	Dakur
	Kutumkata-3	Kakra

3.3 Multiplication of Endophytic Fungi

Each collected endophytic fungi was cultured in potato dextrose agar (PDA) medium, sometimes in combination with yeast extract (0.1–0.2%). To get pure culture and avoid contamination five plates were used for one endophytic fungus with control plate. After getting pure culture it was used for subculture and transferred into potato dextrose broth (PDB) medium for extraction of secondary metabolites and preserved for further use.

3.4 Preservation of Endophytes

Endophytes in the pure culture were preserved on the slant at -86°C and each tube was labeled with code number of the host plant and isolate code with date of isolation. Replicate were made for each isolates and PDA media was used according to the need of the organisms.

3.5 Preliminary test of bioactivity

The endophytic fungi were tested for production of agar-diffusible and volatile inhibitory substances against both pathogenic fungi *in vitro*. An agar plug (5 mm in diameter) taken from the margin of actively growing colonies of endophytic fungi on PDA was placed face down on one side of a plate containing PDA, incubated for 3 days at 25°C, and a similar plug of each pathogen was placed 3 cm apart on the other side of the plate. After 5 days of incubation at 25°C, the diameter of the pathogen colonies was measured and compared with the control (pathogen alone).

3.6 Extraction of Secondary Metabolites

The strains that showed potentiality in the preliminary testing were subjected to this process for further extraction of the metabolites.

Potato Dextrose Broth (PDB) is considered to be the best media for fermentation of fungal endophytes. For extraction a fungal strain that covered the surface of the inoculated petri dish was cut into small pieces of 1.5 cm × 1.5 cm and transferred with a sterile loop into 500 mL Erlenmeyer flask containing either 200 mL PDB. The flasks were incubated at room temperature for 10 days with occasional static and rotating condition in shaking incubator. After the incubation period, the cultures were taken out and filtered to remove the mycelia. Then the fungal metabolites were

extracted with ethyl acetate (1: 1 v/v). Furthermore, the volatile organic solvent was evaporated in vacuum evaporator to yield the crude extracts. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) or methanol for antimicrobial and other bioassay and stored at 4° C (Cui et al., 2015; Qiu et al., 2010).

3.7 Analysis of Crude Metabolites

3.7.1 Estimation of Total Phenolic Content

The fungal metabolites were subjected to this assay. The total phenolic contents of the samples were determined with the Folin-Ciocalteau reagent. Different concentrations (200, 100, 50, 25, 12.5, 6.25 µg/ml) of gallic acid equivalent were prepared in 95% ethanol, and the absorbance was recorded at 273 nm with an UV-Vis Spectrophotometer. The standard curve was plotted. 1 ml samples (1mg/ml) were dissolved in 0.5 ml of the Folin-Ciocalteau reagent and 1.5 ml of ddH_2O. Then, 1 ml of 20% sodium carbonate (Na_2CO_3) solution was added. The mixtures were shaken and incubated at room temperature for 2 h in the dark. Water was set as the control. The absorbance was measured at 760 nm. Phenol contents were expressed as mg of gallic acid equivalents (GAEs) per g of extract (Qiu et al., 2010). Samples were measured in three replicates (Maizura et al., 2011).

3.7.2 Total Flavanoid Content Assay

The fungal extracts were tested for total flavonoid content by colorimetric method. The extract (250 µL) was mixed with distilled water (1.25 mL) and sodium nitrite solution (5%, 75 µL). After 5 min incubation at room temperature, aluminum chloride solution (10%, 150 µL) was added. After 6 min, sodium hydroxide (1 M, 500 µL) and distilled water (275 µL) were added to the mixture. The solution was mixed well and incubated at 25°C for 30 min. Absorbance in triplicate was measured at 510 nm against blank. The calibration curve was prepared by preparing quercetin solutions at concentrations 200, 100, 50, 25, 12.5, 6.25 µg/ml in methanol (Chang et al., 2002). The content of flavonoid was calculated on the basis of the standard curve of quercetin and the results were expressed as mg of quercetin equivalents per g of extract (Li et al., 2015).

3.8 Calculation

The following equation was obtained from a standard Gallic acid calibration curve

$$Y = 0.004538X + 9.9e\text{-}005;\ R^2 = 0.9995$$

Where y is the absorbance and x is the concentration of Gallic acid (µg/ml).

