Hidden Fungi, Emergent Properties: Endophytes and Microbiomes

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Abstract
Endophytes are microorganisms that live within plant tissues without causing symptoms of disease. They are important components of plant microbiomes. Endophytes interact with, and overlap in function with, other core microbial groups that colonize plant tissues, e.g., mycorrhizal fungi, pathogens, epiphytes, and saprotrophs. Some fungal endophytes affect plant growth and plant responses to pathogens, herbivores, and environmental change; others produce useful or interesting secondary metabolites. Here, we focus on new techniques and approaches that can provide an integrative understanding of the role of fungal endophytes in the plant microbiome. Clavicipitaceous endophytes of grasses are not considered because they have unique properties distinct from other endophytes. Hidden from view and often overlooked, endophytes are emerging as their diversity, importance for plant growth and survival, and interactions with other organisms are revealed.
INTRODUCTION

Our work as scientists is often reductionist. To test a hypothesis about the pathogenicity of a particular fungus on a particular plant, for example, we need an experiment in which the only variables are the things we want to test. Other factors are carefully controlled, and if they can’t be controlled, often ignored. This tight focus is necessary to test our hypothesis, but it can make us forget the tremendous complexity of biological systems.

We think of plants as organisms. However, all plant pathologists know that each plant is in fact a complex community and that every plant in a natural or agricultural setting is colonized by a diversity of microbes, both on its outer and inner surfaces. Endophytes, organisms that live inside plant tissues, are part of the microbial community of every plant. It is not always clear, however, if and how these microbial communities affect plant health and function.

Anton de Bary, the German botanist who is considered the father of plant pathology, coined the term endophyte in 1886 to describe microorganisms that colonize internal tissues of stems and leaves (169). This definition was later revised to specify that infections caused by endophytes are asymptomatic, that roots as well as shoots may be colonized, and that an endophyte may not remain an endophyte throughout its life cycle. This broad definition implies that in addition to mutualistic and commensalistic symbionts, endophytes could include latent pathogens, latent saprotrophs, and early stages of colonization by mycorrhizal fungi and rhizobia (Figure 1). Functional mycorrhizal fungi, however, are explicitly excluded, as they are specialized for nutrient transfer from sources outside of the root (24) and are phylogenetically distinct from most groups of endophytes (9, 123). The term endophyte is thus defined by location and does not address the nature of the relationship with the plant, unlike mycorrhiza, which specifies a functional relationship.

The potential importance of endophytic fungi became clear in 1975, when Charles Bacon discovered that endophytes of pasture grasses in the family Clavicipitaceae were toxic to cattle (13). Subsequent work showed that both the endophytes and the associated toxicity syndromes were widespread, with an estimated cost of $600 million a year to the livestock industry (77). Clavicipitaceous endophytes of grasses (i.e., systemic endophytes in the family Clavicipitaceae) are one of the most interesting and economically important examples of plant-fungal interactions, and they have been studied from many perspectives. Although they have inspired much of the research on endophytes, they raise expectations of mutualism, functional significance, and coevolution that are often unjustified when applied to other groups of endophytes (14, 42, 124).

Plant-associated fungi are usually divided into five main functional groups: mycorrhizal, pathogenic, epiphytic, endophytic, and saprotrophic fungi (Figure 1). Most studies focus on only one of these groups, and few consider interactions among them, or between fungi and bacteria. In this review, we summarize examples that show the functional importance of endophytes. We also argue for the urgent necessity to use more integral approaches to study plant-associated microbial communities. Systems biology approaches are emphasized because they are providing dramatic new insights into microbial ecology. We focus on a simple question: Are fungal endophytes of sufficient...
practical importance to warrant the attention of plant pathologists, ecologists, and plant biologists in general?

Extensive literature is available on the biology and diversity of endophytes. In this review, we try (as much as possible) to avoid repeating topics covered in previous review articles. Clavicipitaceous endophytes of grasses are excluded from this review because they have special characteristics and have been reviewed extensively in multiple papers and books (19, 31, 130, 153).

METHODS: CULTURING VERSUS DIRECT AMPLIFICATION AND DETECTION OF ENDOPHYTIC FUNGI

Until recently, most studies on endophytes involved first isolating organisms into pure culture in order to identify them. This means that species that do not grow or grow very slowly in culture media were overlooked, and species that grow well in culture were over-represented. This bias can be corrected by direct amplification of DNA from surface-sterilized plant material [also called environmental PCR (polymerase chain reaction)]. However, unlike bacteria, where only one percent of species in some communities are culturable (45), it appears that a relatively high proportion of fungi can be grown in culture. In a study designed to test the differences between culturing and direct amplification, more species of endophytes were revealed by direct amplification, but more ordinal level groups were revealed by culturing (9). Some taxa appeared more diverse with direct amplification, but others appeared more diverse with culturing. Direct amplification studies will probably reveal major new lineages of fungi, but the best strategy is to use a combination of both methods (18).

For both culturing and direct amplification, a number of methodological issues make it difficult to compare results among studies. Morphotypes are often used as a proxy for species, but this method may overestimate diversity: Sphaeropsis sapinea, an endophyte of Pinus sp., has more than five different morphotypes in culture (26, 132). Methodological factors including surface sterilization, sample size, and time of collection can all affect which endophytes are isolated. Also, many studies are imprecise about sampling strategies in terms of the number of plants sampled and number and size of samples per plant (18, 53).

