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Fungal endophytes: modifiers of plant disease

Posy E. Busby[1,2] · **Mary Ridout**[2] · **George Newcombe**[2]

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Abstract Many recent studies have demonstrated that non-pathogenic fungi within plant microbiomes, i.e., endophytes ("endo" = within, "phyte" = plant), can significantly modify the expression of host plant disease. The rapid pace of advancement in endophyte ecology warrants a pause to synthesize our understanding of endophyte disease modification and to discuss future research directions. We reviewed recent literature on fungal endophyte disease modification, and here report on several emergent themes: (1) Fungal endophyte effects on plant disease span the full spectrum from pathogen antagonism to pathogen facilitation, with pathogen antagonism most commonly reported. (2) Agricultural plant pathosystems are the focus of research on endophyte disease modification. (3) A taxonomically diverse group of fungal endophytes can influence plant disease severity. And (4) Fungal endophyte effects on plant disease severity are context-dependent. Our review highlights the importance of fungal endophytes for plant disease across a broad range of plant pathosystems, yet simultaneously reveals that complexity within plant microbiomes presents a significant challenge to disentangling the biotic environmental factors affecting plant disease severity. Manipulative studies integrating eco-evolutionary approaches with emerging molecular tools will be poised to elucidate the functional importance of endophytes in natural plant pathosystems that are fundamental to biodiversity and conservation.

Keywords Microbiome · Disease ecology · Context-dependency · Biocontrol · *Alternaria* · *Cladosporium* · *Fusarium* · *Trichoderma* · *Aureobasidium* · *Penicillium*

Introduction

Plants resist pathogens with induced genetic defenses (Jones and Dangl 2006). As disease develops in susceptible plants, symptoms appear in the host. The severity of these symptoms is thought to depend on host genetic susceptibility, pathogen virulence, and an abiotic environment conducive to infection (i.e., the disease triangle model, Stevens 1960). However, it is now apparent that endophytes (Petrini 1991; Bills 1996; Wilson 1993; Stone et al. 2000) can also influence the severity of disease symptoms (Freeman and Rodriguez 1993; Arnold et al. 2003). In particular, endophytes have been shown to decrease (pathogen antagonism) or increase (pathogen facilitation) plant disease severity in functional assays that include susceptible plants, virulent pathogens, and an abiotic environment conducive to these interactions.

Endophytes are more diverse and abundant than pathogens within the plant microbiome (Ganley et al. 2004), yet their involvement in plant defense is under-appreciated. The objective of our review is therefore to synthesize current knowledge on fungal endophyte disease modification (bacterial endophyte disease modification is reviewed elsewhere: Weller 1988; Raaijmakers et al. 2002; Compant et al. 2005). This foundation can serve as a guide for future research aimed at understanding the ecological and

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✉ Posy E. Busby
busby@post.harvard.edu

[1] Department of Biology, Duke University, Durham, NC 287708, USA

[2] Department of Forest, Rangelands and Fire Sciences, University of Idaho, Moscow, ID 83844-1133, USA

evolutionary significance of endophyte disease modification in nature, and for using endophytes as biocontrols in agricultural plant systems (Dangl et al. 2013; Ledford 2015).

Our review focuses predominantly on ascomycetous fungal endophytes that form localized infections within the leaves, stems and roots of all land plants (Rodriguez et al. 2009). We do not discuss the specialized group of Clavicipitaceous grass endophytes that deters herbivores, among other ecological functions (see reviews by Breen 1994; Carroll 1992; Clay 1988, 1990, 2014). Technical definitions of endophytism have varied over the years (e.g., Wennstrom 1994; Wilson 1995; Saikkonen et al. 1998; Rodriguez et al. 2009). One standard, working definition of endophytism is that "…infections are inconspicuous, the infected host tissues are at least transiently symptomless, and the microbial colonization can be demonstrated to be internal…" (Stone et al. 2000). This definition is generally appropriate, and corresponds to the usual method of isolating endophytes from asymptomatic, surface-sterilized plant tissues. However, it is important to recognize that with this method: (1) epiphytes recalcitrant to surface-sterilization may appear to be endophytes, (2) some fungi found on plant surfaces also exist internally within plants, so rigid distinctions between epiphyte and endophyte are not always useful, and (3) endophytes co-occur with pathogens in diseased plant tissues, so endophyte studies that exclude diseased tissues may also exclude disease-modifying endophytes.

