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Chapter XII

Disentangling Functions of Fungal Endophytes in Forest Trees

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Abstract

Endophytic fungi are known to be abundant colonizers of the internal tissues of forest trees, but their ecological functions are still largely unknown. Recent studies indicate that endophytes may associate with tree's resistance and tolerance properties, and they are thus potential bio-agents that could be utilized in sustainable forest protection and management. To gain a better understanding of the endophytes' potential role in shaping forest health we need more evidence in the form of ecological studies of endophyte communities, in various tissues, across space, and time.

The recent advances in molecular methods have given us new and effective tools to obtain such data. Studies of endophyte functions are further facilitated with the development of new high through-put screening methods for substrate use and competitive ability. Fungi are known as chemical factories of natural compounds with biological properties. Beside their potential as antagonists against pests and diseases, the tree-associated endophytic fungi therefore also appear as an emerging source of novel biomolecules for industrial or clinical applications outside forestry.

This chapter presents some of the current methodological approaches that are likely to be valuable in studies on endophyte diversity in forest trees, and discusses the goals and impacts of the studies that aim at disentangling the beneficial potential of fungal endophytes in trees. A new concept, bioactive symbiosis, is suggested as a general framework for these studies.

Introduction

The mutualistic potential of plant's endophytic fungi has received considerable attention after Bacon et al (1977) isolated the fungus *Neotyphodium* from pastures where cattles suffered from fescue toxicity syndrome [1]. The fungus' ability to produce ergot alkaloids provides its host with a defensive advantage against herbivores referred to as defensive mutualism [2]. *Neotyphodium* is tightly bound to its host, it gets its nutrients from the plant, and its hyphae grow systemic throughout the grass tissue and infect seeds so that germinating seedlings will inherit the fungal associate. Most research on endophytic fungi has been confined to studies of Clavicipitaceous grass endophytes like *Neotyphodium* (Table 1). This group of fungi consists of relatively few species which are more or less tightly associated with their grass host. Beside anti-herbivory advantages, these endophytes provide their host with other advantages like tolerance to abiotic stress and pathogens [3].

Table 1. The number of scientific papers about grass versus tree endophytes, envisioned through search in Web of Science, Timespan=All Years. Databases=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH

Endoph*	Fung*	Herbivor*	Grass*	Tree*	Hits
X	X	X	X		179
X	X	X		X	39
X	X		X		950
X	X			X	480
X			X		1290
X				X	604

However, the many studies that confirm the mutualistic relationship with clavicipitaceous endophytes have been criticized for all being conducted in well fertilized pasture systems [4]. Under plentiful environmental conditions the costs of hosting the endophyte may not limit plant fitness, and mutual benefits may prevail, whereas the cost benefit balance may swift under less favourable conditions, leading to antagonistic relationship between the plant and the endophyte [5]. Such relational swifts have been described in natural populations of *Festuca rubra*, along gradients of increasing herbivory, nutrient availability, and soil moisture [6].

Collaborative associations between mycobionts and photobionts are known from mychorriza and lichens, and obligate endosymbiosis is also well described at the cell level (i.e. prokaryote origin of mitochondria and chloroplasts). Indeed, some scientists regard collaborative associations between organisms as the main driver of evolution [7]. The endophytic fungi, by definition, live invisibly and symptomless for at least part of their life time within their host [8], and no plants studied so far (including cold season grasses like fescue) have been found to be free of endophytic fungi. Screenings of endophytic communities often show that they exhibit high diversity, representing a wide range of taxonomic groups in varying abundances. There are some reported examples of non-clavicipitaceous endophytes that seem to have anti-herbivory effects, but so far the non-clavicipitaceous studies are too few to support the generality of defensive mutualistic

associations [4] (Table 1). However there is increasing evidence of conditioned relationships between a plant's phenotype and its associated endophytic community [9-11].