Based on the measured absorbance, Gallic Acid Equivalent (GAE) was read (µg/ml) from the calibration line by using the following equation

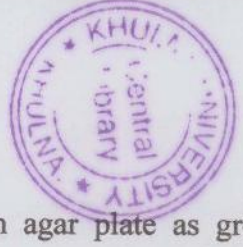
$$\text{GAE} = \frac{\text{Absorbance of sample} - 0.1254}{0.006969}$$

Then total phenolic content (TPC) in fungal extract in Gallic acid equivalents (GAE) was calculated by using the following equation

$$\text{TPC}(\mu\text{g GAE/mg}) = \frac{\text{GAE} \times \text{Dilution Factor}}{\text{Weight of initial Extract}}$$

Total phenolic content value is expressed in terms of µg of Gallic acid equivalent (GAE) per milligram of dry extract, which is a common reference compound.

3.9. Antifungal Test



3.9.1 Broth Microdilution Method

Pathogenic fungi should be cultured 7- to 14-days on an agar plate as growing sporulation. A selective medium (PDA) was used. Three to five well-isolated colonies of the same morphological type from the agar plate were selected. The top of each colony was touched with a wire loop and the growth is transferred to a tube containing 4 mL of suitable broth medium. The broth culture was incubated at 35°C and prepared a 0.5 McFarland standard. The suspension was mixed and diluted so that the final concentration in each well is 5×10^5 CFU/mL. The prongs of the inoculator were delivered 0.01 mL (1: 10 dilution) into each well. MIC panel carefully was inoculated to avoid splashing from one well to another. 10µl prepared microbial suspension was inoculated 50µl 0.9% saline and 40 µl extract or standard solution and incubated the plate at 37°C. The purity of the plate was examined by using reflected

light and then using transmitted light. Sometimes contamination is subtle. If the purity plate shows contaminants, the MIC test cannot be interpreted and should be repeated.

3.10 Identification of the Microorganisms

The identification procedure of endophytic fungi was based on morphology and molecular methods. The morphological characters included cultural characteristics and the morphology of conidia.

3.10.1 Molecular identification based on internal transcribed spacers (ITS)

Isolates of EASF-1, 2, 3 were grown in 250 mL conical flask containing 100 mL potato dextrose broth [20% potato, 2% dextrose (w/v)] for 7 days. The cultures were incubated at 25±1 °C maintaining 12/12 h light and dark period. The flasks were shaken continuously on a rotary shaker (Wisd® laboratory instruments, Ireland) at150 rpm. Approximately 20 g mycelia were harvested and separated by filtration through Whatman No.1 filter paper. They were washed thrice with sterile water and freeze-dried at -86 °C in Ultra low freezer (Thermo scientific, USA) for lyophilizing the sample. The mycelial mass was finely ground in liquid nitrogen and 200 mg of mycelial powder was taken in sterile 1.5 mL eppendorf tube.

PureLink fungal DNA purification kit (Invitrogen by life technologies, USA) was used for DNA extraction as per the manufacturer protocol. Genomic DNA of three isolates were extracted and stored at -20 °C. DNA extracts were run by electrophoresis in 1% agarose gel to confirming the DNA content.

The purified DNA was quantified by using sspectrophotometer (Multiskan GO Microplate, Thermo Fisher Scientific, Germany) at 260 nm wave length. The final concentration of the template DNA was adjusted at 50 ng μL^{-1} for PCR (Kandan, 2013) and stored at -20 °C.

For molecular identification the isolates were selected based on morphological variation specially colony color and conidial color. Internal transcribed spacers regions from the fungal isolates were amplified following polymerase chain reactions. Polymerase chain reactions were carried out in a 25 µL volume consisting of the following components in 0.2 mL thin walled PCR tubes for each isolate.

Table 3.2 Polymerase chain reaction chemicals for the molecular identification

Sl. No.	PCR reaction chemicals	Concentration	Quantity (µL)
1.	Genomic DNA	50 ng μL^{-1}	1.0
2.	Reaction buffer without $MgCl_2$	10 X	2.5
3.	dNTPs	10 mM	0.5
4.	Universal eukaryotic forward primer ITS5 GGAAGTAAAAGTCGTAACAAGG	5 pM μL^{-1}	0.5
5.	Universal eukaryotic reverse primer ITS4-TCCTCCGCTTATTGATATGC	5 pM μL^{-1}	0.5
6.	Taq DNA polymerase	5 U μL^{-1}	0.25
7.	$MgCl_2$	25 mM	1.5
8.	Molecular water (Sigma Aldrich, USA)	-	18.25
	Total volume of reaction mixture		25.0

Amplification was carried out in programmable thermo-cycler (Biometra, Germany) The amplification programme was optimized which involved the following steps for successful amplification of PCR products.

Table 3.3 Thermal profile for molecular identification of the endophyte isolates

Thermal profile		Motives
Temperature (°C)	Duration (minute)	
94.0	02	Hot start
94.0	0.5	Initial denaturation
55.0	01	Primer annealing
72.0	01	Primer extension
Repeated for 40 cycles		
72.0	05	Final extension
4.0	-	Hold

DNA sequence was carried out using genetic analyzer (Applied Biosynthesis, USA) in Apical Scientific Laboratory of Malaysia. Schematic diagram of fungal rDNA gene

encoding 18S, 5.8S and 28S are separated by the Internal Transcribed Sequences presented in Figure 3.1.