Endophytes have been linked to most aspects of plant fitness (Rodriguez et al. 2009), yet a unifying ecological function for this group of fungi has remained elusive. Traditionally, endophytes were viewed as 'latent saprotrophs' or 'secondary pathogens' (Ellis 1972; Funk and Centre 1985; Schulz et al. 1999) with little functional consequence for plant hosts. However, 'latent saprotrophs' and 'secondary pathogens' lack a functional proof equivalent to Koch's Postulates for pathogens. The assumption was that endophytes as latent saprotrophs lie in wait for plant tissues to senesce; or, that given enough time, endophytes as secondary pathogens cause disease (Photita et al. 2004). This latter hypothesis is impossible to conclusively test because observation periods might be too short; even observation periods of months or years might be too short for a true, secondary pathogen in a long-lived host. Support for a new, testable hypothesis is growing: endophytes are largely modifiers of host plant disease severity.

Despite an increasing number of studies, the underlying mechanism by which endophytes modify plant disease severity is often unclear. Induction of host resistance is the first mechanism that is typically considered. Mycorrhizal fungi (Pozo et al. 2002), plant-associated bacteria (Sequeira et al. 1977), viruses (Ross 1961), and nematodes (Kosaka et al. 2001) are already known to contribute to plant defense against pathogens by triggering host resistance, aka systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Wees et al. 2000). Some fungal endophytes have more recently been shown to modulate host plant resistance (Zeilinger and Omann 2007). For example, the fungal leaf endophyte *Colletotrichum tropicale* induces the expression of hundreds of host defense-related genes in *Theobroma cacao*, resulting in greater plant immunity (Mejía et al. 2014). Alternatively, endophytes could suppress host defenses to enable their own infection, thereby making it easier for co-occurring pathogens to infect (Houterman et al. 2008; Bonello et al. 2008).

Endophytes also interact with pathogens directly in ways that can modify the expression of host plant disease. Endophytes can antagonize pathogens via hyperparasitism, competition or antibiosis. Numerous studies have demonstrated such inhibitory effects using in vitro fungal competition experiments, or by exposing pathogens to endophyte metabolites or volatiles (e.g., Heather and Sharma 1987; Pandey et al. 1993; Bailey et al. 2008; Martín et al. 2015). For just one example, species of *Ampelomyces* are mycoparasites that grow internally within powdery mildew hyphae where they suppress mildew sporulation and eventually kill parasitized fungal cells (Kiss 2003). Direct mechanisms responsible for disease modification are likely more diverse those identified to date. For example, phyllosphere fungi could antagonize rust pathogens (belonging to Uredinales, a fungal order of obligate biotrophs) simply by physically interfering with the rust's thigmotropic mechanism of locating stomata (Hoch et al. 1987); rust sporelings that cannot find stomata to penetrate will die on the plant surface. Mechanistic multiplicity in general suggests degeneracy in plant defense, with any one of multiple, dissimilar mechanisms responsible for disease modification (Maleszka et al. 2014).

Interactions between endophytes and pathogens that facilitate disease development are not as well studied. Pathogens could exploit endophyte metabolites to enable their own virulence. Disease-enhancing synergisms between plant viruses (Pruss et al. 1997), and between fungi and herbivorous insects (Caesar 2003) are known, and there are likely similar interactions between pathogenic and endophytic fungi. More generally, it is important to remember that fungi, as eukaryotic microbes, are likely influenced by their interactions with microbes just as eukaryotic macrobes (plants and animals) are.

With few studies addressing mechanisms, our review focuses instead on characterizing the reported outcomes of plant-endophyte-pathogen interactions for disease severity. In particular, we address three specific questions: (1) What

types of ecological interactions are reported among plants, endophytes and pathogens? (2) Do particular fungal endophytes commonly modify plant disease severity? And (3) Under what conditions do endophytes modify disease? We address these questions by reviewing studies that used in vivo manipulative experiments to test fungal endophyte disease modification. In total, we reviewed 85 papers. The endophyte(s) tested in each plant pathosystem are listed along with the outcome of disease modification (i.e., pathogen antagonism, pathogen facilitation, or neutral interaction) in Supplementary Table 1.