Endophytic fungi provide examples of both symbiogenic and more loosely associated plant-fungal mutualistic relationships. This is also captured in the classification by Rodriguez et al. [12] who divide fungal endophytes after host range, colonization and transmission patterns, tissue specificity and symbiotically conferred fitness benefits. Class 1 sensu Rodriguez et al. [12] contains the Clavicipitaceous grass endophytes, which include both systemic, seed transferred, and asymptomatic fungi and non-systemic, ascospore transferred, symptomatic grass endophytes. The systemic endophytes are known to express low taxonomic diversity and high infection rates [3] suggesting that low diversification may be characteristic of endophytes that are more tightly connected with their host plants. If this is a general pattern it should also be relevant for non-clavicipitaceous endophytes, and the tightly associated fungi that Rodriguez et al. [12] group as class 2 should have the highest potential for mutualism. The class 2 endophytes include fungi that colonize below and above ground plant parts extensively and are transmitted vertically or horizontally. Many of them provide documented fitness benefits for the plant, including promoted habitat adaptation, increased biomass, and tolerance against abiotic and biotic stress. These fungi colonize the host plants in diverse ways and appear to be taxonomically less diverse compared to other non-clavicipitaceous groups. In the classification by Rodriguez et al. [12], the remaining non-clavicipitaceous fungi are divided after tissue affinity, and more precisely after whether they colonize above ground (class 3) or below ground (class 4) plant parts. These fungi are transmitted horizontally and are less directly coupled with the host plant, as compared to the class 2 endophytes. However little is still known about the specific life cycles of individual endophyte species, or biodiversity of entire host plant communities; and the taxonomic classification of endophytes is likely to change as our knowledge about these topics grows.

Endophytic Fungi in Trees

Forest trees harbor a plethora of endophytic fungi that live at least a part of their life within the tissues of their host plants without causing symptoms [13-15]. Due to the hidden life of endophytes and their apparently high diversity, as well as the large size and extended life span of forest trees, it has been particularly difficult to address questions related to tree endophyte composition and function. In trees, endophytes are likely to be among the first potential decomposers of dying and dead leaves and wood. Endophytes may therefore significantly affect the dynamics of the decomposition process by affecting the flow of carbon, nitrogen and other nutrients in forest ecosystems. In recent years, the interest for endophytic fungi in forest trees has increased (Fig 1), and while the main emphasis has been on phylogenetic and taxonomic questions, attention has also been given to anti-biotic effects and the production of bio-active compounds [5, 16-18].

Trees are generally rich in ascomycetes and deuteromycetes and the composition of tree's endophyte communities may be related to host species or host clonal properties [19-27], which may be over-layed with the impact of environmental factors [13, 19, 28-29]. Endophytes in trees include known and unknown species with expected functions ranging from mutualistic and antagonistic, to novel or unknown [22, 24]. Although their function, in

most cases, is still unknown, the apparent diversity invites for intelligent screening of these fungi for novel natural products [17-18], and there is a need to develop new fast methods to recognise and classify fungal endophytes [23].

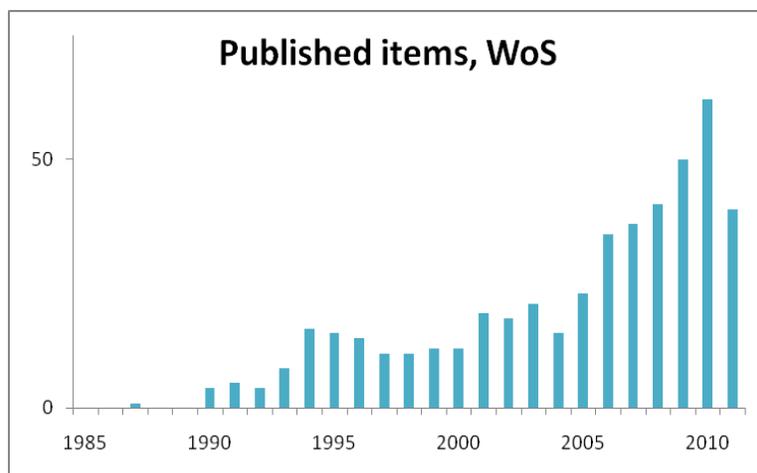


Figure 1. The publication history of 480 scientific papers that contain the words “endoph*”, “fung*”, and “tree”. Search in Web of Science, Timespan=All Years. Databases=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH.