For DNA-based approaches, primer and extraction methods can introduce bias, and there is no consensus on defining a universal barcode region for fungal diversity studies. The internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) is likely to become the official barcoding region for fungi (107), but other regions, such as CO1 (cytochrome c oxidase subunit I) and multilocus barcodes, are still being investigated (131, 141). Other unresolved issues include defining cut-off percentages for BLAST (Basic Local Alignment Search Tool) searches or methods for identification at the species level (7), and how to control for misidentified sequences in public databases (108). Lack of cohesion in the research community has prevented a consensus on these issues, another reason why an integrated picture of endophyte diversity and distribution remains elusive.

Quantifying fungal biomass in plant tissues is also problematic. Real-time PCR techniques vary greatly depending on the type of fungus as well as cell size, number, and type, and require extensive calibration for each species using pure cultures (158). They usually focus on one or a few taxa and are unwieldy for studies of microbial communities. Concentration of ergosterol, a sterol characteristic of fungal membranes, is another way to measure fungal biomass in plant tissues, but it does not distinguish among fungal taxa (110).

METHODS: MICROBIOMES AND SYSTEMS BIOLOGY APPLIED TO ENDOPHYTIC FUNGI

Next-generation sequencing technologies and systems biology allow simultaneous exploration...
Metagenomics: the study of nucleic acids extracted from environmental DNA samples, including all organisms therein

Figure 2
A strategy for more inclusive and integrative studies of plant microbiomes.

Systems biology uses a multidisciplinary approach to study the multiple, complex interactions of and between organisms. It is providing new insights about the role of plant microbiomes in plant adaptation, evolution, and response to global climate change (81, 102). Systems biology approaches are also showing how plant microbiomes can interact with herbivores and insects (43, 75, 111).

These interactions may result in emergent properties that arise from the interplay of genes, proteins, organelles, multiple microbial species, the host plant, and the environment. Thermotolerance is an example of an emergent property that results from interactions among a virus, an endophyte, and plant roots. A virus infecting the endophyte Curvularia protuberata appears to be necessary for thermotolerance to Dichanthelium lanuginosum (panic grass) in geothermal soils of Yellowstone National Park; neither the plant nor the endophyte can survive temperatures above 38°C, but together they can survive intermittent temperatures of 65°C for 10 days (101, 126). Similarly, tomato and panic grass plants colonized by Fusarium sp. survive at 65°C, whereas neither partner alone survives temperatures above 40°C (129).

The use of microbiosensors, quantum dots (Q-dots), and microarrays is also providing new...
insights about function and response of microbiomes (Figure 2). Microbiosensors are microbes that have been genetically engineered to express reporter genes, either continuously or in response to a particular stimulus. The expression of these reporter genes (e.g., nitrate- or carbon-sensitive reporter genes) can be monitored in situ and can facilitate long-term monitoring of microbiomes using specific organisms. Q-dots are nanoscale semiconductors that fluoresce in different colors depending on their size and can be linked to organic compounds to track their movement. These nanoparticles show promise for studying the role of endophytic fungi in nutrient uptake and translocation. Q-dots were used to demonstrate the uptake of organic nitrogen by a saprotroph and its transfer by mycorrhizal fungi to annual bluegrass (171).

Comparative genomics, metagenomics, transcriptomics, and proteomics are changing our perception of plant-microbial interactions (41, 140). Next-generation sequencing technologies are becoming more accessible in terms of cost, allowing in-depth surveys of microbiomes. A study of epiphytic and endophytic communities in Quercus leaves using 454 sequencing (18,020 sequences) revealed a community dominated by Ascomycota (93.4% of the sequences) and more than 700 fungi (84). Microarrays for the grass endophytes Neotyphodium and Epichloë and the plant pathogen Fusarium graminearum are allowing high-throughput transcriptome studies to evaluate expression of specific genes of plants and fungi. They also facilitate discovery of novel genes and mechanisms of interaction among host plants and fungal symbionts (44, 65). These technologies will provide fundamental information that can be used not only with model systems, but other plant symbionts as well. Another technology of great potential is the use of RNAi knockdown mutants to study function of particular genes in the establishment of symbiotic associations. Transformants of the endophyte Piriformospora indica made using RNAi showed a direct link between phosphate transporters and improved plant nutrition (173).

In addition, a growing number of genomes are available for plants and mycorrhizal and endophytic microbes. Genomic studies in Populus (poplar trees) have demonstrated that fungal endophytes respond to genetic differences in individual plants and plant responses to environmental cues. The production of condensed tannins in Populus is regulated at the genetic level and affects directly fungal endophytic infections depending on the ontogenic differences in individual plants, tissue types, and the phenotypic plant responses to environmental factors (15). Genomic analysis of bacteria from poplar roots reveals potential roles in drought resistance and plant protection in poplar (164).

The availability of fungal genomes, including mycorrhizal fungi such as Laccaria bicolor and Glomus intraradices, plant pathogens such as Magnaporthe grisea, Ustilago maydis, F. graminearum, and Stagonospora nodorum, and the almost completed genome of the clavicipitaceous endophyte Neotyphodium lolii is invaluable for understanding the role of mycobiomes in plant survival, establishment, and response to global climate change. However, the majority of fungal genomes available represent human or plant pathogens or fungi with industrial or biofuel potential, and there is a clear necessity to prioritize genomes to advance endophytic research. Comparative genomics, metagenomics, transcriptomics, and proteomics will help to unravel the complexity of plant-microbial interactions (140).

Still, a number of technical challenges are pending. Fungal metagenome studies are limited because with current technologies plant DNA cannot be separated from fungal DNA; the high concentration of host plant DNA makes the much larger but much more dilute fungal metagenome difficult to sequence with adequate coverage. We are also limited by our capacity to manage large datasets and to conduct automated classification, and by insufficient functional gene annotations for current genomes. More quantitative proteomics and
metabolomics approaches also need to be implemented for fungi.

