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Bioprospecting for Microbial Endophytes and Their Natural Products

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Abstract

Endophytes are microorganisms that reside asymptomatically in internal tissues of all higher plants. There is growing interest amongst the researchers about this group of organisms because these are promising sources of biologically active agents. They are potential sources of novel natural products for scope of utilization in the pharmaceutical industry, agriculture, and in environmental applications. Many researches have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry. So far, a great number of novel natural products possessing antimicrobial activities have been isolated from endophytes. It is believed that screening for antimicrobial compounds from endophytes is a promising way to overcome the increasing threat of drug resistant strains of human and plant pathogen. Antimicrobial metabolites isolated from endophytes belong to diverse structural classes, including: alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids. These would provide the opportunity to utilize endophytes as a new source for production of antibiotics.

Key words: Endophytes; Natural products; Life cycle; Bioprospecting; Biodiversity; Bioremediation

Introduction

The term 'endophyte' (Gr. *endon*, within; *phyton*, plant) was first coined by de Bary (1866). They are microorganisms that inhabit living healthy plant tissues without causing any apparent manifestation of symptoms, and live in mutualistic association with plants for at least some part of their life cycle (Bacon and White, 2000). Initially the term endophyte broadly included organisms from foliar pathogens to mycorrhizal root symbionts. Later, fungi producing visible disease symptoms were excluded from this category even though all pathogenic fungi penetrate the host tissue and exist endophytically (Carrol, 1986). The term "endophyte" has undergone transformations though there still is considerable disagreement as what constitute an endophyte. Endophytes are extremely ubiquitous and it is hypothesized that the vast majority of plant species in natural ecosystems (if not all of them) harbor endophytes (Rodriguez *et al.*, 2009). All the species of plants studied to date are expected to harbour at least one such organism, nevertheless, endophyte-plant relationships are not well understood. Endophytes belong to diverse groups of bacteria, fungi, actinomycetes etc. (Bandara *et al.*, 2006). The most frequently isolated endophytes are fungi (Tayung *et al.*, 2008). Endophytic fungi are estimated to be represented by at least one million species residing in plants (Dreyfuss *et al.*, 1994). Usually, the fungal endophytes belong to Ascomycetes, Deuteromycetes and Basidiomycetes (Petrini 1986, Dayle *et al.*, 2001, Rakotoniriana *et al.*, 2008). As many as 110 different fungal species have been isolated from their coniferous hosts (Tayung *et al.*, 2008). The class and species of the fungi depend upon the host plants they are associated with. Krabel *et al.*, (2013) have hypothesized that the environmental conditions probably trigger the wood associated endophytic fungi to change from a mutualist to a virulent parasite. Research on endophytes dated back to over one hundred years (Petrini, 1986). During this period, several aspects of endophyte biology were thoroughly studied, including the diversity, taxonomy, reproduction, host ecology and effects on the host (Saikkonen *et al.*, 1998). They produce secondary metabolites, enhance hardiness of host plants, Provides resistance to fungal diseases by producing antimicrobial compound *in situ*. Increase plant resistance to haribivores and enhance plant competitive abilities. Because natural selection favors the evolution of beneficial endophytic strains, several endophytes were found to secret secondary metabolites that protect plants against herbivore (Robert *et al.*, 2004), insect (Spiering *et al.*, 2005), pathogens (Arnold 2003), abiotic stress (Waller *et al.*, 2005)

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and thus, endophytes represent a promising source of novel, biologically active metabolites for pharmacological and agricultural applications (Dreyfuss *et al.*, 1994; Schulz *et al.*, 2002). They can be used as biocontrol agents (Waller *et al.*, 2005).

Biochemical research revealed that a wide variety of natural products can be obtained from endophytic microbes (Schulz *et al.*, 2002; Strobel and Daisy, 2003). Natural products from endophytic fungi were observed to inhibit many pathogenetic organisms including bacteria, fungi, viruses and protozoans. Idris *et al.*, 2013, isolated *Cladosporium* sp. *Aspergillus* sp from *Kigelia Africana*, which inhibit *Bacillus* , *subtilis*, *Staphylococcus aureus* and *Escherichia coli* (I. Z. D. > 20 mm). Cercosporin, an effective anti-parasitic agent, has been isolated from the endophytic fungus *Mycosphaerella* sp associated with the plant *Sychotria horizontalis* in panama (Moreno *et al.*, 2011). Many antiviral agents were reported from endophytic fungi; two novel compounds cytonic acid A and B have been isolated from the endophytic fungus *Cytonaema* sp.

These compounds are inhibitor of human cytomegalovirus (hCMV) protease (Guo *et al.*, 2000). Due to host-endophyte coevolution, some plants that produce bioactive natural products have associated endophytes that produce the same natural products (Tan *et al.*, 2001). Since the microbial sources of bioactive compounds are easier and more economical for large-scale production than plant sources, the discovery that rare, valuable plant products might also be produced by their endophytic microorganisms is of special pharmacological interest (Strobel *et al.*, 2003).

A famous example is the anticancer agent 'Taxol' that is found in yew tree species (*Taxus sp.*). Stierle *et al.* (1993) have isolated and characterized a novel taxol producing fungus *Taxomyces andreanae*, from *Taxus brevifo*lia. Thus, when searching for novel, endophyte-based drugs, a particularly fruitful approach would be to survey traditional medicinal plants for the bioactive metabolites that may be produced by their associated endophytes (Verma *et al*, 2007; Huang *et al.*, 2008; de Siqueira, 2011). This review, therefore, focuses on the biology of endophytic organisms, their evolution and bioprospecting for natural products.