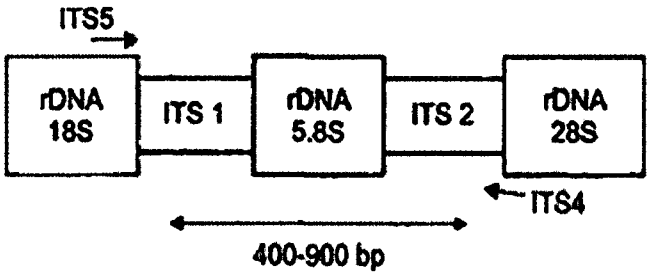


Figure 3.1 Schematic diagram of the fungal rDNA gene cluster

(Gene encoding 18S, 5.8S and 28S Ribosomal RNA subunit are separated by the Internal Transcribed Sequences; Brasileiro *et al.*, 2004).

The amplified products were resolved by electrophoresis on 1% agarose gel stained with ethidium bromide (at 0.5 μg mL^{-1}) run at 60 volts in Tris Acetate EDTA buffer (1.0× TAE) for one hour. Resulted amplicon profiles of all the isolates across the primers were visualized under UV Tech Gel documentation System with computer program (Biometra gel documentation, A Biodoc Analyze 2.2 version, Germany). A molecular weight marker 1 kbp (Direct load, Sigma Aldrich, USA) was used for sizing of the amplicons.

3.10.2 ITS rDNA sequence analysis

DNA sequencing of PCR products was done by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The rDNA homology searches were performed using the Basic Local Alignment Search Tool (BLAST) program through the internet server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, USA). Sequences and accession numbers for compared isolates were retrieved from the genbank database.

All centrifugation steps are carried out in a benchtop microcentrifuge. Various speeds are required for different steps, so please check your microcentrifuge specifications to ensure that it is capable of the proper speeds. All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

3.11 Evolutionary relationships

The evolutionary history was inferred using the Neighbor-Joining method (Saitou, and Nei., 1987). The optimal tree with the sum of branch length = 0.58902598 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein. 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1182 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, et al., 2018).

3.12 Statistical analysis

The calculated data were statistically analyzed on various parameters using SPSS-(Statistical Package for Social Science), Version 16.0, programe. The means for all the treatments were calculated and analyses of Linear regression. Graphs were prepared using Graphpad Prism.

applying the Folin–Ciocalteu reagent method. Manila et al. (2016) showed the highest antioxidant activity ranging from 58 mg/g to 60 mg/g GAE total phenolics from *Chaetomium sp.*, *Aspergillus sp.*, *Aspergillus peyronelii* and *Aspergillus niger* strain. Vienna et al. (2017) reported 0.544 mg/ g equivalent to pyrogallol total phenolic content of ethyl acetate crude extract.

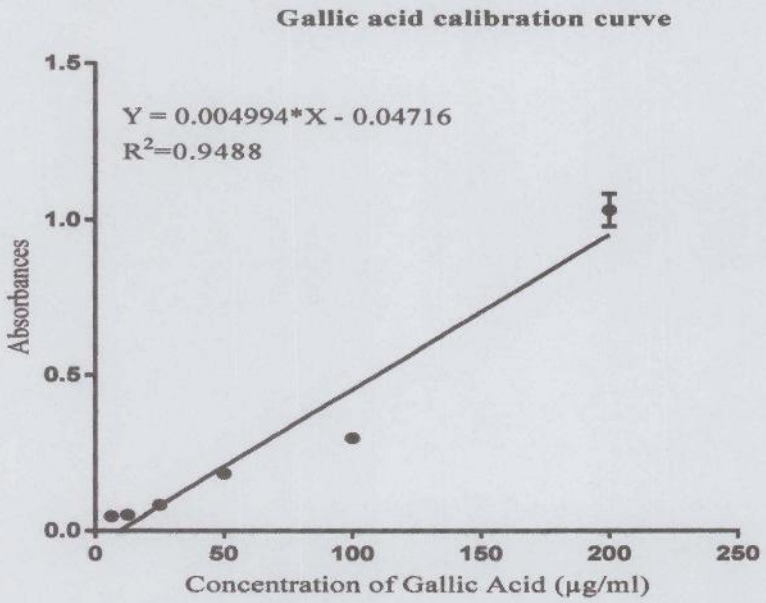


Figure 4.1 Calibration Curve of Standard for Determination of Total Phenolic Content