**ENDOPHYTES AND FUNGAL DIVERSITY**

Although less than 100,000 fungal species have been described, there are probably many more, perhaps 1,500,000 (48, 71). Some mycologists have argued that endophytes comprise a large, although hidden, component of fungal biodiversity (6, 10, 130). Every plant species has a community of endophytes—perhaps 90 species in a single tropical tree leaf (18) and more than 50 different genera associated with roots of an arid grassland species (123). Colonization rates vary: from <1% to 41% of 2 mm² leaf pieces in three major plant lineages in boreal and arctic ecosystems (74) to 90% in leaves in tropical forest trees (98). However, some endophytes are restricted to single cells and tissues in the leaf; endophytes in different tissues may not interact (152). This potential goldmine of undescribed biodiversity has kept mycologists interested in endophytes and the issue of host specificity, particularly in tropical plants.

Fungal endophyte communities in many plants are dominated by various classes, including Dothideomycetes, Sordariomycetes, Leotiomycetes, Eurotiomycetes, and Pezizomycetes (74, 84). Zygomycota and Basidiomycota fungi also occur as endophytes, with Agaricales common in grasses (73, 88, 123), and Russulales, Polyporales, and Agaricales common in woody tissues and roots (146).

Some endophytes are host-specific. Assuming that the number of host-specific species is constant, the total number of endophytic species can be extrapolated from the number of plant species (21, 71). Given that plant diversity is highest in the tropics, endophyte diversity might also be highest in the tropics. A recent meta-analysis found that leaf endophytes are indeed more species-rich in the tropics than in temperate regions (7). However, a recent study showed a different pattern in which diversity of root endophytes of a single plant, *Bouteloua gracilis*, increased with latitude in North America (73) (Figure 3). This indicates the need for more extensive surveys of plant organs to evaluate distribution patterns and diversity of endophytic fungi across large geographical scales.

**Figure 3**
Continental scale distribution of fungal endophytes in a widely distributed host. Bars show the fungal community in roots of the grass *Bouteloua gracilis* at sites ranging from Jalisco, Mexico, to Saskatchewan, Canada. Bars are labeled with site name and Shannon index of diversity (H). Identifications were based on 795 cloned internal transcribed spacer sequences. Data from Herrera et al. (73).
VARIABILITY OF ENDOPHYTES IN SPACE, TIME, AND FUNCTION

Communities of endophytic fungi can vary greatly in a single host species in different sites, climates, seasons, and environments (29, 53, 97, 127, 170). Mycobiome composition may depend on multiple factors, including plant host, plant density, nutrient availability, environmental conditions, and interactions with external microbiomes (e.g., soil fungi and bacteria).

Variation can be observed at all levels. Differences in endophytic communities in a single host species can increase with distance (8) or show no significant variation (73, 88). Leaves, roots, and woody stems of a single plant often differ greatly in the dominant members of their endophytic communities (reproductive structures have been less widely studied) and may even show functional differences (30, 57, 73, 119). For example, leaves, stems, and roots of alfalfa plants are colonized by distinct fungi that produce different ranges of secondary metabolites (Figure 4) (166). These differences in endophytes between roots, stems, and leaves may reflect differences in external environment as much as biological differences among organs and tissues: In epiphytic orchids (genus Lepanthes) in which leaves and roots are equally exposed to air and light, significant differences were not observed in their endophytic communities (20). Even within a single plant, different leaves or roots may differ significantly in community composition (52, 98). Single leaves of a tropical forest tree, Manilkara bidentata, showed fine scale variation of endophyte isolation rates and identity (Figure 5). In this respect, plants are genetic mosaics, because each organ may have a unique combination of genes in its microbiome (72). More studies are needed on seasonal variation and succession to determine functional importance of changes in the mycobiome of the plant.

In the following sections, we discuss how endophytes overlap and interact with mycorrhizal fungi, pathogens, saprobes, and mutualistic symbionts that offer protection against drought, heat, pathogens, and herbivores, and interactions among guilds.

ENDOPHYTES VERSUS EPIPHYTES

Endophytes are often contrasted with epiphytes, which live on external plant surfaces (136). In practice, the distinction is that epiphytes can be washed off plant surfaces or be inactivated by surface sterilization, usually with sodium hypochlorite and ethanol to break surface tension, whereas endophytes cannot. Thus, an epiphyte that survives surface sterilization and grows in culture might be assumed to be an endophyte (9). Although there are few studies comparing phylloplane and endophytic fungal communities of the same leaves, comparisons within pine and coffee leaves indicate that endophytic communities are distinct from epiphytic ones, even though they may live less than a millimeter apart (93, 136).

Temporally as well as practically, the distinction between endophytes and epiphytes is often arbitrary. Many horizontally transmitted endophytes presumably start growing on the surface of the leaf before penetration. Also, endophytes may become epiphytes when internal tissues are exposed, and may help protect the exposed tissues from the environment. In shoot tip–derived tissue cultures of Pinus sylvestris, calli were found to be covered by hyphae of the endophytes Hormonema dematioides, Rhodotorula minuta, and associated biofilms (117). How such endophytes coordinate function, interact with other microbiome biofilm components, and affect plant fitness needs further exploration.