Evolution of endophyte–plant symbioses

All fungi invading plant foliage have an asymptomatic period in their life cycle that varies from imperceptibly short period (e.g. pathogens) to a life time (e.g. *Neotyphodium* endophytes in grasses). The endophyte is found in the embryo of infected seeds. They grow in to emerging leaves as the seed germinates. They remain concentrated in the base of the plants and not in roots. The endophyte grow up the stem and in to the seed head of the reproductive plant. Researchers believe that Endophytes may have developed genetic systems to communicate between themselves and the host plant (Borges *et al.*, 2009). Another probability of their evolution is their long-term coexistence within their hosts which resulted in a co-evolutionary process enabling these organisms to acquire interesting capabilities, such as ability of some of them to synthesize biologically active substances similar to the secondary metabolites produced by their hosts (Wang and Dai, 2011). This feature if utilized with the help of biotechnology could solve many problems we are facing today. Endophytes have been recognized as outstanding sources of novel bioactive compounds (Strobel, 2003). Some produce volatile organic compounds that benefit host plants by providing additional lines of defense against pathogens (Macías-Rubalcava *et al.*, 2010). Morath *et al.* (2012) advocated that the small gas-phase molecules be utilized through biotechnology because of their ability to produce a broad spectrum of aromatic compounds, including pleasant VOCs having useful agricultural and industrial properties (Zhi-Lin *et. al.*, 2012). Endophytic fungus- grass associations are generally treated separately from parasitic, pathogenic and saprophytic interactions and are viewed as mutualistic associations. Benefits to the partners are rarely symmetric and conflicting selection forces are likely to destabilize them. Endophyte–host interactions are based on mutual exploitation. There are, however, unanswered questions like how genetic diversity of the fungus and phenotypic plasticity in fungal life history traits, genetic combinations between the fungus and host, and the fungus and host individually or in concert as a phenotypic unit, respond to the changing selection pressures.

All plants are infested with microbes and may be symptomless i.e. epiphytes or endophytes and represent balanced state of symbiosis or with symptoms i.e. diseased. They live asymptomatically and intercellularly within plant tissues. Both fungi (most frequently isolated) and Bacteria inhabit the plants. Relationship with host may be symbiotic or mutualistic. Some believe they may be aggressive saprophytes or opportunistic pathogens. This state represents the majority. The second category represents unbalanced state of symbiosis and are termed as pathogens. In endophyte plant relationships endophytes gain i.e. get shelter and nutrients this costs plants nutrients and resources. The plant gains in terms of growth promotion due to enhanced nutrient uptake, increased tolerance to harsh environments e.g. drought tolerance, induced resistance to pests and diseases.

The majority of published works indicate that endophytic fungi can be regarded as plant-defending mutualists producing biologically active alkaloids responsible for the evolution of the endophytic life-style of these fungi (Clay and Schardl, 2002). It, however, appears that the key factors responsible for evolution of the endophytic life-style of fungi are more complex involving multi-species interactions, multiple levels of causation and multidirectional flows of influence, and are influenced by stochastic events, such as abiotic and biotic environmental conditions, that drive the life histories of coevolving fungi and host plants (Fig.1) (Saikkonen *et. al.,* 2004).

Over the past 20 year much has been studied about a unique symbiotic interaction

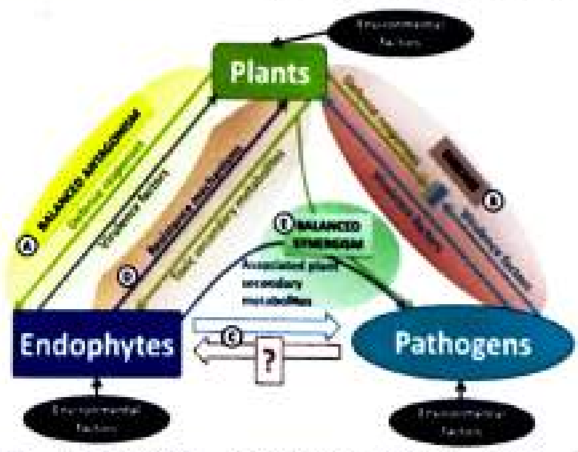

Fig. 1: Possible relationship between plant and microorganisms leading to evolution of endophytes (A) Balanced antagonism hypothesis (B) Plant disease caused by pathogenic fungi; (C) Endophyte-pathogen reciprocity. The question mark (?) indicates that this phenomenon might not be universal, and further research is necessary for verification. (D). Endophyte survival strategy; (E) Balanced synergism (Adapted from Saikkonen *et. Al.,* 2004).

between fungal endophytes and grasses. The fungi (Clavicipitaceae, Ascomycota) grow intercellularly and systemically in aboveground plant parts. Asexual endophytes of cool-season grasses that get vertically transmitted have been repeatedly derived from sexual species that abort host inflorescences. The phylogenetic distribution of seed-transmitted endophytes is strongly suggestive of cocladogenesis with their hosts and the molecular data suggest that many seed-transmitted endophytes are interspecific hybrids. Superinfection may result in hyphal fusion and parasexual recombination. Most endophytes produce one or more alkaloid classes that likely play some role in defending the host plant against pests. Hybridization may have led to the proliferation of alkaloid-production genes among asexual endophytes, favouring hybrids. The ergot alkaloid ergovaline, lolitrems, and lolines are produced by only a single sexual species, *Epichloe festucae,* but they are common in seed-transmitted endophytes, suggesting that *E. festucae* contributed genes for their synthesis. Asexual hybrids may also be favoured by the counteracting of the accumulation of deleterious mutations (Muller's rachet). Endophyte infection can provide other benefits, such as enhanced drought tolerance, photosynthetic rate, and growth. Estimates of infection frequency have revealed variable levels of infection with especially high prevalence in the subfamily Pooideae. Longitudinal studies suggest that the prevalence of seed-transmitted endophytes can increase rapidly over time. In field experiments, infected tall fescue suppressed other grasses and forbs relative to uninfected fescue and supported lower consumer populations. Unlike other widespread plant/microbial symbioses based on the acquisition of mineral resources, grass /endophyte associations are based primarily on protection of the host from biotic and abiotic stresses.

