Tansley review

Fungal endophytes: diversity and functional roles

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Summary

All plants in natural ecosystems appear to be symbiotic with fungal endophytes. This highly diverse group of fungi can have profound impacts on plant communities through increasing fitness by conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption, or decreasing fitness by altering resource allocation. Despite more than 100 yr of research resulting in thousands of journal articles, the ecological significance of these fungi remains poorly characterized. Historically, two endophytic groups (clavicipitaceous (C) and nonclavicipitaceous (NC)) have been discriminated based on phylogeny and life history traits. Here, we show that NC-endophytes represent three distinct functional groups based on host colonization and transmission, *in planta* biodiversity and fitness benefits conferred to hosts. Using this framework, we contrast the life histories, interactions with hosts and potential roles in plant ecophysiology of C- and NC-endophytes, and highlight several key questions for future work in endophyte biology.

I. Introduction

Since the first description of symbiosis as 'the living together of dissimilar organisms' (De Bary, 1879), an array of symbiotic lifestyles have been defined based on fitness benefits or impacts to macroscopic hosts and microscopic symbionts (Lewis, 1985). Collectively, more than 100 yr of research suggests that most, if not all, plants in natural ecosystems are symbiotic with mycorrhizal fungi and/or fungal endophytes (Petrini, 1986). These fungal symbionts can have profound effects on

Table 1 Symbiotic criteria used to characterize fungal endophytic classes

| | Clavicipitaceous | Nonclavicipitaceous | | |
| | Class 1 | Class 2 | Class 3 | Class 4 |
Criteria				
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits*	NHA	NHA and HA	NHA	NHA

*Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat of origin. Habitat-adapted (HA) benefits result from habitat-specific selective pressures such as pH, temperature and salinity.

plant ecology, fitness, and evolution (Brundrett, 2006), shaping plant communities (Clay & Holah, 1999) and manifesting strong effects on the community structure and diversity of associated organisms (e.g. bacteria, nematodes and insects; Omacini *et al.*, 2001). The fossil record indicates that plants have been associated with endophytic (Krings *et al.*, 2007) and mychorrhizal (Redecker *et al.*, 2000) fungi for > 400 Myr and were likely associated when plants colonized land, thus playing a long and important role in driving the evolution of life on land.

Unlike mycorrhizal fungi that colonize plant roots and grow into the rhizosphere, endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves, emerging to sporulate at plant or host-tissue senescence (Sherwood & Carroll, 1974; Carroll, 1988; Stone *et al.*, 2004). In general, two major groups of endophytic fungi have been recognized previously, reflecting differences in evolutionary relatedness, taxonomy, plant hosts, and ecological functions (Table 1): the clavicipitaceous endophytes (C-endophytes), which infect some grasses; and the nonclavicipitaceous endophytes (NC-endophytes), which can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies, conifers, and angiosperms. To date, most reviews have focused only on C-endophytes (e.g. Clay & Schardl, 2002), or have examined C- and NC-endophytes together (but see Arnold, 2007), often treating NC-endophytes with distinctive life histories as a single group. This bias is partially a result of the significant agricultural impacts of C-endophytes (described in section II) and a dearth of knowledge about the ecological significance of NC-endophytes. However, NC-endophytes are highly diverse fungi that represent at least three distinct functional groups (Table 1) based on an array of life history characteristics and their ecological significance. The goal of this review is to describe an operational framework for clarifying the taxonomy and ecological roles of endophytes, and for addressing key research questions, in each functional group.

C-endophytes (hereafter, Class 1 endophytes) represent a small number of phylogenetically related clavicipitaceous species that are fastidious in culture and limited to some cool- and warm-season grasses (Bischoff & White, 2005). Typically these endophytes occur within plant shoots, where they form systemic intercellular infections. Clay & Schardl (2002) recognized three types of clavicipitaceous endophytes, ranging from symptomatic and pathogenic species (Type I) to mixed-interaction and asymptomatic endophytes (Types II and III, respectively). In this review we discuss all three types of C-endophyte with a focus on Type III C-endophytes, because they – like the NC-endophytes treated here – grow within plant tissues without manifesting symptoms of disease.