ROOT ENDOPHYTES AND INTERACTIONS WITH MYCORRHIZAE

Root endophytes colonize healthy plant roots without the nutrient transfer interfaces commonly observed in mycorrhizae (25, 137). Root endophytes are common, but they have been neglected for many years in comparison with
Endophyte | Frequency | Antifungal metabolites
--- | --- | ---
Phome medicaginis | 54% | Brefelcin A
Alternaria sp. | 12% | (not yet identified)
Cercospora sp. | 1% | (not yet identified)
Epicoccum sp. | 1% | (not yet identified)
Colletotrichum trifolii | 40% | Mevinolin
Fusarium spp. | 69% | Lateritin

2-(3,4-dihydroxy-phenyl)ethanol

**Figure 4**
Differences in most common endophytes among leaves, roots, and shoots of alfalfa (*Medicago lupulina* or *Medicago sativa*). Shown from left to right, plant organs, common endophytes, frequency, and secondary metabolites with antifungal activity. Metabolites were detected in vitro and not in planta. Nonetheless, organs of a single plant may differ in both fungal endophytes and bioactive secondary metabolites. Adapted from Weber & Anke (166).

DSE: dark septate endophyte
AMF: arbuscular mycorrhizal fungi

mycorrhizae; only recently have they been considered fundamental components of ecosystem models (35, 62). Because some important root endophytes do not sporulate in culture, the use of molecular techniques has been important for identification (123, 163) and has confirmed that many of these endophytes coexist with other functional groups in the microbiome (Figure 6). For example, dark septate endophytes (DSEs) occur in mycorrhizal roots of *Pinus halapensis* and *Rosmarinus officinalis*, along with ectomycorrhizal and arbuscular mycorrhizal fungi (AMF), respectively (61), and with AMF in *Pedicularis* roots (95).

Because of problems with taxonomy and identification, some root endophytes are commonly described in the literature using generic names: DSEs, hyphomycetes-endophytes, or hyaline hyphal endophytes. However, these terms are based on morphological characteristics and colonization patterns, and group together phylogenetically diverse fungal taxa.
Here, we provide a short summary of both groups.

DSEs are common, widely distributed and diverse endophytes, found in 600 phylogenetically diverse host species and including fungi from different phyla (82). One DSE group, the Phialocephala fortinii s.l.–Acephala applanata species complex, colonizes roots in multiple plant hosts in Europe and North America (63, 64, 86, 106).

DSEs are easily distinguished from mycorrhizal fungi because their hyphae are darkly pigmented due to the presence of melanin. They coexist with mycorrhizal fungi, pathogens, and other endophytes (Figure 6) (61, 82, 85, 122, 123).

DSEs are commonly observed in roots of plants in stressful and nutrient-limited environments (17, 70, 88, 95, 123), which suggests they may facilitate plant establishment and survival. Their broad geographic and host distributions indicate low specificity. For example, P. fortinii has been found in mutualistic association with plants from Pinaceae, Cyperaceae, Ericaceae, Salicaceae, and Rosaceae (82).

An increase in nutrient content and growth was observed for Carex, Pinus contorta, and Vulpia ciliata plants that were inoculated with DSEs (70, 85, 106). The DSE Heterocanium chaetospira forms a functional mycorrhizal symbiosis with Brassica campestris (Chinese cabbage); the fungus can transfer nitrogen to and receive carbon from the plant (162). The Brassicaceae are usually described as nonmycorrhizal, so this association with DSE suggests that mycorrhizae are too narrowly defined. The mechanisms of how these fungi contribute to acquisition of nutrients, protection, and plant growth are still not well understood, but the ability of DSEs to decompose complex organic matter indicates that some may act as satrotrophs (28).

Hyaline hyphal endophytes and hyphomycetes are also common in plant roots (23, 165). Aquatic hyphomycetes are commonly found in riparian and coastal ecosystems. For example, multiple species of aquatic hyphomycetes were isolated from roots from riparian plants including grasses, pteridophytes, and Cupressaceae trees (148). Like DSEs, hyphomycetes coexist with other types of endophytes, mycorrhizae, and other fungi that are normally associated with marine and freshwater environments (4), but less is known about their roles.

Some endophytes may form mycorrhiza-like structures. Roots of the orchid Dendrobium nobile inoculated with an endophyte, Leptonidium, formed structures similar to pelotons and showed increases in plant height, biomass, and stem diameter, all characteristic of the

**Figure 5**

Fine-scale distribution of fungal endophytes at the level of individual leaves. Each row shows a leaf of Manilkara bidentata cut into 1 × 2 mm pieces. Results show a high level of endophytic colonization (>90% of leaf pieces), a high diversity of fungi in a single leaf, and marked differences in communities among individual leaves in a single population. Adapted from Lodge et al. (98).
recognized mycorrhizal interactions in orchids (76). Work with model systems has suggested that root endophytes activate specific metabolic pathways that facilitate transfer of nutrients. For example, the external hyphae of *P. indica* in corn roots express a phosphate transporter [propylisopropyltryptamine (PiPT)] (173).

Functions that overlap with those of mycorrhizal fungi (e.g., nutrient transfer, protection, internal-external colonization) show the need to integrate endophytic and mycorrhizal fungal research. In many mycorrhizal studies, endophytes are ignored or reported as contaminants.

**ARE SOME ENDOPHYTES LATENT PATHOGENS?**

A common theme in discussions of endophytes is that they could be latent or quiescent pathogens. A change in the host or environment may trigger pathogenicity in an endophyte that was previously asymptomatic. Whether or not an interaction becomes pathogenic depends partly on the time scale in which it is observed; even mutualisms can be viewed as series of brief, reciprocal, antagonistic interactions (138, 139).