"Endophyte" is a generic term for any organism that lives inside of a plant, analogous to an epiphyte living on the plant surface. There has been semantic disagreement over usage of the term endophyte, with the suggestion that the word implies a mutualistic relationship that may not exist and that other words may be better (Wennstrom, 1994). For example, some plant pathogens like smuts may exist internally and asymptomatically within host plants for many years before they finally become evident.

Nevertheless, a large variety of heterotrophic organisms exist internally within plants, at least during part of their life cycle, without producing any disease symptoms. Most attention has been focused on fungal endophytes that exist in leaves, stems, and reproductive organs of host plants. The straight forward technique of surface sterilizing a leaf or leaf segment and plating it out on nutrient agar will generally result in the outgrowth of one or more fungi even if the leaf was asymptomatic. Asymptomatic, endophytic fungi may be ubiquitous in the plant kingdom, reviling insects in their species diversity (Carroll 1988; Arnold *et al.* 2000). But we know relatively little at present about the distribution and diversity of endophytes in different plant groups and plant communities, outside of a few well studied examples. Grass Endophytes Grass endophytes may constitute a monophyletic clade with the fungal family Clavicipitaceae (Ascomycota; Kul dau et al. 1997), but even with recent data it remains unclear if this is a monophyletic clade. Clavicipitaceous fungi include parasites of the grass family (and occasionally sedges) that can form pathogenic or mutualistic relationships with their hosts. Three of the four tribes infect only grasses or sedges, while the fourth tribe, Cordycipieae (genus Cordyceps), is pathogenic on insects or other fungi (Kuldau *et al.*, 1997). The tribe Clavicipeae (i.e., Claviceps) parasitizes a wide range of grasses where it forms infections of single grass florets and replaces the seed with individual sclerotia. These are the well-known ergot pathogens that produce toxic ergot alkaloids (Groger, 1972). The most diverse tribe is the Balansieae, consisting of several genera forming systemic infections of host grasses that also produce alkaloids (Bush *et al.*, 1997). One genus (*Epichloe*) has spawned a diversity of asexual forms (*Neotyphodium* species) that have radiated in association with cool-season grasses in the subfamily Pooideae (Schardl, 1996). Our primary focus is on these associations, reflecting the substantial research efforts devoted to this group. Growth in the Host Growth of clavicipitaceous endophytes in grasses exhibits several distinctive features. Growth is systemic throughout the aboveground tissues of their hosts (fig. 1A). Sparsely branched hyphae grow parallel to the long axis of plant cells in intercellular spaces where they likely subsist on sugars and amino acids released into the apoplast. Infections are perennial such that plants will remain infected throughout their life span; although sectoring and loss of infection in particular segments of host plants can be occasionally observed. During host flowering, the fungus grows into ovules and seeds or it proliferates to form a fruiting body. Molecular evidence suggests that most endophyte hosts are infected by only a single fungal genotype (Kover *et al.*, 1997; Meijer and Leuchtmann, 1999). Multiple infections can occur occasionally and may be highly significant because they afford the opportunity for fungal hybridization (Schardl *et al.*, 1994; Tsai *et al.*, 1994). Experimental inoculations with multiple strains show that all but one strain is eventually excluded at either the whole plant or tiller level (Wille *et al.*, 1999; Christensen *et al.*, 2000). In other endophytic associations with nongrass hosts, infections are typically highly localized and may consist of only a few epidermal cells. One leaf or one plant may be infected by dozens or hundreds of distinct fungal species (An *et al.*, 1992; Saikkonen *et al.*, 1998; Arnold *et al.*, 2000).

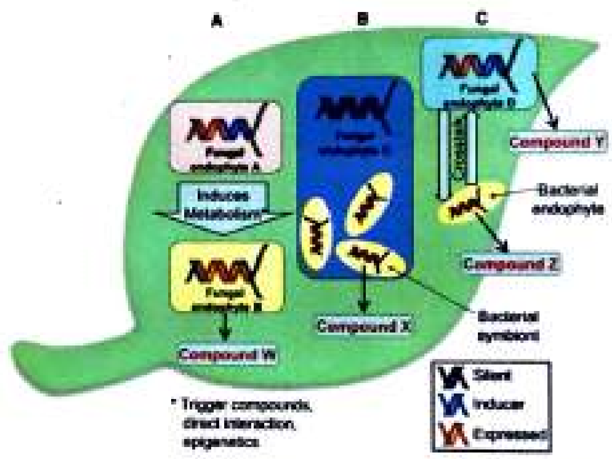

Fig.2 Communication sharing amongst different endophytic organisms leading to production of different molecules (A) Fungus-fungus crosstalk; (B) Fungus-bacterial endo-symbiont crosstalk (Adapted from Saikkonen *et. al.*, 2004)

Endophyte–host interactions are based on mutual exploitation

All fungi invading plant foliage have an asymptomatic period in their life cycle that varies from an imper-ceptibly short period (e.g. pathogens) to a lifetime (e.g. *Neotyphodium endophytes* in grasses). Endophytic fungus–grass associations are generally treated separately from parasitic, pathogenic and saprophytic interactions and are viewed as mutualistic associations. However, endophyte–host interactions are based on mutual exploitation. Benefits to the partners are rarely symmetric and conflicting selection forces are likely to destabilize them. Unanswered questions are how (i) genetic diversity of the fungus and phenotypic plasticity in fungal life history traits, (ii) genetic combinations between the fungus and the host, and (iii) the fungus and host individually or in concert as a phenotypic unit, respond to changing selection pressures (Saikkonen *et al.,* 2004).