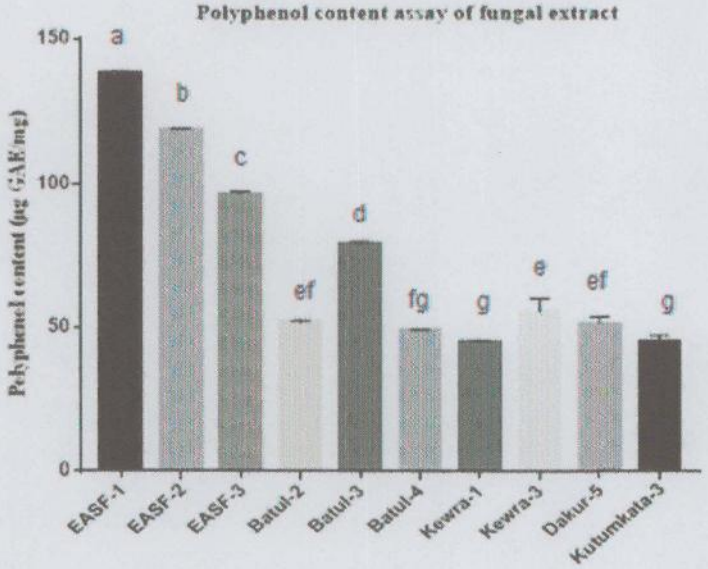


Figure 4.2 Polyphenol content in extract of endophytic fungi

4.2.2 Total Flavonoid Contents (TFC)

Flavonoid content was calculated from the regression equation of the standard plot ($Y=0.0004299x+0.0679$, $R^2=0.9831$) and was expressed as Quercetin equivalents (QE µg QE/mg of extract) (Fig 4.3).

Flavonoids content of ten endophytic fungi at absorbance 1, 2 and 3 showed variation. The highest mean flavonoids content (109.6 µg QE/mg of extract) was observed from EASF-1 endophytic fungi followed by EASF-2, EASF-3 and Batul-3 (83.25 µg QE/mg of extract and 67.73 µg QE/mg of extract and 63.08 µg QE/mg of extract, respectively). While, Batul-4 endophytic fungi had the lowest amount of flavonoids (4.113 µg QE/mg of extract). Moreover, Batul-2, Kewra-1, Kewra-3, Dakur-5 and Kutumkata-3 contained 15.76, 12.65, 31.27, 17.3 and 32.05 µg QE/mg of extract of flavonoids, respectively (Table 4.2, Fig. 4.4).

Novia et al. (2018) reported that among the four crude extracts, leaf contained the highest (31.05 ± 0.35 mgQE/g) amount of total flavonoid content compounds followed by stem (21.82 ± 0.46), seed kernel (13.21 ± 1.35) and then root (12.55 ± 0.08 mg QE/g) and Aryal et al. (2019) reported that the greatest flavonoid content was observed with extracts of *P. oleracea* (39.38 ± 0.57 mg quercetin equivalents (QE)/g) as measured by an aluminium chloride colorimetric method, while the least was recorded for *I. aquatica* (6.61 ± 0.42 QE/g).

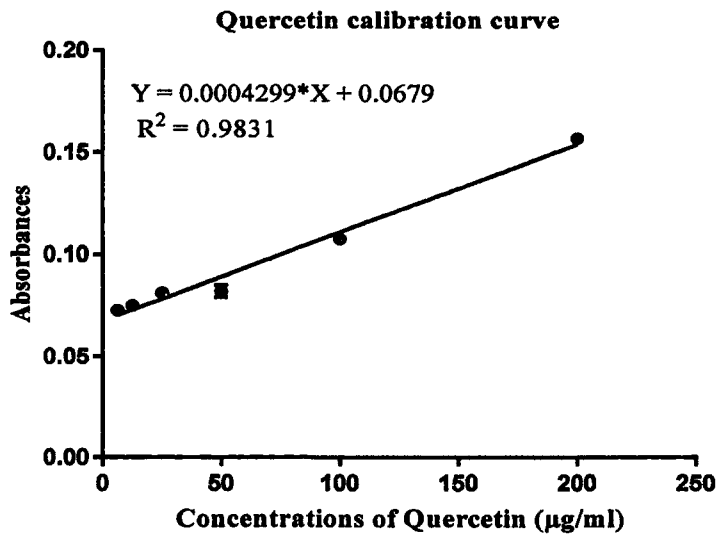


Figure 4.3 Calibration Curve of Standard for Determination of Total Flavonoid Content