No tight association with a systemic fungus similar to that of *Neotyphodium* in the cold season grasses has so far been reported for trees, and unequivocal examples of a shared defence strategy are sparse. However, several studies have suggested that endophytic fungi may negatively affect development and survival of insect herbivores, e.g. in Douglas fir (*Pseudotsuga merziesii*) [30], White elm (*Ulmus americana*) [31], Scots pine (*Pinus sylvestris*) [32], Garryara oak (*Quercus garryara*) [33-34], and Emory oak (*Quercus emoryi*) [35]. Furthermore the results of Arnold et al. [22] indicate that a limit in pathogen damage on *Theobroma cacao* was related with the presence of fungal endophytes, and Newcombe et al. [36] reported limited rust damage when poplar leaves had been pre-infected with endophytes. Other studies, however, suggest a lack of direct endophytic effects on tree’s resistance (for example in *Betula spp.* [29, 37]), different effects to different herbivore species (*Q. garryara*, [35]), or negative effects on parasitoids implying that endophyte presence can associate with a positive feed-back on herbivore survival (in *Q. emoryi* [38]). In general, the literature seems to support a view that tree endophytes are indicators of host properties, either by association or possibly as part of shaping them.

Endophytes receive nutrients from their host and therefore basically have a parasitic life style, but at the same time the endophyte share fitness advantages with its host, and what strengthens the plant may consequently also benefit the endophyte. If an endophyte does not affect host interactions with other organisms it may be considered neutral (Fig 2a). If on the other hand the endophyte takes active part in plant defence this may happen in two distinctively different ways: either through direct interactions with the antagonist (Fig 2b), or indirectly through enhancement of the plant’s tolerance or ability to defend itself (Fig 2c). In the case of simple, well described systems like *Neotyphodium* the endophyte component may be manipulated and studies can be performed to disentangle the nature of the interaction. In

the case of diverse communities of fungi like those found in trees, studies may have to be performed at the community level before single effects may be recognized and studied specifically. There is some evidence that entire communities of endophytes may be associated with specific tree species, genotypes and environments including tree specific phenolic profiles (Bailey et al. 2005, Albrechtsen et al. 2010) [25, 27].