Although knowledge of the ecology, life history and phylogeny of endophytic fungi has accumulated rapidly during the past two decades, basic questions about the evolutionary origin, speciation and ecological role of endophytes remain largely unanswered [Clay *et al.,* 2002]. Although the term 'endophyte' has been controversial since it appeared [De Bary *et al.*, 1866, Petrini, 1991, Wilson, 1995], it has become synonymous with mutualism. However, recent studies show that the ecological role of even systemic grass endophytes can be complex and labile. Functionally, in terms of interactions with their host, different fungi are scattered throughout phylogenetic lineages [Clay *et al.*, 2002, Saikkonen *et al.*, 1998; Faeth *et al.*, 2002]. Moreover, defense of the host plant via endophyte mycotoxins, the most often cited mechanism of mutualism, discovered in agronomic grasses, seems rare in most native grass– and tree– endophyte interactions (Saikkonen *et al.*; 1998, Faeth, 2008; Ahlholm, 2002; Faeth, 2002). Nonetheless, the majority of published studies are still based on the conventional wisdom that endophytic fungi are plant-defending mutualists, with fungus-produced, biologically active alkaloids as key to the evolution of the endophytic life-style of these fungi (Clay, 2002). We propose that key elements for the evolution of the endophytic life-style of fungi are more complex, and involve multi-species interactions, multiple levels of causation and multidirectional flows of influence, and are influenced by stochastic events, such as abiotic and biotic environmental conditions, that drive the life histories of coevolving fungi and host plants.

Genetic diversity of the fungus and phenotypic plasticity in fungal life history traits

A number of pasture and turf grass species form mutually beneficial symbiotic associations with endophytic fungal species. Within the fescue grasses, diploid meadow fescue (*Festuca pratensis* Huds.) interacts with *Neotyphodium uncinatum* while allohexaploid tall fescue (*Festuca arundinacea* Schreb.) has been reported to associate with *Neotyphodium coenophialum* and two other morphologically distinct taxa (*Festuca arundinacea* taxonomic groups 2 and 3 [*Fa*TG-2 and *Fa*TG-3]). The evolutionary history of hexaploid tall fescue is complex, as part of a species group with varying ploidy levels and exhibiting distinct eco-geographical morphotypes. To evaluate both naturally occurring variation and host grass taxon specificity, diversity was determined in collections representing multiple meadow fescue and tall fescue accessions. Initial screening with a minimal set of endophyte-specific simple sequence repeat (SSR) genetic markers detected endophyte incidence in 33% of 701 tested accessions.' Subsequent analysis identified N. coenophialum genotypes within continental and rhizomatous hexaploid and octoploid tall fescue [F. arundinacea sub sp. atlantigena (st.-Yves) Auquier] accessions. Festuca arundinacea taxonomic group 2 and FaTG-3 endophytes appeared to be restricted to Mediterranean hexaploid and decaploid tall fescue [F. arundinacea cirtensis (St.-Yves) Gamisans] hosts. Endophytes of meadow fescue were confirmed as belonging to N. uncinatum. This study has elucidated host specificity of fescue endophyte taxa and supported models for host–symbiont coevolution. A substantial number of candidate novel endophytes have been identified that are suitable for metabolic characterization and deployment by inoculation in fescue breeding programs (Ekanayake et al., 2011)

Lima *et al.,* 2012, reported thirty-nine endophytic fungi identified as *Colletotrichum* spp. associated with Brazilian pepper tree or aroeira (Schinus terebinthifolius Raddi. Anacardiaceae)in Parana state, Brazil. These endophytes were identified by morphological ' and molecular methods, using PCR taxon-specific with CaInt/ITS4, CgInt/ITS4, and Col1/ITS4 primers, which amplify specific bands in *C. acutatum, C. gloeosporioides lato sensu*, and *Colletotrichum boninensis*, respectively, and by DNA sequence analysis of the rDNA internal transcribed spacer region (ITS1, 5.8S, ITS2). We also assayed the presence of dsRNA particles in *Colletotrichum* spp. isolates. Combining both morphological characters and molecular data, we identified the species *C. gloeosporioides*, C. *boninense*, and *C. simmondsii*. However, we found a high genetic variability intraspecific in *C. gloeosporioides* which suggests the existence of several other species. Bands of double-stranded RNA (dsRNA) were detected in three of thirty-nine isolates. Identity of these bands was confirmed by RNAse, DNAse, and S1 nuclease treatments for the isolates LGMF633, LGMF726, and LGMF729. This was the first study reporting these particles of dsRNA in *C. gloeosporioides*.

Phenotypic plasticity in fungi was observed on phenotypic changes in the colony morphology of the fungus *Aureobasidium pullulans* (Slepecky *et al.,* 2009). The variation in colony form is shown to depend on (i) the types of single carbon substrates (sugars and sugar alcohols) used in the growth medium, (ii) colony age, (iii) incubation temperature, (iv) light cycle and (v) substrate type. Expanding colonies grow in a developmental sequence that show synchronize growth phase shifts as well as unusual transitions from homogeneous to sectored, yeast to mycelial and giant to micro colonial growth forms. Epigenetic influences on phenotypic switches are suggested to be potential causes of form changes. The fungus *Aureobasidium pullulans* reversibly forms different types of colonies depending on the substrate and temperature on which it is grown, that is its environment. This property coupled with other natural attributes suggests that this microorganism could serve as a model for investigating a diversity of problems on the causes of phenotypic plasticity.