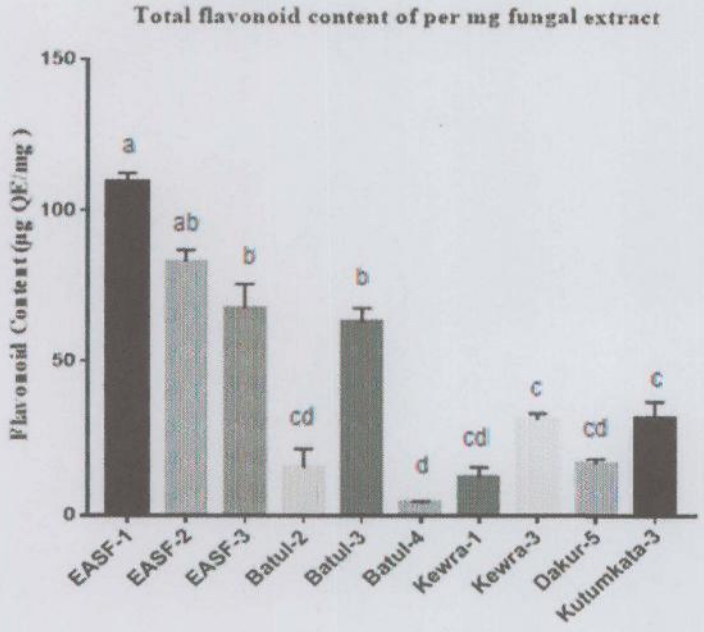


Figure 4.4 Flavonoid content of the extract obtained from endophytic fungi

4.3 Antifungal activity of endophytic fungi

Endophytic fungi (EASF-1) showed the antifungal activity against *Bipolaris sorokiniana* (Wheat), *Fusarium oxysporum* (Tomato), *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana). The Minimum inhibitory concentration (MIC) was found at 3 (mg/mL), fungistatic concentration (FS) at 3 (mg/mL) and fungicidal concentration (FC) at more than 3 (mg/mL) against *Bipolaris sorokiniana* (Wheat) on the other hand The Minimum inhibitory concentration (MIC) and fungistatic concentration (FS) was similar which was 0.375(mg/mL)and fungicidal concentration (FC) at 3 (mg/mL) against*Fusarium oxysporum* (Tomato). Whereas, MIC was >3 mg/mL in case of *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana) as such no FS and FC was found.

Endophytic fungi (EASF-2) showed the antifungal activity against *Fusarium oxysporum* (Tomato) and *Alternaria solani* (Tomato). Against *Fusarium oxysporum*, EASF-2 revealed the MIC and FS of 3 mg/mL and FC of >3 mg/mL but the MIC and FS was 1.5 mg/mL and FC was >3 mg/mL against *Alternaria solani* (Tomato). On the otherhand, EASF-2 revealed >3 mg/mL MIC and no FS and FC against *Bipolaris sorokiniana* (Wheat) and *Colletotrichum musae* (Banana).

EASF-3 showed the antifungal activity against *Bipolaris sorokiniana* (Wheat), *Fusarium oxysporum* (Tomato), *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana). In this case, Minimum inhibitory concentration (MIC) was found at 1.5 (mg/mL), Fungistatic concentration (FS) at 1.5 (mg/mL) and fungicidal concentration (FC) at more than 3 (mg/mL) against *Bipolaris sorokiniana* (Wheat), While the Minimum inhibitory concentration (MIC) was found at 0.75 (mg/mL), Fungistatic concentration (FS) at 0.75 (mg/mL) and fungicidal concentration (FC) at 3 (mg/mL) against *Fusarium oxysporum* (Tomato). Moreover, the Minimum inhibitory concentration (MIC) was observed at 3 (mg/mL), Fungistatic concentration (FS) at 3 (mg/mL) and fungicidal concentration (FC) at more than 3 (mg/mL) against Alternaria solani (Tomato) and The Minimum inhibitory concentration (MIC) was found at 0.375 (mg/mL), Fungistatic concentration (FS) at 0.375 (mg/mL) and fungicidal concentration (FC) at 1.5 (mg/mL) against Colletotrichum musae (Banana).

Batul-2, Batul-3 and Kewra-3 did not reveal the antifungal activity against four tested pathogens and MIC was > 3 (mg/mL) of fungal concentration. Similar results were found in case of Kutumkata-3 except against *Colletotrichum musae* where, MIC and FS were 1.5 (mg/mL) and FC > 3 (mg/mL).

Endophytic fungi (Batul-4 and Kewra-1) showed the antifungal activity against *Bipolaris sorokiniana* (Wheat) exhibiting MIC 3 (mg/mL) and 1.5 (mg/mL), respectively and did not reveal any antifungal activity against other pathogenic fungi.

Dakur-5 was effective against *Bipolaris sorokiniana* (Wheat) and *Alternaria solani* (Tomato) at 3 (mg/mL) MIC however, not so efficient against other two pathogenic fungi.