A full range of interactions from antagonism to facilitation

Agricultural plants are the subjects of the majority of studies in the endophyte disease modification literature, greatly outnumbering wild and invasive plant studies (Supplementary Table 1). Given that domesticated plants represent only a small fraction of global plant biodiversity (approx. 124 crop species—Gallai et al. 2009—within a kingdom of approx. 450,000 species—Pimm and Joppa 2015), the focus on this group clearly reflects a bias toward studying economically important plants. Furthermore, the agricultural incentive to develop biocontrol agents that *reduce* crop disease severity may result in a tendency to report pathogen antagonism more frequently than pathogen facilitation or neutral interactions. And indeed, pathogen antagonism was by far the most commonly reported ecological interaction in the reviewed studies overall, with studies of agricultural plants in particular reporting pathogen antagonism more frequently than studies of wild and invasive plants (Supplementary Table 1).

Despite the potential for bias in a literature dominated by agricultural plants, the reviewed studies demonstrate a full range of interactions—from antagonism, to neutral interactions, to facilitation—among plants, fungal endophytes and pathogens (Supplementary Table 1). In nature, all types of interactions can be found in a single plant pathosystem. For example, leaf endophytes of *Fallopia japonica* (Japanese knotweed) can facilitate (*Phomopsis* sp.), antagonize (*Alternaria* sp. and *Phoma* sp.), or have no effect (*Collectotrichum* sp. and *Pestalotiopsis* sp.) on the severity of a leaf rust disease caused by *Puccinia polygoni-amphibii* var. *tovariae* (Kurose et al. 2012). Similarly, in *Populus trichocarpa*, the black cottonwood of the Pacific Northwest, fungal leaf endophytes can facilitate (by 2×), antagonize (by up to 40×), or have no effect on the severity a leaf rust disease caused by *Melampsora* × *columbiana* (Fig. 1, Raghavendra and Newcombe 2013; Busby et al. 2015). In the *P. trichocarpa* leaf microbiome the pathogen antagonists identified include species of *Cladosporium*, *Trichoderma*, *Chaetomium*, *Penicillium*, *Truncatella*, *Ulocladium* and *Stachybotrys*. Several of these endophytes are dominant foliar fungi in wild *Populus* trees across the Pacific Northwest (e.g., species of *Cladosporium* and *Trichoderma* are among the top 15 operational taxonomic units, or OTUs, of 968 total, Busby et al. 2015), but others (e.g., *Stachybotrys*) are rare. The root endophyte *Morchella*, also commonly found in plants of the Pacific Northwest (Baynes et al. 2012), can likewise reduce *Melampsora* rust disease severity (Busby unpub. data, Fig. 1). The only pathogen facilitator identified was *Epicoccum nigrum*, again, a common leaf endophyte of *P. trichocarpa* (Busby et al. 2015) and many other plants (Vázquez de Aldana et al. 2013). Overall, in this wild plant pathosystem endophytes are not only capable of modifying plant disease severity, but they also appear to be common enough in nature to be ecologically important.

Diversity of disease modifiers

Endophyte disease modification in *F. japonica* and *P. trichocarpa* is representative of a broader pattern found across all studies: a taxonomically diverse group of commonly occurring fungal endophytes can modify disease severity (Supplementary Table 1). With few studies reporting pathogen facilitation, it is premature to search for taxonomic trends. However, several fungal genera are commonly reported as pathogen antagonists: species of *Trichoderma* (Carisse et al. 2000; Narisawa et al. 2002; Andrews et al. 1983; Elad et al. 1980; Grosch et al. 2006; Jones et al. 2014; Kandula et al. 2015; Larran et al. 2016; Danielsen and Jensen 1999; Martinez-Medina et al. 2014; Larkin and Fravel 1998; Mousseaux et al. 1998; Morago-Suazo et al. 2011; Michereff et al. 1995; Pandey et al. 1993; Hanada et al. 2008, 2010; Raghavendra and Newcombe 2013; Romeralo et al. 2015; Busby et al. 2015), *Aureobasidium* (Andrews et al. 1983; Wachowska and Glowacka 2014; Pandey et al. 1993; Brame and Flood 1983; Romeralo et al. 2015), *Fusarium* (Narisawa et al. 2002; Lee et al. 2009; Rodriguez-Estrada et al. 2012; Larran et al. 2016; Larkin and Fravel 1998; Pandey et al. 1993; Perello et al. 2002; Arnold et al. 2003; Hanada et al. 2010), *Penicillium* (Narisawa et al. 2002; Waqas et al. 2015; Pandey et al. 1993; Hanada et al. 2010; Ridout and Newcombe 2015; Busby et al. 2015), *Chaetomium* (Andrews et al. 1983; Larran et al. 2016; Dingle and McGee 2003; Istifadah and McGee 2006; Danielsen and Jensen 1999; Perello et al. 2002; Busby et al. 2015), *Bionectria* (Rodríguez et al. 2015; Hue et al. 2009; Morago-Suazo et al. 2011; Cota et al. 2008; Yohalem 2004; Borges et al. 2015) and two yeast genera, *Pichia* (De Melo et al. 2015; Masih and Paul 2002; Kefialew and Ayalew 2008) and *Candida* (Mekbib et al. 2011; de Capdeville et al. 2002; Usall et al. 2000). Some of