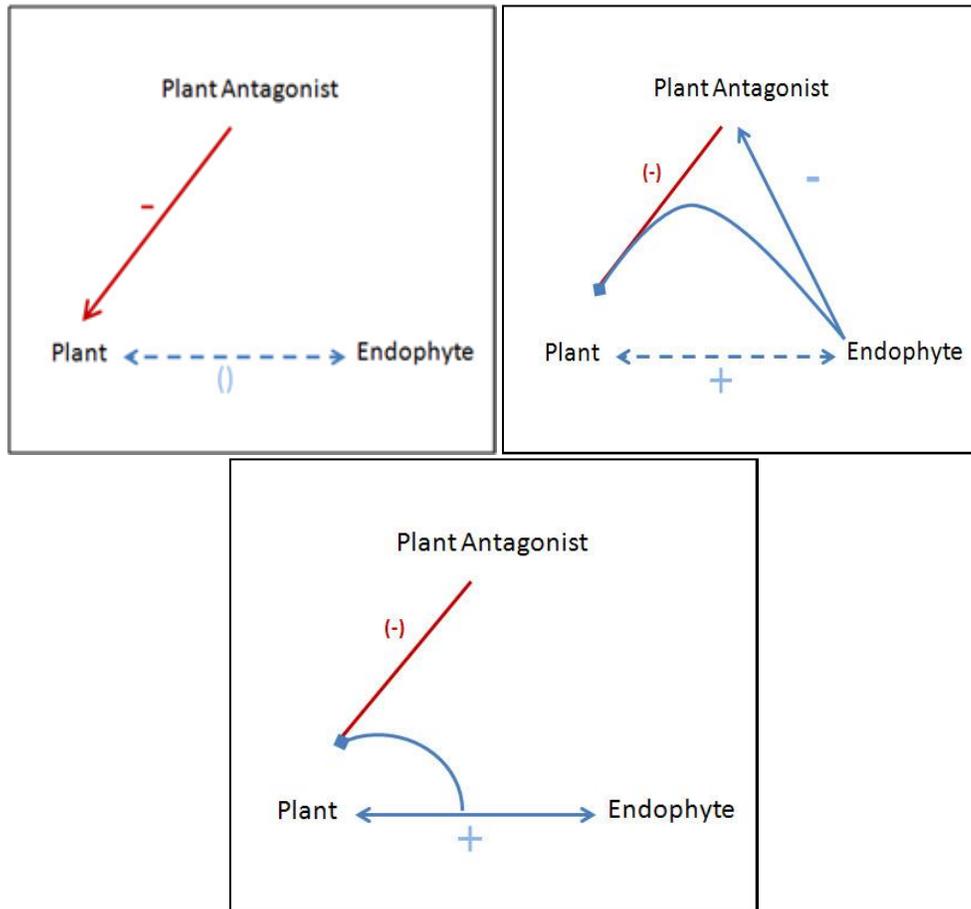


Figure 2. Defensive mutualism is an example of indirect plant defence, when the plant collaborates with another organism to obtain resistance benefits. The presence of endophytic fungi in plant tissues may be neutral when the endophyte does not have any impact on the plants interaction with other organisms (a). Defensive mutualism is when the endophyte causes resistance advantages for the host plant. This may happen if the endophyte directly limits the antagonist (b) or if it enhances the plants resistance properties and thus indirectly limits the negative effect of the antagonist (c).

Defining the Endophyte Communities with New Methods

Endophyte communities have conventionally been studied by culture-dependent methods where surface sterilization of tissues and subsequent growth of isolates on nutrient-rich media has been followed by morphotyping on the basis of colony characters and examination of

spores (e.g., [22]), in some cases combined with Sanger-based DNA-sequencing [27]. Using this approach, large numbers of endophytes can be detected and the method provides a technically uncomplicated and cost-effective method to compare differences in the diversity of certain endophyte classes across plant materials, or to examine the presence of target fungi in a sample. Clearly, our current knowledge about the endophyte communities is greatly indebted to the skillful use of this approach in earlier studies (e.g. [22, 39]). However, researchers using the culture-dependent approach are also frequently pointing out the obvious limitations of the method. The basic limitation is that many endophytes are nonculturable on standard media or even on a selective media, remain sterile, or may escape the analysis in other ways, e.g. due to their extremely slow growth rate. Therefore, a comprehensive characterization of endophyte communities in trees may rarely be successful using this approach alone.

The field of characterizing entire microbial communities is, however, under rapid development, and next-generation sequencing (NGS) techniques are rapidly replacing traditional Sanger based techniques [40-42]. The instrument capacity has exploded over the past decade and what was a major sequencing task only a few years ago is now achievable for standard capacity facilities [41]. Metagenomics is a culture-independent analysis that uncovers sequence-based information of collective microbial genomes contained in an environmental sample [42]. The metagenomic analysis of microbial communities are based on DNA or RNA extraction which are used for different purposes: DNA libraries may be used for bioprospecting, and DNA sequences for assessment of taxonomic diversity and metabolic potential; RNA extracts may provide information about community functions and active community members [43].