Among the ten endophytic fungi it was evident that EASF-3 was the most effective fungi against four tested fungal phytopathogen.

This pathogen was preliminary named as EASF-3 but data on molecular identification confirmed that the pathogen was *Trichoderma harzianum*, which was an effective biocontrol agent. *Trichoderma species* are known to be effective antagonists of several plant pathogens. *Trichoderma species* have been increasingly used as biological control agents and a few of the isolates are commercially available (Henis et al., 1984). Isolates of *Trichoderma spp.* can produce lytic enzymes (Haran et al.,

1996). *Trichoderma* species are known to produce a number of antibiotics, such as trichodermin, trichodermol, *harzianum* A and *harzianolide* (Simon and Sivasithamparam, 1988). *T. harzianum* also produce antifungal compounds viz, azaphilone and butenolide that show marked in vitro inhibition of R. solani (Vinale et al., 2006). Grondona et al (1997) reported on the antifungal activity of *T. harzianum* against soil borne pathogens with 50% inhibition on all pathogens.

Table 4.1 Antifungal activity tested of ten endophytic fungi against four pathogenic fungi

Codes of Fungi	***Bipolaris sorokiniana***			***Fusarium oxysporum***			***Alternaria solani***			***Colletotrichum musae***		
	MIC (mg/mL)	FS (mg/mL)	FC (mg/mL)	MIC (mg/mL)	FS (mg/mL)	FC (mg/mL)	MIC (mg/mL)	FS (mg/mL)	FC (mg/mL)	MIC (mg/mL)	FS (mg/mL)	FC (mg/mL)
EASF-1	3 ± 0	3 ± 0	>3	0.375 ± 0	0.375 ± 0	3 ± 0	>3	-	-	>3	-	-
EASF-2	>3	-	-	3 ± 0	3 ± 0	>3	1.5 ± 0	1.5 ± 0	>3	>3	-	-
EASF-3	1.5 ± 0	1.5 ± 0	>3	0.75 ± 0	0.75 ± 0	3 ± 0	3 ± 0	3 ± 0	>3	0.375 ± 0	0.375 ± 0	1.5 ± 0
Batul-2	>3	-	-	>3	-	-	>3	-	-	>3	-	-
Batul-3	>3	-	-	>3	-	-	>3	-	-	>3	-	-
Batul-4	3 ± 0	3 ± 0	>3	>3	-	-	>3	-	-	>3	-	-
Kewra-1	1.5 ± 0	1.5 ± 0	3 ± 0	>3	-	-	>3	-	-	>3	-	-
Kewra-3	>3	-	-	>3	-	-	>3	-	-	>3	-	-
Dakur-5	3 ± 0	3 ± 0	>3	>3	-	-	3 ± 0	3 ± 0	-	>3	-	-
Kutumkata-3	>3	-	-	>3	-	-	>3	-	-	1.5 ± 0	1.5 ± 0	>3

All result here represented with mean ± SEM, n=3

MIC=Minimum inhibitory concentration

FS= Fungistatic concentration

FC= Fungicidal concentration

"-"= data not avaiable

4.4 Molecular Identification of endophytic fungi

Molecular identification was done to identify Mangrove associated three endophytic fungi Namely EASF-1, EASF-2 and EASF-3 as they provided better antifungal activity. Molecular identification of endophytic fungi was done following barcode to ensure the species. Gene sequencing of the fungi has effectively result to identify and conform from Gene bank. In the present investigation, sequenced endophytic fungi were identified as *Paecilomyces tenuis*, *Talaromyces sp* and *Trichoderma harzianum,* because the identified fungi were showed respectively 99.70%, 99.33% and 100% similarity with the Gene bank (Table 4.6). Result of this study proved that identified fungi are matched with *Paecilomyces tenuis*, *Talaromyces sp* and *Trichoderma harzianum* following Gene bank references are KX621968.1, LT558969.1 and MG707197.1. The name of endophytic fungi is determined as *Paecilomyces tenuis*, *Talaromyces sp* and *Trichoderma harzianum.*

Table 4.2 ITS rDNA sequence of EASF-1 (*Paecilomyces tenuis*), EASF-2 (*Talaromyces sp*) and EASF-3 (*Trichoderma harzianum*)