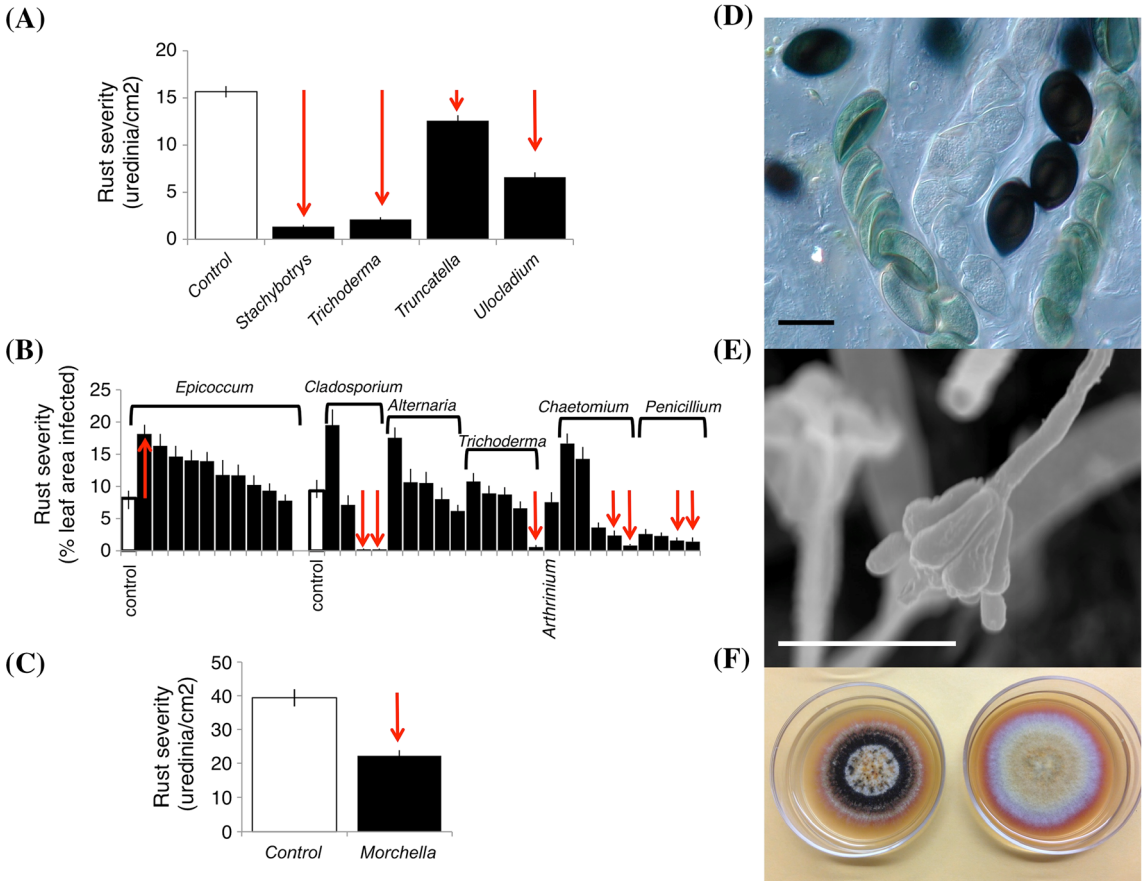

Fig. 1 Fungal leaf and root endophytes modify *Melampsora* × *columbiana* rust disease severity in *Populus trichocarpa*. In one study, leaf endophyte species within *Stachybotrys*, *Trichoderma*, *Truncatella* and *Ulocladium* all reduced disease severity (**a**) (Raghavendra and Newcombe 2013). In a subsequent study, a taxonomically and geographically more diverse group of fungal leaf endophytes had a broader range of effects, from rust antagonism to facilitation (**b**) (Busby et al. 2015). The commonly occurring root endophyte *Morchella* can also reduce rust disease severity (**c**) (Busby unpub. data). *Open bars* are controls; *error bars* are standard error; *red arrows* emphasize the direction (i.e., pathogen antagonism or pathogen facilitation) of statistically significant disease modification. Images of endophytes in *right panels* are: ascospores of *Chaetomium* sp. found in association with *Melampsora* leaf rust (**d**), asexual conidia of the pathogen antagonist *Stachybotrys* (**e**), and pure cultures of the pathogen facilitator *Epicoccum nigrum* on PDA (**f**). *Scale bars* in **d**, **e** are approximately 10 um