Part of the difficulty in distinguishing endophytes from pathogens is that many endophytes are closely related to pathogens. In many cases,
morphological comparisons do not provide sufficient resolution (e.g., *Colletotrichum gloeosporioides*), but the use of multiple gene sequence analysis has facilitated the differentiation of related endophytes and pathogens (132). For example, a common, nonpathogenic endophyte was subject to quarantine because of its relationship to *Guignardia citricarpa*, causal agent of citrus black spot (12).

A rigorous study concluded that endophytes are not simply latent pathogens: When endophytes of western white pine (*Pinus monticola*) were isolated and identified by ITS sequencing, many were in the same families and genera as important pathogens (especially Rhytismataceae and Mycosphaerellaceae) (54). However, the most similar sequence to each endophyte never came from a pathogen of *P. monticola*. Instead, many endophytes were most closely related to, but distinct from, pathogens of other species of pine. These endophytes may be separate, less-pathogenic lineages derived from pathogens or pathogens of other hosts that landed on a nonhost plant. The authors preferred the first explanation.

The hypothesis that some endophytes are not pathogens, but derived from pathogens, is supported by experimental work on *Colletotrichum magna*. This fungus is another example of a pathogen of one group of plants (cucurbits) that can be an endophyte of other plants. A pathogenic strain was converted to a nonpathogenic strain by mutation of a single gene, and some of the mutants even had mutalistic effects (125, 128). Also, isolates of *Colletotrichum* from Panama and China show a large range of activities from endophytic to pathogenic (8, 72, 132).

The reverse is also true: Pathogens can arise from endophytes. *Arabidopsis* plants colonized by single gene mutants of *P. indica* grew more slowly and produced fewer seeds than uncolonized plants or plants colonized with the wild type (80). External factors, such as changes in plant gene expression, habitat, nutrient status, or stress, can also make nonpathogenic endophytes pathogenic (80).

However, other examples suggest that some endophytes are indeed latent pathogens. For example, some of the most common endophytes in asymptomatic wheat leaves were wheat pathogens, suggesting latency (90). Endophytes of weeds may be pathogens of nearby crops, complicating control of the pathogens (145) and establishment of quarantine guidelines. Similarly, a DSE from pine was also a root pathogen of cucurbits (61). Relatives of pathogens may be found as endophytes in different ecosystems: *Moniliophthora* sp. was isolated and amplified directly many times from healthy roots in a dominant grassland in New Mexico, and was related to one of the most devastating pathogens of cacao (73, 88, 123). In *Musa acuminata* only a few endophytes were pathogenic in leaf pathogenicity assays, but those were closely related to known pathogens (116). A shift in host may also trigger pathogenicity: *Colletotrichum tropicale*, a common endophytic in Panamanian trees, has also been isolated as a pathogen in old leaves of *Persea americana* and from *Annona muricata* fruits (132). The lack of a clear distinction between endophytes and pathogens complicates efforts to inoculate plants with endophytes in the field (see section on Field Applications).

**BIOCONTROL OF PATHOGENS AND INSECT PESTS BY ENDOPHYTIC FUNGI**

Control of insects and fungal pathogens can be accomplished by different mechanisms: by antagonism, including competition, parasitism, or production of secondary metabolites; by induction of host defenses; or by stimulation of host growth and vigor (2). These mechanisms overlap, and a single endophyte may employ several of them. Most studies have proposed the induction of host defenses, specifically mechanisms of systemic acquired resistance (SAR). SAR is often mediated by production of salicylic acid, jasmonic acid, ethylene, and a variety of pathogenesis-related (PR) proteins (160). Endophytes may also produce secondary
metabolites that directly inhibit insects or pathogens, or produce elicitors that stimulate the plant to produce secondary metabolites. Furthermore, a single endophyte may offer protection from both fungal pathogens and insects. For example, *Beauveria bassiana* inhibits both fungal pathogens and insects, mostly by production of secondary metabolites, and *Lecanicillium* spp. and *Trichoderma* spp. are both mycoparasites and insect parasites, although they also produce inhibitory metabolites (68, 112).

**Pathogens**

Endophytic fungi are effective biological control agents against a variety of fungal pathogens. In most of the following examples, the endophyte is inoculated before exposure to the pathogen (11). The control is endophyte-free plants; these can be difficult to generate and maintain, especially in trees, because infection by air- and rain-borne inoculum must be prevented. In cacao, multiple studies have demonstrated that endophytic fungi can reduce damage by important pathogens, including *Phytophthora palmivora*, *Moniliophtora roreri*, and *Moniliophtora perniciosa* (8, 11, 104). The endophyte *Gliocladium catenulatum* can reduce up to 70% the incidence of witches’ broom disease in cacao (133). In wild banana (*M. acuminata*), two endophytes (*Cordana* sp. and *Nodulisporium* sp., out of a total of 723 cultures) showed potential activity against *Colletrotrichum*, evaluated as a reduction in radial growth of the pathogen and growth inhibition by the production of secondary metabolites (109). The endophyte *Gliocladium catenulatum* can reduce up to 70% the incidence of witches’ broom disease in cacao (133).

The root endophyte *P. indica* can mediate resistance to root and leaf pathogens in multiple plant species. *P. indica–Arabidopsis* interactions have been used as a model to study response mechanisms of plant-endophyte systems to plant pathogens (142). This fungus is restricted to the root cortex but can induce resistance to leaf pathogens through a systemic signaling between plant and fungus. *P. indica* induces systemic resistance in *Arabidopsis* to *Golovinomyces orontii*, a powdery mildew, by activating the jasmonate signaling pathways (150). A related endophyte, *Sebacina vermifera*, increased the growth of *Nectiana attenuata* but increased the susceptibility to herbivorous insects through the inhibition of ethylene synthesis and jasmonic acid pathways (16).