Species Name	ITS rDNA sequence
Paecilomyces tenuis (KX621968.1) Code name EASF-1	GTACAAGTTTCCGGATCAAGCCTGTCGGCGGGAACATGATTCCTTCCGCGG TGTACGCTTATCGAAGCCTCTGCAGCCACGCAAGTGGTAGGCCCGGGCGAC TCTAAACAACCCGGTGCCGCTGCAAGTCCGCGCCCCCTCCCGGGACGTGGG CGACACTTTTGAATTGACGGGGACACCCTAACGCCGAACGCACCAAGCCG TGCCGGGAAACCGCACGGGGGCCCATGCGAAAGACATGGGGGACGGTTAC AGACGATTCGGATGACCTGCCACGGCAGCGAAATGGGCAATCCGCAGCGA AGCCCCTACGGCCTCCTGCCTGCGCGGCGGGAAGGCTACGGGGAACGTTC ACAGACTAAGTGGAAGTGGGTGGGATGCCGAATCCTGCTTAAGATATAGT CGGGCCCCCTGGGAGACCAGGGGGGCAAGTACACTGCTCGTGCGTGTGTA AGCTGTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTCCG CTCTGGGCCCAACCTCCCACCCGTGTCTCTTGCGTACTTTGTTGCTTTGGCG GGCCCACTGGGTCACTCCGGTCGCCGGGGGGCGCTATGCTCCCGGGCCCGT GCCCGCCAGAGCACCCCTGTGAACCCTGATGAAGAGAGGCTGTCTGAGTC CCACGATAATCGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGT GAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGC ATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCGCGGCTTGTGTGTTGGGC GTGGTCCCCCTGTCTTTGGCGGGGACCTGCCCGAAAGGCAGCGGCGACGTC CCGCCTAGTCCTCGAGCGTATGGGGCTCTGTCACGCGCTCGGGAGGGACTG GTGGGCGTTGGTCACCCCTTATTCTTTCTACGGTTGACCTCGGATCAGGTAG GAGTTACCCGCTGAACTTAGCATCA

Species Name	ITS rDNA sequence
Talaromyces sp (LT558969.1) Code name EASF-2	TGGAAGTAAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGA TCATTACCGAGTGCGGGCCCTCGCGGCCCAACCTCCCACCCGTGTCTCTCT CTACCTGTTGCTTTGGCGGGCCCACCGGGGCCACCCGGTCGCCGGGGGACG TCCGTCCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGAACCCTGATGAAGA TGGGCTGTCTGAGTCGAATGAAAATTGTCAAAACTTTCAACAATGGATCTC TTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA ATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCT GGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCCCG GCTTGTGTGTTGGGCGTGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGC GACGTCCGTCTGGTCCTCGAGCGTATGGGGCTTTGTCACTCGCTCGGGACG GATCGGCGGAGGTTGGTCACCACCACAGTTTTACCACGGTTGACCTCGGAT CAGGTAGGAGTTACCCGCTGAACTTAAGCATATCATAA

Species Name	ITS rDNA sequence
Trichoderma harzianum (MG707197.1) Code name EASF-3	TTCCTCCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATCC GAGGTCAACATTTCAGAAGTTGGGTGTTTAACGGCTGTGGACGCGCCGCGC TCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACC GCCACTGTATTTCGGAGACGGCCACCGCCAAGAGGCAGGGCCGATCCCCA ACGCCGACCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGC ATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGAT TCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCAT CGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTCGAA ACGCCTACGAGAGGCGCCGAGAAGGCTCAGATTATAAAAAACCCGCGAGG GGGTATACAAAAAGAGTTTTGGTTGGTCCTCCGGCGGGCGCCTTGGTCCGG GGCTGCGACGCACCCGGGGCAGAGATCCCGCCGAGGCAACAGTTTGGTAA CGTTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGG TTCACCAACGGAGACCTTGTTACGACTTTTTACTTCC

Table 4.3 Closest relatives of the endophytic fungal isolates

No.	Sample Code	Matched Gene bank ID	% of the Similarity	Gene bank Query coverage	Analyzed/ Proposed Identification of the Sample
1.	EASF-1	*Paecilomyces tenuis* (KX621968.1)	99.70%	96%	*Paecilomyces tenuis*
2.	EASF-2	*Talaromyces sp.* (LT558969.1)	99.33%	100%	*Talaromyces sp.*
3.	EASF-3	*Trichoderma harzianum* (MG707197.1)	100%	100%	*Trichoderma harzianum*

4.5 Evolutionary Relationships

From the evolutionary relationships, It was observed that endophyti fungi EASF-1, EASF-2 and EASF-3 are closely related to all other fungi which are include in the Figure 4.5 but they are very closely related to *Paecilomyces tenuis* (KX621968.1), *Talaromyces sp.* (LT558969.1) and *Trichoderma harzianum* (MG707197.1) respectively.