these fungal genera, like *Trichoderma*, include species that are well known to antagonize plant pathogens via mycoparasitic interactions and to trigger SAR (Perazzolli et al. 2008; Segarra et al. 2007; Harman et al. 2004). In addition, yeast endophytes are increasingly recognized as antagonistic against postharvest pathogens (Spadaro and Gullino 2004). In contrast, endophytes within other genera, like *Fusarium*, are less familiar pathogen antagonists.

The eight fungal genera found to commonly antagonize pathogens occur in five different fungal orders within the phylum ascomycota (Hypocreales, Eurotiales, Sordariales, Dothideales and Saccharomycetales) and thus clearly illustrate that disease modification is an ecological function that is not narrowly phylogenetically constrained. Even ascribing ecological function at the genus level may prove difficult when taxonomic groups like *Fusarium* and *Penicillium* include pathogen antagonists, pathogen facilitators, and pathogens themselves (Rodríguez et al. 2015; Rodriguez-Estrada et al. 2012). Moreover, the ecological function of endophytes may also vary within a fungal species. For example, isolates of *Trichoderma atroviride* varied in pathogen antagonism toward three different pathogens of ryegrass: *Rhizoctonia solani*, *Pythium ultimum* and *Sclerotinia trifoliorum* (Kandula et al. 2015). Similarly, strains of several different endophyte species varied in antagonism (*Cladosporium tenuissiumum*, *Chaetomium funicola*) and facilitation (*E. nigrum*) toward *Melampsora* rust in *P. trichocarpa* (Busby et al. 2015). Such variation could reflect intraspecific variation in endophyte function, or interspecific variation that is not resolved by the ITS locus (Kõljalg et al. 2013). Both greater taxonomic resolution of endophytes and more studies in wild plant pathosystems are needed before pathogen antagonism and facilitation can be mapped onto fungal and plant phylogenetic trees.

Context dependency of disease modification

Another theme to emerge from recent literature is that endophyte modification of plant disease is context-dependent, depending on the abiotic and biotic environment, host plant, and/or pathogen. In other words, inoculation with the same endophyte can result in different effects on disease severity. Unfortunately, few studies explicitly address context-dependency by manipulating factors potentially affecting endophyte disease modification while holding others constant. Thus our discussion is less retrospective than prospective (see also *Research moving forward*).

Environmental factors such as temperature, pH and humidity influence fungal activity (Cook and Baker 1983), and thus they should also influence fungal endophyte disease modification. For example, *Trichoderma* sensitivity to soil moisture influenced the strength of disease modification in one study (Jones and Bienkowski 2015), and *Candida* sensitivity to atmospheric conditions influenced its antagonistic effects on an apple pathogen in another study (Usall et al. 2000). In a field study, xylem endophytes varied in their antagonism of the Dutch elm disease pathogen across study years, possibly reflecting abiotic environmental effects on endophyte disease modification, although the biotic environment could not be ruled out (Martín et al. 2015). Because endophytes interact with pathogens in the context of taxonomically diverse plant microbial communities (Vorholt 2012; Christian et al. 2015), this biotic environment must play an important, if under-appreciated, role in endophyte disease modification. Indeed, endophytes themselves are part of the biotic environment. Simply by manipulating the order of endophyte arrival into the microbial community, Adame-Álvarez et al. (2014) demonstrated that the ecological function of an endophyte can shift from pathogen antagonism to pathogen facilitation. In another example, manipulating soil microbiota altered the outcome of leaf rust disease severity in a wild flax host (Tack et al. 2015). More distantly related microorganisms within the biotic environment, for example fungal-feeding nematodes and slime molds, may also alter endophyte disease modification by consuming disease-modifying endophytes.