Fungi are notable for their ability to switch growth forms in response to environmental stimuli (Rayner and Coates 1987). Most likely fungi rely on the capacity to make these shifts to achieve survival, dispersal and reproductive advantages, and no doubt their success at these fundamental processes helps explain their recognition as a kingdom. The ability of fungi to alter forms and shift to different modes of living has been of interest to mycologists because of their importance to understanding fungal molecular biology, ecology and evolution as well as their utility in industry and their role in both infection and biological control. One fungus that assumes many different shapes (i.e. it is pleomorphic) and lives in a wide variety of habitats is *Aureobasidium pullulans*. This fungus has been recovered from diverse surfaces types, especially the phylloplane (Andrews *et al* 2002, McGrath and Andrews 2007, Andrews and Harris 1997, Woody *et al* 2007). Examples of other surface sources include glass (Schabereiter-Gurtner et al 2001), painted material (Shirakawa et al 2002), as well as rocks and marble (Urzı *et al* 1999, 2001). It is found in soil, freshwater and saltwater, ice (Zalar *et al* 2008) and is commonly recovered from the atmosphere (e.g. Shelton et al 2002, Lugauskas *et al* 2003, Griffin et al 2003, Samson et al 2004) and above (i.e. the Mir space station, Alekhova *et al* 2005). Unusual sources of *A. pullulans*, often as a contaminant, include for example samples containing ancient DNA (Hauf *et al* 1995), aviation fuel (Rauch et al 2006), spacecraft (La Duc *et al* 2003) and damaged nuclear reactors (Zhdanova *et al* 2000). *Aureobasidium pullulans* is involved as the principal colonizer initiating biodeterioration (e.g. plasticized polyvinyl chloride, Webb *et al* 2000) has been used as an indicator of environmental pollution (Deshpande *et al* 1992) and is implicated in human disease (Taylor *et al* 2005). The pleomorphic characteristic of fungi (Savile 1969) is also known as "phenotypic plasticity", that is the ability of any organism to respond to environmental signals by altering morphology, physiological state or behavior (West-Eberhard 1989). This ability is widespread among taxa and has been studied extensively primarily because of its importance to an organism's ability to survive and propagate. The function that describes the range of phenotypes produced by a single genotype in a suite of environments is called a "reaction norm" and is a concept generally adopted by geneticists studying evolution and ecology (Pigliucci 1996). Because over their lifetimes organisms occur in changing environmental conditions they are expected to have reaction norms that scale to the variable environment they inhabit and thus the individual is expected to be phenotypically plastic. What determines the shape of the reaction norm and how the change from one phenotype to another occurs are central questions in molecular, evolutionary and ecological genetics. Pigliucci (1996) discusses two broad approaches to studying phenotypic plasticity. One is statistical, which uses the tools developed by students of quantitative genetics. The major limitation of this method is that the assumptions underlying the theory are often too simple and as a consequence inferences about genetic mechanisms can be unrealistic. The second approach is a mechanistic study of the genes involved in phenotypic plasticity. The initial phase of this approach is to use a genetic screen designed to detect plasticity genes or genetic networks involved in the transition from one phenotype to another. To facilitate this type of work model plants and animals, such as Arabidopsis thaliana or Drosophila melanogaster, often are employed as experimental organisms. Even though microorganisms have been used for studies in plasticity (Promislow 2005, Stomp *et al* 2008), their great potential for understanding the mechanism of phenotypic plasticity have not been generally recognized, especially in fungi (Jennings 1993, Andrews 1992, Bago *et al* 2004). Bacterial colonies growing on the surfaces in Petri dishes show differentiated structures that result from a complex series of morphological events. The geometry of bacterial colonies can be a consequence of swarming, chemotactic auto-aggregation, self-engineering, intercellular communication, nutrient gradients and stress (Shapiro 1995, Ben-Jacob and Levine 2006). Shapiro (1998) emphasized the need to consider a bacterial population as a multicellular organism with complex signalling systems that result in coordinated behaviours. These emergent phenotypes of single cells growing together and communicating affect survival, movement and reproduction, all of which can be beneficial and thus evolve. Pattern formation in bacteria, such as *Bacillus subtilis* (Mimura *et al* 2000), exemplify the degree of phenotypic plasticity that can occur in microorganisms grown in relatively simple culture conditions. In this case colony morphology show characteristics of "phase transitions" where there are abrupt changes from one morphologic type to another along nutrient and agar density gradients.

Genetic combinations between the fungus and the host

Even highly mutually beneficial microbial-plant interactions, such as mycorrhizal- and rhizobial-plant exchanges, involve selfishness, cheating and power-struggles between the partners, which depending on prevailing selective pressures, lead to a continuum of interactions from antagonistic to mutualistic. Using manipulated grass-endophyte combinations in a five year common garden experiment, we show that grass genotypes and genetic mismatches constrain genetic combinations between the vertically (via host seeds) transmitted endophytes and the out-crossing host, thereby reducing infections in established grass populations. Infections were lost in both grass tillers and seedlings in F1 and F2 generations, respectively. Experimental plants were collected as seeds from two different environments, i.e., meadows and nearby riverbanks. Endophyte-related benefits to the host included an increased number of inflorescences, but only in meadow plants and not until the last growing season of the experiment. Our results illustrate the importance of genetic host specificity and transgenerational maternal effects on the genetic structure of a host population, which act as destabilizing forces in endophyte grass symbioses. Genetic mismatches may act as a buffering mechanism against highly competitive endophyte-grass genotype combinations threatening the biodiversity of grassland communities (Axelrod *et al.*, 1981) and these mismatches should be acknowledged, particularly in breeding programmes aimed at harnessing systemic and heritable endophytes to improve the agriculturally valuable characteristics of cultivars (Bronstein, 1994).