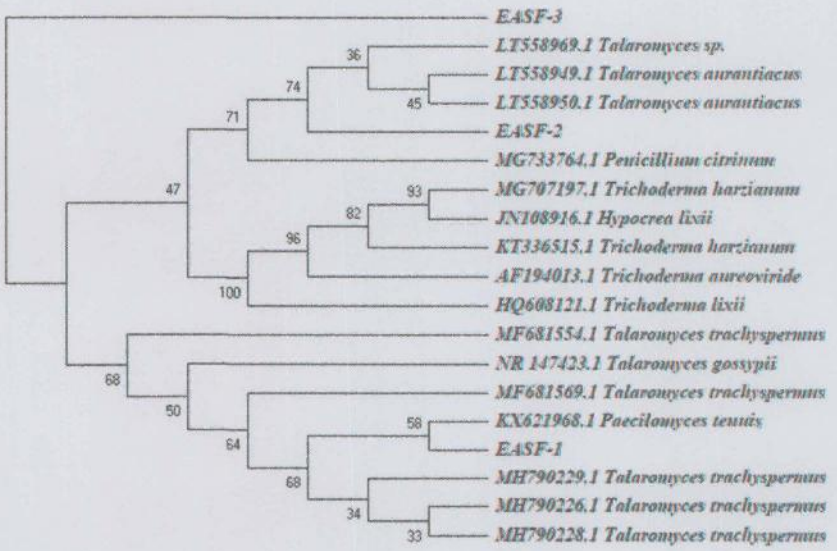


Figure 4.5 Evolutionary relationships of taxa

CHAPTER VI

SUMMARY AND CONCLUSION

Endophytic fungi from Mangrove plants exhibit excellent bioactivities such as antifungal and their natural products are highly active. This proves the holiest approach of this plant in traditional activity against pathogenic fungi such as *Bipolaris sorokiniana* (Wheat), *Fusarium oxysporum* (Tomato), *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana). Considering the significance of endophytic fungi, the present experiment was undertaken to determine the total phenol and antifungal activity of these endophytic fungi. The experiment was conducted in the plant protection laboratory, agrotechnology discipline and and Pharmaceutical Chemistry Laboratory, Pharmacy Discipline, Khulna University, Khulna during the period from February 2018 to February 2019.

35 endophytic fungi tested preliminary agaist pathogenic fungi by Block Method. Among them ten endophytic fungi namely, EASF-1, EASF-2, EASF-3, Batul-2, Batul-3, Batul-4, Kewra-1, Kewra-3, Dakur-5 and Kutumkata-3 showed antifungal activity.

Among ten endophytic fungi, EASF-1 endophytic fungi collected from Sundarbans showed the highest TFC (109.6 µg QE/mg of extract) and TPC (138.4 µg GAE/mg of extract) followed by EASF-2, EASF-3. The Batul-4 and the Kewra-1 endophytic fungi showed the lowest (4.113 µg QE/mg of extract) amount of TFC and TPC (45.15 µg GAE/mg of extract).

In case of antifungal activity, EASF-1 showed activity against *Fusarium oxysporum* (Tomato) and *Bipolaris sorokiniana*, EASF-2 showed the antifungal activity against *Fusarium oxysporum* (Tomato) and *Alternaria solani* (Tomato). Endophytic fungi (EASF-3) showed the antifungal activity against four tested pathogens.

Molecular identification was done to identify Mangrove associated three endophytic fungi namely EASF-1, EASF-2 and EASF-3 as they provided better antifungal activity. Molecular identification of endophytic fungi was done following barcode to ensure the species. Sequencing of the ITS resion fungi has effectively result to identify and conform from Gene bank. In the present investigation, sequenced endophytic fungi EASF-1, EASF-2 and EASF-3 were identified as *Paecilomyces*

tenuis, *Talaromyces sp* and *Trichoderma harzianum,* respectively because the identified fungi showed similarity 99.70%, 99.33% and 100% respectively with the Gene bank.

From the above experiment it can be concluded as follows:

- EASF-1 endophytic fungi collected from Sundarbans showed the highest TFC (109.6 µg QE/mg of extract) and TPC (138.4 µg GAE/mg of extract) followed by EASF-2, EASF-3.
- The Batul-4 and the Kewra-1 endophytic fungi showed the lowest amount of TFC (4.113 µg QE/mg of extract) and TPC (45.15 µg GAE/mg of extract), repectively.
- Among the ten endophytic fungi it was evident that EASF-3 was the most effective fungi against four tested fungal phytopathogen (*Bipolaris sorokiniana* (Wheat), *Fusarium oxysporum* (Tomato), *Alternaria solani* (Tomato) and *Colletotrichum musae* (Banana)).
- According to molecular identification the sequenced endophytic fungi were EASF-1, EASF-2 and EASF-3 and identified as *Paecilomyces tenuis*, *Talaromyces sp* and *Trichoderma harzianum,* respectively.

CHAPTER VII

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