In addition to the environment, the other two legs of the disease triangle—the host plant and pathogen—also matter for fungal endophyte disease modification (i.e., when the same endophyte isolate is tested in different plant pathosystems its effects on disease severity can vary). The best examples of host/pathogen combinations jointly affecting endophyte disease modification are found in highly specific, biotrophic rust and powdery mildew pathosystems (Nischwitz et al. 2005; Kiss 2003). Studies controlling endophyte isolate and plant host have also demonstrated that the identity of the *pathogen* alone matters for endophyte disease modification. Pandey et al. (1993) tested fifteen leaf endophyte species against two different pathogens of *Psidium guajava*: *Pestalotia psidii* and *Colletotrichum gloeosporioides*. While fourteen of fifteen were antagonistic against *C. gloeosporioides*, only nine were antagonistic against *P. psidii*. In another study, Perello et al. (2002) tested nine leaf endophyte species against four different necrotrophic wheat pathogens: *Zymoseptoria tritici*, *Alternaria triticimaculans*, *Bipolaris sorokiniana*, and *Drechslera tritici-repentis*. All nine endophytes were antagonistic against *Z. tritici* and *D. tritici-repentis*, eight were antagonistic against *B. sorokiniana*, and four were antagonistic against *A. triticimaculans* (Perello et al. 2002). In contrast, *Glomus* root endophytes had the same antagonistic effect against two pathogens (*Embellisia chlamydospora* and *Fusarim oxysporum*) of *Vulpia ciliata* (Newsham et al. 1995).

There are several explanations for why the same endophyte might differentially affect the severity of disease caused by two different pathogens (holding host plant constant). For example, plant recognition of an endophyte may prime a plant for defense against a pathogen (Redman et al. 1999), and this response may be more robust for closely related endophyte-pathogen pairs. Basic biological differences between pathogens could also result in distinct ecological interactions with endophytes. Biotrophic pathogens parasitize living plant cells whereas necrotropic pathogens feed on dead plant cells. Due to the biotrophic nature of rusts and powdery mildews, pathogen antagonists that act via antibiosis and/or mycoparasitism may be more likely to impact biotrophic pathogens, whereas pathogen antagonists that act via competition for nutrients in the apoplast may be more likely to impact necrotrophs.

Finally, controlling both endophyte and pathogen isolates can demonstrate that the identity of the *host plant* alone matters for endophyte disease modification. For example, Martín et al. (2015) reported that endophytes differentially modified disease caused by the pathogen *Ophiostoma novo-ulmi* in different genotypes of Dutch elm. However,endophyte effects on plant disease severity do not always depend on plant genotype (Raghavendra and Newcombe 2012; Busby et al. 2013). Genotypic differences in plant defenses could explain endophytes' differential effects if endophytes modulate defenses (Mejía et al. 2014). Or endophytes may colonize particular plants more efficiently than others (Arnold and Lutzoni 2007), and thereby interact more strongly with pathogens in those plants. If the host plant does matter, pathogens that alternate on different hosts may be differentially modified by the same endophyte when residing in different hosts.

Studying endophyte disease modification: past, present and future

Despite an agricultural focus in the endophyte disease modification literature, and a potential bias toward reporting pathogen antagonism, looking across all plant systems it is clear that disease modification is an ecological function shared by a diverse group of commonly occurring fungal endophytes. This emerging view of endophyte function has two significant implications: (1) it suggests that the disease triangle model would be improved by incorporating endophytes, and the broader plant microbiome, and (2) it suggests endophytes are not only involved in plant disease, but are also implicated in disease-based processes that are fundamental to biodiversity (Klironomos 2002; Benítez et al. 2013), conservation (Borer et al. 2007) and sustainable agriculture (Ledford 2015). Here, we discuss specific areas for future research addressing basic questions in plant-endophyte ecology that will also have direct and immediate agricultural applications. We begin with a discussion of methods for studying endophyte disease modification, highlighting new tools and techniques that will be invaluable in the pursuit of this research.

Methods for testing endophyte disease modification

Before testing fungal endophyte disease modification, the species involved in the tripartite plant-endophyte-pathogen interaction must be known. This task is typically most challenging for fungal endophytes because the majority of species of fungi have either never been described and/or belong to higher order taxa under revision. Traditional methods for approximating the identity of endophytes include isolating the endophyte in pure culture, determining its diagnostic, morphological features, and obtaining DNA sequence data. Sequencing multiple loci (e.g., ITS rDNA, LSU rDNA, beta-tubulin gene) can improve taxonomic resolution among closely related endophytes and help to resolve questions regarding inter- versus intraspecific variation in endophyte function.