Mutualistic interactions between microbes and plants are viewed as a ubiquitous cooperation conferring reciprocal benefits to the partners. However, even seemingly highly mutualistic interactions (e.g. between plants, mycorrhizal fungi and/or rhizobia) are inherently unstable, because reciprocal cooperation is based on mutual exploitation and thus costs and benefits are rarely symmetric to the partners (Axelrod *et al.*, 1981, Bronstein, 1994, Smith et al., 1997, Saikkonen,1998, Kiers et al., 2008, Saikkonen. 2004, Cheplick et al., 2009]. Consequently, microbialplant interactions, like any other biological interspecific interaction [Axelrod *et al.*, 1981, Bronstein, 1994, Smith et al., 1997, Saikkonen et al.,1998, Kiers et al., 2008, Saikkonen et al., 2004, Cheplick et al., 2009, Pellmyr et al.,1994, Herre et al.,1998, Stadler et al., 2005, Thompson et al., 2005, Sachs et al., 2006], involve selfishness, cheating and power-struggles between the partners, thus forming a continuum of interactions from antagonistic to mutualistic [Kiers et al., 2008], with an occasional breakdown in mutualism [Sachs *et al.*, 2006]. The symbiosis between endophytes and grasses is generally considered to be a classic example of microbe-plant mutualism driving grassland communities [Omacini et al, 2001], as well as those food webs subsisting upon them [Omacini et al, 2001, Saikkonen et al, 2006]. The close link between endophyte fitness and its host grass is presumed to align the interests of both partners towards a mutually beneficial cooperation [Saikkonen et al., 2004, Cheplick et al., 2007, Saikkonen et al., 2006,, Clay et al., 2002], a view which seems to be supported by empirical evidence. In this highly integrated symbiosis, hyphae grow intercellularly and asymptomatically throughout the above-ground tissues of the host grass. Through growing into the developing inflorescence and seeds, the fungus is vertically transmitted from maternal plant to offspring. Evolutionary evidence of strictly asexual Neotyphodium and sexual Epichloe¨ endophytes suggests that such vertical transmission is concomitant with a reduced ability for contagious spreading by asexual or sexual spores and genetic host specificity (Clay et al., 2002). Because the fitness and distribution of a fungus largely depends on host fitness (Saikkonen et al., 2004), any mutualistic cooperation providing a selection advantage to the host plant also benefits the fungus. Conversely, reciprocal benefits from the fungus to the host plant, such as increased growth, resistance to biotic and abiotic stresses and enhanced competitive abilities (Saikkonen, 2005), further support the idea of endophyte-grass mutualism (Clay et al., 2002). Nevertheless, in most endophyte-grass interactions partner benefits and symbiotic dependence are asymmetric (Saikkonen, 2004). Symbiosis is essential for an endophyte because during its systematic growth the fungus subsists entirely on and within the host grass and vertical transmission via host seeds is the primary mode of fungal distribution (Clay et al., 2002). By contrast, the symbiotic relationship remains only conditional to the host plant, as plant fitness does not necessarily depend on the fungus (Saikkonen et al., 1998, Saikkonen et al., 2004, Saikkonen et al., 2006). In fact, in some environments symbiosis may even be maladaptive (Ahlholm et al., 2002, Faeth et al., 2003). For example, in endophyte species capable of sexual reproduction, the production of its fruiting body is costly to the host in terms of prevented flowering (Schardl *et al.*, 2004). Furthermore, in completely asexual endophyte strains, the adaptive value of symbiosis to the host grass appears to vary among fungal strains, being more pronounced in nutrient-rich environments (Saikkonen et al., 2006), as well as being dependent on plant-plant interactions in grassland communities (Clay *et al.*, 1999, Lehtonen *et al.*, 2015) and trophic interactions in food webs (Clay *et al.*, 1999, Rudgers *et al.*, 2008, Saari *et al.*, 2002, Saikkonen *et al.*, 2010). Accordingly, the infection incidence of grass species and populations appears to be highly variable spatiotemporally (Saikkonen *et al., 2000,* Jensen et al., 2004, Wei et al., 2006, Wali et al., 2007, Saari et al., 2009), reflecting how fungus and host alike respond to changing selection pressures, either individually or as a phenotypic unit (Saikkonen et al., 2004).Here, we use endophyte manipulation trials and a five year common garden experiment to test the importance of genetic compatibility to endophyte-grass symbiosis.

Genetic compatibility was examined in three transgenerational phases from the parental plant generation to those of the F1 and F2 generations; first at the initial encounter of the fungus and the grass, then in the success of the vertical transmission of the fungus to the vegetative propagules (tillers) and offspring of the host grass. The reasoning is that the asymmetric dependence of the endophyte and the host grass may lead to (Axelrod et al., 1981) host plant sanctions against less beneficial fungal strains in prevailing selective pressures and (Bronstein et al., 1994) the loss of the vertically transmitted fungus, which is continually confronted with new genetic combinations in the out-crossing host population. This is because the endophyte genotype remains unchanged in the plant lineage whilst plant genotypes are blended through recombination over time (Saikkonen et al., 2004). This could lead to a genetic mismatch between the fungus and the host, thus destabilizing the symbiosis and constraining the diversity of successful genotype-genotype combinations of the vertically transmitted endophytes and the host grasses.

Role of endophytes

Different works carried out so far regarding the role of endophytes in host plants indicate that they can stimulate plants growth, increase disease resistance, improve plant's ability to withstand environmental stresses and recycle nutrients (Sturz and Nowak 2000; Strobel 2002; Johri 2006). Endophytes that reside in leaves and stems of plants contribute to the host's successful survival. The array of alkaloids and other chemicals synthesized by the endophytes endow the plant with more resistance to nematodes (worms), insect herbivores and livestock (Schulz et al. 2004). Besides these, endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan and Zou 2001; Strobel and Daisy 2003). Many of these compounds are bioactive and the range includes alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols as well as some chlorinated compounds. There is a need to investigate fungal endophytes from medicinal plants because it has been hypothesized that these plants harbor some distinct and rare microbes that mimic the chemistry of their respective hosts and synthesize identical bioactive natural products or derivatives that are more bioactive than the one produced by the host. Strobel and Daisy (2003) have necessitated the need to study plants growing in unique environmental settings having ethno medicinal uses, extreme age or interesting endemic locations because they are expected to harbor novel endophytes that may produce unique metabolites having diversified applications. Many scientists believe that plants growing in lush tropical rainforests, where competition for light and nutrients is severe, are most likely to host the greatest number of bioactive endophytes than temperate parts of the worlds (Owen and Hundley 2004). The indigenous communities have been using medicinal plants in different ways for the treatment of various diseases, which in turn has resulted in scientific discoveries, with a wealth of literature on plant extracts and their biological activities. Wang et al. (2007) have demonstrated that the endophytes isolated from these plants are excellent producer of strong fungicidal and bactericidal metabolites.