In other examples, *P. indica* uses sophisticated mechanisms to induce host cell death in order to establish a mutualistic relationship with barley roots (39). The presence of *P. indica* attenuates the expression of a barley BAX inhibitor gene. This gene is known to inhibit plant cell death and fungal colonization was inhibited in transgenic barley that expressed the gene.

**Insect Pests**

Horizontally transmitted endophytes are highly variable in their effects on shoot-feeding insects (69), but several studies have demonstrated potential in this area. Three of fifty foliar fungal endophytes isolated from *Picea rubens* (red spruce) needles showed toxicity against *Choristoneura fumiferana*, the eastern spruce budworm (156). *Beauveria bassiana*, *Lecanicillium lecanii*, and *Aspergillus parasiticus* are entomopathogens that have been identified as endophytes in various plants and can be successfully inoculated in the field (60). Positive results with *B. bassiana* and *L. lecanii* include a decrease in reproduction and leaf consumption by the aphid *Aphis gossypii* and a decrease in the growth of nymphs of the Australian plague locust *Chortoicetes terminifera* following inoculation with the fungi (67). Endophytes can also offer protection from other plant pests:
Meira geulakonigii colonizes grapefruit peel and protects against mites (113). Inoculation with Fusarium oxysporum reduced populations of nematodes in roots of banana and tomato plants (144).

Of course, unsuccessful attempts at biocontrol are much less likely to be published than successful ones. Although the cited studies show impressive potential for using endophytes for biocontrol in crop plants and forest trees, it should not be assumed that all endophytes are effective at plant protection (42).

Field Applications

The fluidity of lines between endophytes and other functional groups, as shown in the previous sections, raises questions about the safety of field applications of endophytes for biocontrol. Will changes in the environment (e.g., nutrient availability or gene expression in the host) shift the fungus from mutualistic to pathogenic? Will the endophyte be pathogenic in other, nearby plants? Will it harm beneficial insects as well as pests? Will it produce mycotoxins? Because all of these actions have been shown to occur in some endophyte-host combinations and not occur in others, these questions should be considered for each particular application. For example, there are commercial formulations of clavicipitaceous endophytes of grasses that protect turfgrasses from insect pests (33). These products would be undesirable when applied to pasture grasses, however, because the same secondary metabolites that deter insects are toxic to livestock.

In some cases, inconsistency in pathogenicity versus mutualism could simply reflect poor taxonomic knowledge of the fungi (3, 82). In Colletotrichum it can be difficult to distinguish Colletotrichum theobromicola, which causes anthracnose in leaves, from Colletotrichum ignatum and C. tropicale, which infect leaves without causing disease but cause rot in ripe fruits (132). Comparisons between greenhouse and in vitro experiments also show inconsistent results: When P. contorta was inoculated with P. fortinii, open pot culture experiments and in vitro culture assays differed in response in terms of biomass and foliar nutrient concentration (86).

It is clear that more comprehensive surveys are needed to document relationships of endophytes with congeneric saprotrophs and pathogens. In addition, the complexity of interactions among endophytes, pathogens, insects, and plants shows the difficulty of predicting outcomes for plant protection (59, 79). Pilot studies on these interactions also need to be scaled up to field experiments. We think advances in the biology of endophytes, combined with growing interest in biocontrol and integrated pest management, will lead to a growing number of commercial applications.

Another potential use of endophytes, at least in the case of those endophytes that are vertically transmitted, is vectors of genes intended to improve plant performance; transformation of the endophyte may be easier than transformation of the plant (33).

ENDOPHYTES AS SOURCES OF NOVEL SECONDARY METABOLITES

Endophytes are fertile ground for drug discovery (149). The number of patents that use endophytes for the production of secondary metabolites or with biologically important activities has increased dramatically in the past 20 years (Figure 7) (118). Classes of bioactive metabolites obtained from endophytes include alkaloids, cytochalasins, polyketides, terpenoids, flavonoids, and steroids (66). Activity targets include bacteria, fungi, and cancer cells; cellular targets include cell division, glucose transport, HIV-1 protease, and the cytoskeleton (60, 71, 151).

One of the best-known and most curious examples is that of paclitaxel, the first billion dollar anticancer drug (154). Paclitaxel was so successful in the 1990s that the survival of the source plant, the yew Taxus brevifolia, was threatened by overharvesting. An endophyte of T. brevifolia, Taxomyces andreanae, was also found to produce paclitaxel, presumably the
result of lateral gene transfer from host to fungus (151). Other examples of potentially useful metabolites from endophytes abound. *Fusarium subglutinans*, an endophyte in stems of a perennial twining vine, *Tripterygium wilfordii*, produces subglutinol A and diterpene pyrones with immunosuppressive activity (91, 155). Another *Fusarium* showed activity against intravascular thrombosis, a main cause of cardiovascular diseases (172). Endophytes clearly have potential for bioprospecting.

Interesting and important examples of endophyte secondary metabolism are still being discovered. Several groups of Convolvulaceae, including common *Ipomoea* species and cultivars (e.g., morning glories), are notorious for the ergot alkaloids in their seeds, compounds related to LSD (115). It has recently been discovered that these alkaloids are produced by endophytic fungi in the Clavicipitaceae (89). The fungi are unculturable, but their presence is revealed by direct sequencing. The endophytes are vertically transmitted through the seed, and fungicide-treated plants have neither the fungi nor the alkaloids (1). The proportion of the plant metabolome derived from or affected by endophytes is unknown.