Sequencing methods have advanced in the past few years from Sanger-sequencing DNA barcode regions (e.g., ITS rDNA) of individual isolates to sequencing DNA barcode regions for entire fungal endophyte communities using next-generation DNA sequencing (NGS) (Zimmerman and Vitousek 2012; Bonito et al. 2014). While these new methods may appear to be ideal for the characterization of complex, cryptic endophyte communities, extreme care is needed in the interpretation of NGS datasets (Nguyen et al. 2014). Far too often algorithm-based assignments are used to loosely link community members to fungal taxa in databases with poor endophyte representation (e.g., UNITE). Moreover, given limitations on the taxonomic inferences that can be drawn from the amplicons typically sequenced, and contamination in NGS datasets (Nguyen et al. 2014), it is only by combining these datasets with traditional methods for identifying fungi that community members can be confidently assigned to known taxa, or described as new.

Once isolated and assigned to taxa, proper maintenance of endophytes in culture is essential for reliable, repeatable tests of disease modification. Serial subculturing can alter the functionality of even asexually reproducing cultures. This has been shown for pathogens (Newcombe et al. 1990), and is likely true for endophytes as well. Endophyte isolates should therefore be: (1) archived immediately after isolation, (2) minimally sub-cultured, and (3) maintained as pure, single asexual genotype, cultures. Storing agar plugs of fungal isolates in tubes of sterile distilled water can maintain viability over long periods of time (Richter 2008). Alternatively, re-isolation of endophytes from inoculated plants may serve to 'refresh' an isolate's ecological function. Finally, deposition in established culture collections (e.g., CBS-KNAW Fungal Biodiversity Centre or ATCC: The Global Bioresource Center) is needed to ensure availability to other researchers.

Experimental approaches to demonstrating disease modification involve first inoculating plants with a fungal endophyte and then with a pathogen. Disease severity in endophyte-treated plants is then compared to severity in a control group. It is important to realize that demonstrating disease modification falls short of defensive mutualism, which additionally requires showing that host fitness is proportionally higher than non-host fitness in the presence of the pathogen relative to pathogen-free conditions (Clay 2014). While few studies take this extra step (Martinez-Medina et al. 2014; Rodríguez et al. 2015), it will be important for future studies addressing how disease-modifying endophytes impact plant ecology and evolution.

While the majority of reviewed studies included in vivo glasshouse assays only, coupling in vitro, in vivo and field tests of endophyte disease modification in different environments is the most robust approach for studying endophyte disease modification. An in vitro assay is often used as a preliminary screen of many endophytes. Of studies that have coupled in vitro and in vivo experiments (Adame-Álvarez et al. 2014; Andrews et al. 1983; de Capdeville et al. 2002; Martín et al. 2015; Grosch et al. 2006; Khastini et al. 2012; Masih and Paul 2002; Mekbib et al. 2011; Moraga-Suazo et al. 2011; Pandey et al. 1993; Sasan and Bidochka 2013; Stadler and von Tiedemann 2014; Hue et al. 2009), several found discrepancies between the two assays (de Capdeville et al. 2002; Martín et al. 2015) and thus revealed the importance of using live plants when testing endophyte disease modification. Even fewer studies

additionally tested endophytes in field experiments (Stadler and von Tiedemann 2014; Hue et al. 2009). Across in vitro, in vivo and field experiments, Hue et al. (2009) found consistent antagonistic effects of Clonostachys rosea on fusarium head blight of wheat. In contrast, Stadler and von Tiedermann (2014) found that *Microsphaeropsis ochracea* antagonized the soilborne pathogen *Verticillium longisporum* only under controlled in vitro and in vivo laboratory conditions, but not in the field. Field tests are thus clearly valuable for determining whether endophyte disease modification is robust to abiotic and biotic environmental variation, and for evaluating whether ecological interactions are locally adapted to environments.