Edophyte diversity

Great diversity of microbes are isolated from different healthy parts such as leaves, stem, fruits and roots of the ethnomedicinal plants (Ahmed *et al,* 2012). Majority of these endophytes isolated are fungi followed by bacteria and a few actinomycetes (Table 1). Ahmed *et al,* (2012) isolated a total of 5 endophytic fungal strains (LBBR01, LBBR02, LBBR03, LBBR04 and LBBR05) from Baru and screened them for antimicrobial activity by disk diffusion method. This test is accepted by the FDA (Food and Drug Administration) and it is established as standard by NCCLS (National Committee for Clinical Laboratory Standards). Caruso *et al.* (2000) isolated 150 fungal and 71 actinomycete endophytes from the internal tissues of woody branches, shoots and leaves of different plants of *Taxus baccata* and *Taxus brevifolia.* Arnold *et al.* (2000) isolated 418 endophyte morpho species from 83 healthy leaves of *Histeria concinna* and *Ouratea lucens* in a low land tropical forest of central panama, and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity. Similarly, Jalgaonwala *et al.* (2010) isolated 78 bacterial and 142 fungal endophytes from aerial and underground parts of various medicinal plants. Teerayut *et al.* (2009) isolated 194 fungal endophytes from wild medicinal plants of Thailand. Santhosh *et al.* (2011) isolated 41 endophytic fungi from 195 samples of healthy leaves and stem of a red listed endangered medicinal plant *Coscinium fenestratum.*

Bioprospecting

Bioprospecting is defined as the systematic search for new sources of chemical compounds, genes, proteins, microorganisms and other products that have potential economic value present in our biotic resources, traditional knowledge often assist the bioprospecting process.

Problems that we face today include multi drug resistance, infectious microorganisms e.g. *Staphylococcus, Mycobacterium, Streptococcus* that have bcome ressitant to existing chemicals, appearance of diseases like AIDS, SARS etc, ancilliary infections due to weak immune system and infection by opportunistic pathogens like *Aspergillus* spp, *Crytococcus* spp, *Candida* spp. These infections are common in immunocompromised patients. Protozoal and nematodal infections like maleria, trypanomiasis, filariasis etc. Environmental and health problems due to indiscriminate use of agrochemicals. Do we need new medicines/agrochemicals to fight these problems? The answer is yes.The new chemicals, however, should have the characters like high affectivity, low toxicity, natural and minor or no environmental impact. Basis of modern medicines include combinational

Table 1: Different endophytic microorganisms isolated from different host plants

Host Plant	Endophytes isolated	Reference
Erythrina crista-galli	**Bacteria**: *Arthrobacter citreus, Corynebacterium insidiosum, Enterobacter dissolvens, Pseudomonas fluorescens* **Yeasts**: *Nematospora coryli, Schizosaccharomyces octospora, Sporobolomyces roseus* **Fungi**: *Aspergillus ochraceus Absidia glauca, A. glauca,, Paecilomyces variotii, Penicillium islandicum, Penicillium notatum, Zygorhynchus moelleri*	Weber *et al.*, 2005
Withania somnifera (L.) Dunal	**Ascomycota**: *Chaetomium bostrycodes, Eurotium rubrum, Melanospora fusispora,* **Deuteromycota**: *Aspergillus awamori, Aspergillus auricomus, Aspergillus flavus, Aspergillus niger, Aspergillus pulvinus, Aspergillus terreus, Aspergillus terreus var. aureus, Aspergillus terricola, Aspergillus thomii, Cladosporium cladosporioides, Alternaria alternate, Curvularia oryzae, Drechslera australiensis, Fusarium moniliforme, Fusarium semitectum, Myrothecium roridum, Penicillium corylophilum, Penicillium sp., Phoma sp.*	Khan *et al.*, 2010
Solanum rubrum	**Hypomycetes**: *Aspergillus versicular, Aspergillus fumigatus, Aspergillus niger, Aspergillus sydowi, Aspergillus fonsecaeus, Curvularia lunata, Curvularia geniculate, Penicillium purpurogenum, Penicillium lanosum, Penicillium oxalicum, Trichoderma viridae, Trichoderma lignorum* *Coelomycetes: Colletotrichum sp*	Jena *et al.*, 2013
Morinda pubescence	**Hypomycetes**: *Aspergillus clavatus, Aspergillus fumigatus, Aspergillus versicular, Aspergillus sydowi, Aspergillus flavus, Asperigillus sp., Curvularia lunata, Curvularia interseminata, Curvularia subulata, Penicillium purpurogenum, Penicillium albidum, Trichoderma koningi, Cladosporium herbarum, Nigrospora zimmermann, Nigrospora sphaerica, Torula sp.* **Ascomycetes**: *Chaetomium dolichotrichum, Chaetomium globosum* *Coelomycetes: Colletotrichum sp*	Jena *et al.*, 2013

chemistry, automated synthesis of structrally related small molecules and revolves around certain basic chemical structure besides, screening by machines. Natural products have untold diversity of chemical structures. The chemical diversity of endophytes is unparalleled by even the largest combinatorial database. It requires lot of time to pick, and choose a biological source. Isolation of active natural products, decipher their structure, the natural product may serve as lead molecule whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. The traditional approaches in medicines include use of natural products. Chinese are the largest user of traditional medicines. They have 5000 plants and plant products in their pharmacopia. Tribal groups in our country also use myriad of plants and plant parts for treating various diseases. 3000 years ago fungi grown on roasted green corn were used to treat intestinal ailments. In 800 AD Papaver sominiferum was used as anestheitc and pain reliever. These products were used without the knowledge of mechanism of action and chemical nature of bioactive compounds. The plant products, in general enhanced the quality of life, reduced pain and suffering and provided relief. Metabolites and /or byproducts from microorganisms/ plants/ animals. World's best known and universelly used medicine Aspirin (Salicylic acid) is derived from Glycoside salicin found in plants like Salix and Populus. World's first billion dollar drug 'Taxol' is a natural product from Taxus sp. Microbes source of bioactive natural products. This idea was conceived after Pasteur discovered that fermentation was caused by living cells. Discovery of Penicillin from Penicillium notatum. This opened the way of discovery and application of microbial metabolites with activity against both plant and human pathogens.