Recent years have witnessed an explosive increase in metagenomic studies applied to fungal communities, based on the pyrosequencing technology. For example, Jumpponen and Jones [44] used 454-pyrosequenced [45] DNA-tagged [46, 47] PCR amplicons to study the fungal richness, diversity and community composition in the *Quercus macrocarpa* phyllosphere. A striking result of most of these analyses is the richness of uncultured taxa in the environmental samples, and methodology has been developed to set standards for both sampling and subsequent analyses [40, 48-49]. The progress in metagenomics is highly relevant for disentangling endophyte function in trees, and information on taxonomic diversity and associations are suddenly accessible through these high through-put methods. However, even the NGS approach is not free of errors due to artefacts and innate biological traits. In connection to validation of NGSs, and pyrosequencing in particular, concerns have been raised about problems associated with, e.g., nucleic acid extraction, primer selection and PCR, cell number and biomass in the samples as well as copy number instability within and between fungal species. All these factors may bias and skew the data and limit the utility and relevance of the information that can be extracted from these data [50]. Furthermore, the generally nonstandardized presentation of the NGS data is complicating the interpretation and comparison across studies [48]. It should also be kept in mind that targeting of the NSG analysis to endophyte communities is still dependent on selection of appropriate tissue samples. The use of DNA barcoding that employs short gene regions, e.g. internal transcribed spacer regions 1 and 2 (ITS) as the standard fungal DNA barcoding gene e.g. [51], or multilocus barcode approach [52], is currently broadly used as a sequence-based method to identify fungi. Yet, the identification of “unknown” fungi on basis of sequence data is strictly

dependent on the availability of reliable voucher specimens [53] and quality of query tools and databases.

In addition to characterization of endophyte diversity, knowledge about functional properties is also highly needed, i.e. niche use strategies. The substrate-utilization profiles of isolated tree endophytes can be effectively recorded, e.g., using a phenotype microarray platform. This method is based on redox chemistry in a 96 well microtiter format, employing cell respiration as a universal reporter [54-55]. Briefly, a standardized cell suspension of each tested isolate is inoculated into the wells of microtiter plates, which have been loaded with pre-configured sets of organic substrates (specific arrays, i.e., plates, for carbon (C), nitrogen (N), phosphorus (P) or sulphur (S) compounds are commercially available). The development of color in wells correlates with the ability of cells to oxidize the compounds and is recorded over an incubation period with the help of a fully automatic system. The data is analyzed with a bioinformatics software. The method thus allows a high-throughput screening and comparison of phenotypes at cellular level. Using methods such as phenotype microarrays it is possible to gain detailed information about the substrate-use profiles of different fungi, which will help us to assess not only the competitive interactions between endophytes, but also determine which traits of forest trees may best provide specific qualitative or quantitative information about endophyte diversity.

Studies on Endophytes of Forest Trees: Knowledge Gaps and Links to Forest Management

As discussed above, endophytic fungi are likely to have diverse functions in trees, e.g., as enhancers of tree resistance to different stress factors or as a pool of potential opportunistic pathogens, respectively. These functions can be due to the general abundance and diversity of the endophyte community, or they may be attributed to the presence and frequency of specific endophytes. In either case, it is essential to understand how endophyte communities are formed, maintained and limited under different conditions and within different tissues of trees, and to what degree, and how, the different endophytes differentiate niches provided by the tree tissues. This understanding may significantly add to our ability to apply silvicultural practices (e.g. fertilization, intelligent choice of regeneration materials, maintenance of forest and stand structures) in a sustainable way, and where possible, to utilize endophytes to promote forest health and tree vitality.