In addition to manipulative tests, observational studies can provide additional insights into endophyte disease modification. In the past, such studies may have been avoided due to the complexity of microbial communities. However, DNA sequencing can now be used to characterize fungal endophyte communities and fungal interactions. For example, Tack et al. (2015) used terminal restriction fragment length polymorphisms (TRFLP) to characterize differences in soil bacteria and fungi in two field sites. They then conducted manipulative experiments to test if these differences affected the outcome of leaf rust disease severity. In another study, Busby et al. (2015) coupled experimental tests of endophyte disease modification with a NGS molecular field survey to evaluate the abundance and distribution of disease-modifying fungi in nature. Not only were several of the experimentally identified disease-modifying fungal endophytes common in wild trees, but NGS data also demonstrated that some of the endophytes correlated with disease severity in the direction predicted by experiments (Busby et al. 2015). The combination of manipulative experiments and molecular surveys bolstered the argument that endophytes play an important role in disease modification in nature. More precise tools, like qPCR, can further improve the resolution of such relationships. However, there are also limits to combining manipulative experimental approaches with indirect, sequence-based approaches. Primer bias may prevent amplification of particular disease modifying endophytes, and thus their observational study via next-generation amplicon sequencing. Alternatively, disease modifying endophytes identified by observational NGS studies may be un-culturable, and thus unavailable for in vivo testing.

Research moving forward

Fungal endophytes form taxonomically and functionally diverse communities in all plants (Rodriguez et al. 2009), yet the majority of these communities remain unknown to science. While a few large research efforts address how endophyte community structure varies among hosts and environments (e.g. NSF Dimensions of Biodiversity and GoLife projects), only by coupling such descriptive, correlative studies with manipulative tests of disease modification can we address the hypothesis that disease modification is an ecological function of fungal endophytes in wild plant pathosystems. Moreover, such studies will be poised to evaluate whether pathogen antagonism is indeed the overwhelming outcome of endophyte disease modification, or whether antagonism is simply over-reported in the literature (Supplementary Table 1).

Perhaps the most resounding theme to emerge from the literature that requires further study is the high degree of context-dependency. However, this should not come as a surprise given context-dependency in other plant-fungal symbioses. For example, mycorrhizal fungi are sometimes but not always beneficial to their plant hosts (Johnson et al. 1997), and fungal endophytes confer salt and drought tolerance to plants only in environments characterized by elevated salinity and aridity (Rodriguez et al. 2008). Given context-dependency, we need explicit questions and hypotheses to test when and where endophytes modify plant disease. For example: Is endophyte disease modification environmentally constrained? One hypothesis is that pathogen antagonism occurs only in environments characterized by elevated disease pressure, where antagonists confer a fitness advantage to plants. Related questions are: How does host genotypic variation influence endophyte colonization and disease modification? And is the ability to modify disease phylogenetically constrained within particular fungal lineages? Answers to these and other questions will help to determine whether endophyte disease modification is adaptive or incidental.

Presently, the ecological and evolutionary implications of plant-endophyte-pathogen interactions are not well understood. This is in part because few natural plant pathosystems have been studied. But more fundamentally, these complex interactions are likely to be as challenging to understand as mycorrhizal mutualisms (Kiers and van der Heijden 2006; Kiers et al. 2011; Selosse et al. 2006), and for the same reasons. Disease antagonists might be defense mutualists, but in systems studied to date, neither the plant, nor the pathogen, nor the endophytic antagonist is entirely exclusive with respect to partners. Just as 'cheaters' exploit the mycorrhizal mutualism by taking from the host while providing little in return, there may be endophytes that do the same. Pathogen facilitators that increase disease severity may, for example, obtain as many nutrients from the plant host as antagonists, while undermining defense. A more immediate challenge is to understand whether disease modifiers are interacting primarily with the plant or with the pathogen or with both. Fortunately, advanced genetic tools (e.g., genome wide

associated studies, RNA-Seq, isogenic plants with altered immune systems) are now available (Lebeis et al. 2015; Kruske et al. 2015) to answer this question in the context of manipulative studies.

Finally, and without a doubt, the development and deployment of endophyte technologies for reducing crop plant disease will require a sophisticated understanding of plant genotype-microbiome-environment interactions. Importantly, "environment" includes the diverse plant microbiome where fungal endophyte disease modification occurs. Much of this knowledge will come from new research directions that have already been discussed. However, addressing specific questions in the context of breeding programs and disease management strategies will also be essential. For example, how has artificial selection for desirable traits, including disease resistance, affected endophyte communities and endophyte disease modification? Additionally, how do fungicides and fertilizers affect the formation of beneficial symbioses? Synthetic chemicals are known to inhibit formation of effective nodules with N-fixing bacteria (Fox et al. 2007); do fungicides likewise inhibit pathogen antagonists? Answers to these and related questions will help to determine the feasibility and potential benefits of using pathogen antagonists in domesticated plant systems. In parallel, environmental assessment of non-host impacts must also be carefully evaluated.

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