### ARE SOME ENDOPHYTES LATENT SAPROTROPHS?

Many authors have proposed that some endophytes are latent saprotrophs that get a head start, becoming active on the death of the host organ. A saprotroph able to colonize living tissues will be able to start growing as soon as the tissue senesces, gaining a competitive advantage over other saprotrophs that arrive later. In the initial stages of decomposition, the fungi will have rapid access to sugars and other nutrients easy to assimilate that will not be available to later arrivals. In addition, senescent plants could provide stimuli or opportunities for sporulation absent from living plant tissues. On a community level, there are diffuse benefits to this head-start saprophytism: It allows rapid turnover of nutrients (85).

This hypothesis of endophytes as latent pathogens was supported by a phylogenetic study that compared relationships of endophytes and saprotrophs isolated, respectively, from healthy and decaying leaves and twigs of *Magnolia liliifera* (120). In the common endophyte genera *Colletotrichum/Glomerella*, *Fusarium/Nectria*, *Phomopsis/Diaporthe*, *Guignardia*, and *Corynespora*, endophytic and saprotrophic isolates are closely related, supporting the hypothesis of latent saprotrophism. No saprotrophic *Xylaria* was isolated, thus for *Xylaria* the hypothesis could not be evaluated. In the case of *Xylaria*, endophytic and saprotrophic species in the same area, and even on the same host, may be distinct (96).

Other studies also support the saprotroph head-start concept. Over 20% of root endophytes in blue grama were close relatives of saprotrophic and coprophilous fungi (123). In fungal communities in litter of *Pinus edulis, Juniperus monosperma*, and *Populus deltoides* in New Mexico, several clades showed high similarity with endophytic fungi reported for arid grasslands and other environments (51, 123, 121). In this case, it is possible that endophytes represent saprotrophs that landed in nontarget hosts, as discussed above for pathogens. The three litter types differed significantly in composition...
of their fungal communities, suggesting specificity.

Metabolic activity typical of saprotrophs has been demonstrated in several groups of endophytes. The endophyte *Hymenoscyphus ericae* produces extracellular enzymes with cellulolytic, hemicellulolytic, pectinolytic, and ligninolytic activity in vitro, able to penetrate plant cell walls to access complex humic clusters (27). The capacity to degrade complex organic molecules can be viewed as indirect evidence of latent saprotrophism.

Dual roles as endophyte and saprotroph have also been observed for *Phoma medicaginis*, a dominant endophyte in alfalfa plants (*Medicago lupulina* and *Medicago sativa*) that accelerates growth and sporulation after death of the host. In addition, the fungus produces brefeldin A, a metabolite that shows antifungal activity against other fungi, facilitating the transition from an endophytic to saprotrophic life style (Figure 4) (167). Similar adaptations were demonstrated for *Phomopsis*: In Australia, *Phomopsis leptostromiformis* can colonize lupins as an endophyte but grows extensively as a saprotroph after plant death (168). *P. leptostromiformis* is also known to produce a large number of mycotoxins that facilitate its establishment. The ability of the fungus to compete for the substrate in early decomposition stages facilitates its reproduction and sporulation (30, 65).

The contrary case, facilitation by endophytes of saprotrophic fungi, has also been demonstrated. Leaves of *Camellia japonica* colonized by the endophytic fungus *Coccomyces* sp. are more accessible to other decomposers than leaves without it (111). Similarly, interactions among endophytes (*Phomopsis* and *Xylaria*) and two saprotrophic fungi (*Mycena polygramma* and *Phanerochaete*) affect the decay rate of twigs of Japanese beech (47). These studies reveal endophytes as important elements in decomposition. However, little is known about how much overlap exists between saprotrophic and endophytic communities, the mechanisms that trigger the transition from endophytic to saprotrophic life styles in a senescent plant or in litter, and the regulatory pathways that control expression of the enzymes during decomposition versus endophytic or pathogenic colonization.

**ENDOPHYTES IN PHYTOREMEDIATION**

Bacterial endophytes can make plants more effective at tolerating and degrading xenobiotic compounds in the soil. For example, plants in soil contaminated with petroleum had endophyte communities with higher frequencies of hydrocarbon-degrading bacteria than plants on uncontaminated soil (143). Plants inoculated with VM1330, an engineered endophytic bacterium, showed increased tolerance to toluene and decreased transpiration of toluene to the atmosphere (105). *Festuca* grasses infected with endophytic clavicipitaceous fungi removed more polycyclic aromatic hydrocarbons from oil-contaminated soils than uninfected plants (147). A study of *Lolium perenne* inoculated with the endophyte *Acremonium loli* showed an increase in plant resistance to zinc toxicity (22). Similarly, mycorrhizal fungal communities are known to vary in their response to heavy metals and also to facilitate plant survival in contaminated soils (56). Phytoremediation enhancement could be a result of improvement in nutrition, protection against pathogens or direct degradation or accumulation of heavy metals in specific structures (56, 159). Few phytoremediation studies have focused on endophytic fungi or complete microorganisms to date, but given the utility of fungi in bioremediation, the potential is clear.

**ENDOPHYTES AND PLANT RESPONSES TO CLIMATE CHANGE**

Given that plant microbiomes influence plant establishment, resistance to environmental stress, and survival, it is expected that they influence the response of plants and ecosystems to climate change (134). The study of how plant-fungal systems respond to environmental cues is complicated by the fact that plant microbiomes are dynamic and show large seasonal
variation and host variation even among sympatric plant species (34, 37, 83, 97). For example, in sub-Arctic plants colonized with AMF and DSE, *Ranunculus acris* showed high rates of colonization by both throughout summer, *Trollius europaeus* had lower colonization, and in *Alchemilla glomerulans* colonization by DSE fungi decreased during the summer (135).