Transmission of Class 1 endophytes is primarily vertical, with maternal plants passing fungi on to offspring via seed infections (Saikkonen *et al.*, 2002). Colonized plants usually harbor one dominant fungal isolate/genotype (Wille *et al.*, 1999). Class 1 endophytes frequently increase plant biomass, confer drought tolerance, and produce chemicals that are toxic to animals and decrease herbivory (Clay, 1988). However, the benefits conferred by these fungi appear to depend on the host species, host genotype and environmental conditions (Saikkonen *et al.*, 1999; Faeth & Sullivan, 2003; Faeth *et al.*, 2006).

NC-endophytes are highly diverse, representing a polyphyletic assemblage of primarily ascomycetous fungi with diverse and often poorly defined or unknown ecological roles. NC-endophytes have been recovered from every major lineage of land plants, and from all terrestrial ecosystems, including both agro-ecosystems and biomes ranging from the tropics to the tundra (Arnold & Lutzoni, 2007). The scale of their diversity, their ecological roles, the insights they provide into the evolution of various ecological modes in fungi, their potential applications, and the ability of many fungi to switch between endophytic and free-living lifestyles (Vasiliauskas *et al.*, 2007; Macia'-Vicente *et al.*, 2008a; Selosse *et al.*, 2008) are becoming more apparent, engendering growing enthusiasm from mycologists, ecologists, physiologists and applied scientists.

NC-endophytes can be differentiated into three functional classes based on host colonization patterns, mechanism of transmission between host generations, *in planta* biodiversity levels, and ecological function (Table 1). Although all three classes have broad host ranges, Class 2 endophytes may grow in both above- and below-ground tissues. By contrast, Class 3 and 4 endophytes are restricted to above-ground tissues and roots, respectively. Colonization of host tissues also differs:

Class 3 endophytes form highly localized infections, while Class 2 and 4 endophytes are capable of extensive tissue colonization. In general, the diversity of Class 2 (Rodriguez *et al.*, 2008) endophytes in individual host plants is quite limited, whereas the diversity of Class 3 endophytes within a host plant or tissue can be extremely high (e.g. > 20 species recorded from a single tropical leaf; Arnold *et al.*, 2003). The diversity of Class 4 endophytes within individual plants has not been sufficiently evaluated. Differences in *in planta* biodiversity of Class 2 and 3 endophytes may reflect differences in host colonization and transmission patterns: although members of both classes are transmitted horizontally, Class 2 endophytes also are transmitted vertically via seed coats, seeds or rhizomes.

One unique aspect of Class 2 endophytes is their ability to confer habitat-specific stress tolerance to host plants (Rodriguez *et al.*, 2008). Endophyte-conferred fitness benefits are defined here as habitat-adapted if the benefits are a result of habitat-specific selective pressures such as pH, temperature and salinity; or as nonhabitat-adapted if the benefits are common among endophytes regardless of habitat (Table 1). Only Class 2 endophytes have been shown to confer habitat-adapted stress tolerance (Rodriguez *et al.*, 2008). Because most Class 3 and 4 endophytes have not been assessed for the conferring of fitness benefits to hosts, mode of transmission or rhizosphere competence, the composition of these Classes may be more complex than presented here and may be revised in years to come.

In this discussion, we define these endophytic groups and describe their life histories, symbiotic dynamics and ecological functions.