In macro-ecology, the assembly, structure and dynamics of communities has been explained both by neutral theories and by tradeoff-based theories of interspecific competition as explained by Tilman [56] and refs. therein. The interspecific competition for resources is likely to be a central mechanism structuring the endophyte communities in trees, but also demographic stochasticity [56] may play a significant role in shaping of the prevailing endophyte assemblies in forest trees: The annual invasion of deciduous foliage [57] is likely to be dominated by the habitat-specific inoculum potential and therefore the availability of viable propagules in the environment is bound to have a marked impact on the endophyte communities in leaves. The endophyte communities in deciduous leaves should thus be more readily affected by environmental disturbances and resource pulses (e.g. fertilization and thinning operations), and more strictly recruitment limited [56], than the endophyte

communities in the perennial tissues (bark and xylem) of the same trees. The colonization of the perennial tissues is likely to occur cumulatively over longer periods of time. In these tissues, the endophyte diversity may be mainly limited by the competition from resident fungi that occupy the relatively nutrient poor niche or micro-habitat. This effect may especially limit the invasion by fungi that represent the same or similar functional groups than the resident fungi [56].

Little is still known about the biochemical mechanisms that allow the maintenance of endophytes in planta. Once established, the endophytes have to cope with the chemical and physical environment inside the host, including constitutive and induced defensive mechanisms. The characteristic trait of the endophytic life style, i.e., the asymptomatic presence in the host plant, necessitates effective neutralization of plant defensive responses, either by avoidance or suppression. For instance, it is still unclear if the tree genotypes that show high resistance against aggressive fungal pathogens may also deter endophytic fungi. It is, however, apparent that the nature of the endophyte-host interaction can change from neutral to mutualistic, pathogenic or saprotrophic association [5, 14, 15, 37] and depending on the condition most fungi can adopt any one of these life-styles [58]. In a recent study, Alvarez-Loayza et al. [59] investigated the effects of light availability on disease expression by *Diplodia mutila*, a common endophyte/pathogen on the tropical palm (*Iriarteia deltoidea*) that prefers shady conditions and successfully colonizes wet areas. They found that light conditions appear to influence where on the endosymbiont-pathogen continuum *D. mutila* is placed: while high light triggered the pathogenicity, apparently through light-induced production of H₂O₂ that triggers necrosis in the host tissues, low light promoted endosymbiotic development. This response constrained recruitment of endophyte-infested seedlings to shaded understory by reducing seedling survival in direct light [59]. It is conceivable that similar mechanisms, involving the interaction of environmental change and plant biochemistry, can trigger shifts from endophytic to pathogenic life style also in forest trees. This issue is of high contemporary interest for forestry, because the dual role of fungal communities as potential forest pathogens and as important part of the forest's biodiversity directly applies to the concept of sustainable forest management.

Conclusion

Clearly, the effect of fungal communities on stress tolerance and resistance of trees can be substantial. As a habitat-specific epigenetic mechanism [60] the fungal endophyte communities may profoundly challenge any attempts to improve the growth and resistance of trees by manipulation of plant genome or environment. Therefore, we propose that the assembly and dynamics of endophytic communities should not be ignored in silviculture e.g. forest health management [61], and in discussions of forest improvements for example when promoting genetically modified manipulations for desirable traits (GM trees). Through their ability to produce manifold of extracellular chemicals with various biological activities [16, 62] and manipulate host defensive chemistry [63], endophytes may contribute to at least part of the variations that have been recorded in different studies on plant defenses, such as phenolic compounds, and thereby push the plant quality beyond thresholds that matter e.g. to insect herbivores. Only through improved understanding of the ecological and biochemical

mechanisms that regulate the recruitment and establishment of endophytic fungi in trees we can proceed to utilize the fungal communities as an integrated means to improve the health and vitality of forest trees. As a general guideline for future studies, we suggest that the apparently versatile (direct and indirect) functions of endophytes in forest trees could be fruitfully investigated under a concept of bioactive symbiosis, which we define as the ability of endophytes to shape plant resistance, either positively or negatively (depending on the conditions), against antagonists of any kind, including virus, bacteria, fungi, and herbivores.

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