Since the discovery of the world's first billion-dollar anticancer compound - paclitaxel (Taxol) biosynthesized by *Pestalotiopsis microspora* an endophytic fungus of Himalayan yew, interest in studying such endophytes for their medicinal potential has grown tremendously (Strobel, 1996). Natural products (Table 2) from endophytes have a broad spectrum of biological activity and can be grouped into several categories such as alkaloids, steroids, terpenoids, isocumarins, quinones, phenyl propanoids, lignans, aliphatic metabolites, lactones etc. (Zhang *et al.*, 2006). Puri *et al.* (2005) isolated a novel Camptothecin producing endophytic fungus *Entrophosphora inferquens* from an important Indian medicinal plant *Nothapodytes foetida*. *E. inferquens* synthesizes camptothecin having potential immunomodulatory activity. Similarly, Chen *et al.* (2007) isolated an endophytic fungus *Pencillium thomi* from the roots of *Bruguiera gymnorhiza*. The separation of endophytic fungus from the root led to the isolation of a new compound 4', 5 dihydroxy -2, 3 dimethoxy 4(-hydroxy propyl)- biphenyl along with 11 known compounds. Their effect against three human cell lines was also investigated. Cardiac glycosides Digoxin (C41H64O14) and Digitoxin (C41H64O13) were the main important bioactive compounds extracted from *Digitalis lanata* and *Digitalis purpurea* respectively, were also isolated from their respective endophytes [Ahmed et al, 2012]. Similarly, steroidal saponin, diosgenin (C27H42O3) and a glucoside, namely Aucubin (C13 H19 O8 H2O) were isolated from *Dioscorea bulbifera* from *Plantago ovate* as well as from their endophytes (Ahmed *et al*, 2012).

Endophyte and biodiversity

It is hypothesized that the ecosystems having greatest biodiversity seem to be the ones also having greatest number of endophytes. Tropical and temerate rainforests are the most biologically diverse terrestrial ecosystem on earth. They occupy only 1.44% of land mass but harbor 66 % of world's terrestrial biodiversity. Areasof high plant endemism would possess specific endophytes.Biological diversity implies chemical diversity. Chemical inovation exists in ecosystems where evolutionary race survives. Tropical rain forests are remarkable example of this type of environment. Competition is graet, resources are limited and selection pressure is high. Therefore, there is high probability of novel biologically active compounds i.e. chemical evolution. Host influence general metabolism of endophytic microbes. Reasons of production of identical phytochemicals (host and endophytes), Some say it is because of genetic recombination between the two during evolution, Biology of interrelatedness of endophytes not known, It is not exactly known what an endophyte produces in culture and what it may produce in nature, exact relationship and mode of interaction with plants.

Future research prospects and Conclusion

They are important components of terrestrial ecosystem. They are diverse yet fundamental aspects of their interaction with host is not known. Enormous opportunities exist for the recovery of novel fungal forms, taxa and biotypes. Each plant species harbour at least one endophyte but vast majority of plants have not been studied so for their endophyte associates. More and more plants from unique environmental settings, especially those with unusual biology, and possessing novel strategies of survival should be studied with an objective to unraveal novel chemicals to be utilized for human welfare. Some important points like the processes of endophyte-plant interactions and their relationships with their co-evolutionary patterns and response of host-microbe units to changing environments together or separately need to be deciphered for proper understanding of this exciting relationship. Understanding is also to be developed of genetic bases and phenotypic plasticity of traits of the microbe-plant unit, the use of controlled microbe-plant genotype combinations in different environments. If the endophyte-plant interactions follow similar evolutionary and ecological processes as other host mutualists, host-parasite or host-disease interactions also needs explanations. This relationship helps the host to fight stressed situation and is the store house of diverse chemicals that have multifarious utility which can save the humanity from sufferings. Isolation of endophytic fungi from medicinal and other plants may result in methods to produce biologically active agents for biological utilization on a large commercial scale as they are easily cultured in laboratory and fermentor instead of harvesting plants and affecting the environmental biodiversity this can be used as a strategy for plant conservation.

Table 2: Secondary metabolites produced by different endophytic fungi isolated from different hosts

Sl. No	Host Plant	Endophytic fungi	Secondary metabolites	References
1	*Fragraea bodenii*	*Pestalotiopsis jester*	Quinones	Li et al., 2001
2	*Geotrichum sp.*	*Crassocephalum crepidioides*	Isocoumarin derivatives	Kongsaeree et al., 2003
3	*Artemisia annua*	Unidentified fungus CR115	Diterpenes	Brady et al., 2000
4	*Trachelospermum jasminoides* and *Artemisia annua*	*Myrothecium roridum*	Terpenoid	Shen et al., 2006
5	*Taxus chinensis*	*Gliocladium* sp.	Steroids	Zhang et al., 2002
6	*Cynodon dactylon*	*Aspergillus fumigatus* CY018	Steroids	Liu et al., 2004
7	*Murraya paniculata*	*Eupenicillium* sp	Quinazolines	Barros et al., 2005
8	*Catharanthus roseus* (L.) G. Don	*Fusarium oxysporum*	Indole derivatives	Zhang, et al., 2000
9	*Maytenus hookeri*	*Chaetonium globosum*	Indole derivatives	Zhang, et al., 2002
10	*Imperata cylindrical*	*Chaetosphaeridium globosum* IFB-E019	Indole derivatives	Ding et al., 2006
11	*Terminalia morobensis*	*Pestalotiopsis microspora*	Phenols and phenolic acids	Strobel et al., 2002 and Harper et al., 2003
12	*Erythrina crista-galli*	*Phomopsisi* sp.	Lactones	Weber et al., 2004
13	*Melia azedarach* L	*Aspergillus fumigatus*	Tryprostatin	Zhang et al., 2013

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