II. Clavicipitaceous endophytes (Class 1)

Clavicipitaceous endophytes of grasses were first noted by European investigators in the late 19th century in seeds of *Lolium temulentum*, *Lolium arvense*, *Lolium linicolum*, and *Lolium remotum* (Guerin, 1898; Hanausek, 1898; Vogl, 1898). From their earliest discovery, investigators hypothesized a link to toxic syndromes experienced by animals that consume infected tissues. However, these hypotheses were largely untested until Bacon *et al.* (1977) linked the endophyte *Neotyphodium coenophialum* to the widespread occurrence of 'summer syndrome' toxicosis in cattle grazing tall fescue pastures (*Festuca arundinaceae*).

In 1988, Keith Clay proposed that clavicipitaceous endophytes are defensive mutualists of host grasses (Clay, 1988). As this hypothesis became widely known, investigations on endophyte natural history, evolution, ecology, and physiology followed (e.g. Rowan & Gaynor, 1986; White, 1987; Siegel *et al.*, 1989; West *et al.*, 1990; Patterson *et al.*, 1991; Riedell *et al.*, 1991; Breen, 1993; Bush *et al.*, 1997; Saikkonen *et al.*, 1999; Lane *et al.*, 2000; Faeth, 2002; Leuchtman, 2003; Schardl & Moon, 2003; Panaccione, 2005; Rao *et al.*, 2005; Panaccione *et al.*, 2006; Koulman *et al.*, 2007). As a result of the beneficial effects of C-endophytes, turf grass breeders now

offer a variety of cultivars identified as 'endophyte enhanced' (Funk & White, 1997).

1. Evolutionary origins

The Clavicipitaceae (Hypocreales; Ascomycota) include free-living and symbiotic species associated with insects and fungi (e.g. *Cordyceps* spp.) or grasses, rushes and sedges (e.g. *Balansia* spp., *Epichloë* spp. and *Claviceps* spp.) (Bacon & White, 2000). The family is relatively derived within the Hypocreales (Spatafora & Blackwell, 1993; Rehner & Samuels, 1995; Spatafora *et al.*, 2007), a lineage well known for plant pathogens, saprotrophs, and endophytes, many of which produce bioactive compounds. Soil-inhabiting species, which include some of the most geographically widespread taxa in the group, are placed particularly deeply in phylogenetic trees based on molecular sequence data (Spatafora *et al.*, 2007), suggesting that saprobism was common among the earliest members of the family.

Ancestral state reconstructions in a multilocus phylogenetic framework suggest that clavicipitaceous endophytes arose from insect-parasitic ancestors and diversified through a series of inter-kingdom host jumps (Koroch *et al.*, 2004; Spatafora *et al.*, 2007; Torres *et al.*, 2007b). The evolution of endophytic *Epichloë/ Neotyphodium* and *Balansia* is thought to have begun with free-living insect parasites, and then progressed to epibiotic plant biotrophic forms (e.g. similar to *Hypocrella* spp.) that gained access to plant nutrients by (1) first infecting and necrophytizing scale insects and whiteflies and (2) then developing on the stream of nutrients emerging to the surface of the plant through the insect's stylet or stylet wound. Finally, the capacity to infect grass hosts directly arose, with forms that are epibiotic (e.g. *Myriogenospora* spp.) and others that are endophytic.

This scenario highlights a shortcut to biotrophy that did not involve the often-expected transition from virulent plant pathogens to endophytism. Endophytes descended from insect pathogens likely did not possess enzymes or toxins for killing or degrading plant tissues, such that plant defensive mechanisms would not limit their colonization. Nutrient-use studies suggest that evolution of biotrophy and endophytism in this group involved (1) reduction of enzymatic capabilities, (2) increasing dependence on the host plant to provide nutrients for growth, and (3) an apparent increase in the production of particular secondary metabolites beneficial in the symbiosis (e.g. ergot alkaloids; Torres *et al.*, 2007a). As more nuclear genomes are sequenced, detailed comparisons between these life strategies will be possible within common phylogenetic backgrounds. Derivation of C-endophytes from insect pathogens may in part explain why they are adept at the production of toxins that affect insects and other animals: it is likely that the chemical arsenal used by free-living taxa such as *Cordyceps*, which is effective at killing and degrading insects, is similar to or shares major precursors with that used by the endophytes now widespread in grasses.