Despite these limitations, several studies have demonstrated that endophytic communities affect plant responses to various aspects of climate change, such as warming, increase in carbon dioxide, increase in nitrogen deposition, and prolonged periods of drought (87, 49). Warming increased density of some endophytic species in arctic willow (*Salix arctica*) in Canada (49). The association of plants with endophytes can also ameliorate the negative effects of drought. Cacao plants colonized by the endophyte *Trichoderma hamatum* showed delayed gene expression in drought conditions. This could be a result of an improvement in nutrient acquisition, plant growth or signaling pathways when the plant is colonized by the endophyte. Fungal endophytes can also control plant responses to stress through the production of specific molecules: *Paraphaeosphaeria quadriseptata* produces a heat shock protein 90 (HSP90) inhibitor, monocillin I, that enhances heat stress tolerance in *Arabidopsis* (103).

The increase of CO₂ in the atmosphere stimulates photosynthetic activity and plant growth. Mycorrhizal and endophytic microbial communities depend on plant carbon, so elevated levels of CO₂ can also modify plant-microbial interactions. *P. sylvestris* seedlings inoculated with dark septate fungi showed an increased in biomass under elevated CO₂ conditions and an improvement in the use of nutrients (3). Elevated CO₂ stimulated the mycorrhizal root colonization of *Plantago lanceolata* and stimulated growth of endophytic fungi in *Festuca arundinacea*, which affected the distribution of nitrogen between the two plants (32).

Long-term experiments indicate that ecosystem stability is positively dependent on root diversity and biomass, which is in turn influenced by the colonization levels of mycorrhizal and endophytic fungi. Large numbers of species are necessary to support ecosystem functioning, especially where land use is intensive over large areas (99), but our knowledge about the role of endophytic fungi on plant and ecosystem stability is very limited. The establishment of long-term monitoring experiments, and the use of quantitative and standardized methods, is essential to understand the roles of plant microbiomes in responses to climate change.

**CONCLUSIONS**

So do fungal endophytes matter? In many cases they clearly do: We have presented examples in which diverse endophytes affect diverse plants in diverse ways, including in their interactions with fungal and insect pests. However, as noted above, experiments with negative results are less likely to get published than positive ones. A plant at any given moment contains a great diversity of endophytes, some of which may later become important, for better or worse. Most will probably never significantly affect the host as individuals, but they may have impacts as part of the microbiome and functional metagenome. The fact that different organs of a single host contain different communities increases its phenotypic plasticity (72), perhaps an advantage in unpredictable environments. This is itself an emergent property that is possible as a result of the complex interactions between microbiomes and plants.

It is increasingly clear that endophytes overlap with other functional groups of plant-associated microorganisms—for example, the DSEs that form associations that function like mycorrhizae—so part of the importance of endophytes is self-contradictory, as some do not remain endophytes. Our increasing ability to visualize complex interactions in the microbiome will help us understand the roles of fungal endophytes. We believe that there are unknown but important endophytes and effects of endophytes yet to be discovered.

Recent studies on the human microbiome have yielded amazing results. For example, the
bacterial community of the skin and digestive system is unique for each individual (36, 46). Differences among progeny of interspecific hybrids of *Populus* in relationships with endophytes (15) and in endophytes among leaves on a single tree (98) suggest that plants may also have unique microbiomes, probably at the level of cultivars, if not at the level of individuals. As pressures on the global food supply increase because of population growth, increasing affluence, and climate change, agriculture will be forced to do more with less. Plant genetics and breeding are receiving widespread attention as ways to address this need (157). We believe that understanding and manipulating the complex interactions of plant microbiomes, although less obvious, will be equally important.

**SUMMARY POINTS**

1. In interactions with plants and the environment, fungal endophytes show functional overlap and complex interactions with other groups: mycorrhizal fungi, pathogens, saprotrophs, epiphytes, and bacteria.

2. Until recently culturability of endophytes was a limiting factor. Direct PCR from plant tissue allows unculturable species to be detected. Metagenome approaches and other PCR approaches allow simultaneous detection and the study of active components of the whole microbiome.

3. Some interactions among endophytes, other microbiome components, host plants, pathogens, and pests show emergent properties. The study of plant microbiomes as a system allows analysis and integration of these complex interactions.

4. DSEs are found in roots of many plant groups, especially in nutrient-poor boreal soils and arid systems. As a heterogeneous and sometimes dominant group, their importance is increasingly evident and includes mycorrhizal functions in some plants.

5. Some endophytes may be latent pathogens, some may be derived from pathogens, and others may be latent saprotrophs, but many are neither.

6. Endophytes have been shown to protect various plants from a wide range of pathogens and insect pests. Some function by antagonism, inducing host defenses, and/or improving plant health and nutrition.

7. Some endophytes enable plant hosts to survive in hostile environments where neither host nor endophyte can survive alone. This emergent property shows that endophytes could affect plant responses to climate change.

8. Endophytes are a mine for bioprospecting. By extrapolation from numbers of new endophytes and metabolites reported in the past twenty years, many interesting cases are yet to be discovered.

**FUTURE ISSUES**

1. Toward a synthesis era: Can the research community agree on guidelines for isolation, extraction, amplification, annotation, and sequence database curation?

2. How do we identify functional cores of plant microbiomes and predict which endophytes are likely to affect plant health or produce useful secondary metabolites?
3. Which endophytes are metabolically active in the plant, and which are merely hitchhikers?

4. How can we best apply a systems biology approach to complex, simultaneous interactions of the different groups of organisms that affect plant health?

5. How can useful endophytes be applied for pest control or to confer other desired traits on their hosts?

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Errata

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