2. Life history

Epichloë species (anamorphs: *Neotyphodium*) are endophytic symbionts of cool-season grasses (White, 1987). Mycelium occurs in intercellular spaces of leaf sheaths, culms, and rhizomes, and may also be present, if sparsely, on the surface of leaf blades (White *et al.*, 1996; Moy *et al.*, 2000; Dugan *et al.*, 2002; Tadych *et al.*, 2007). When the grass flowers, the fungus grows over the developing inflorescence to form a stroma. The inflorescence primordium remains at an arrested stage of development within the fungal mycelium, and development of the seed head is prevented (i.e. choke disease). Some *Epichloë* species (defined as Type II endophytes by Clay & Schardl, 2002) exhibit stromata only in a proportion of the tillers, allowing partial seed production and thus vertical transmission within seeds; while others (Type I endophytes) produce stromata on all or most of the tillers and any tillers that escape the fungus are free of endophyte infection (White, 1988). *Epichloë* also can produce auxins that alter development of plant tissues (De Battista *et al.*, 1990).

The stromata of *Epichloë* bear spermatia and fungal populations contain two mating types that must be transferred between stromata before perithecia and ascospores develop (White & Bultman, 1987). Symbiotic flies of the genus *Botanophila* (Diptera: Anthomyiidae) act as 'pollinators' of stromata, vectoring spermatia (Bultman & White, 1987; Steinebrunner *et al.*, 2008). Fertilized stromata produce infective ascospores; however, precisely how initial infections occur is only partially understood (Chung & Schardl, 1997; Steinebrunner *et al.*, 2008). Notably, stromata production and the sexual cycle of *Epichloë* occur only on grasses in the northern hemisphere: C-endophytes in the Southern Hemisphere are unable to form stromata and do not reproduce sexually. This suggests that endophyte spread in Southern Hemisphere grasses has occurred through epiphyllous conidial production or by a means other than stromata production, or that Southern Hemisphere endophytes once possessed the capacity to produce stromata but that capacity was lost.

Some species of *Epichloë* have lost the capacity for development of the sexual stage. These have been referred to as asexual or Type III endophytes (Clay & Schardl, 2002) and are classified as species of *Neotyphodium* (White, 1988). No obvious symptoms of Type III endophyte infection are observable at any stage of plant development. These fungi are endophytes of leaves, culms, and rhizomes, and they frequently colonize inflorescence primordia. As inflorescences develop the mycelium grows into ovules, and within seeds it colonizes the scutellum and embryo axis (Philipson & Christey, 1986) before germination. Considerable evidence suggests that Type III C-endophytes have evolved numerous times and are distributed in both the Northern and Southern Hemispheres (White, 1988; Moon *et al.*, 2002; Schardl & Moon, 2003; Schardl *et al.*, 2004).

Type III C-endophytes are transmitted vertically through seeds but many retain an epiphyllous mycelium where conidia form, suggesting the potential for horizontal transmission. Tadych *et al.* (2007) demonstrated that epiphyllous conidia are released from conidiophores only in water, suggesting that they may spread among plants via rain or dew. The likely, but as yet unconfirmed, sites of infection are tillers, where the fungi colonize meristems epiphyllously in plant crowns. Most Type III endophytes produce conidia in culture and perhaps epiphyllously, suggesting that these propagules retain some function. Tiller colonization may result in infection of some ovules and seeds of neighboring plants, but the original mature neighboring plant tissues would not bear endophytic mycelium: C-endophytes grow rapidly in nutrient-rich meristematic tissues but show limited capacity to grow through mature plant tissue (Western & Cavett, 1959; White *et al.*, 1991). In turn, the endophytic mycelium is systemic throughout the above-ground portions of the grass plant because it proliferates in the shoot meristem and is deposited in the intercellular spaces of the newly forming aerial plant parts. By contrast, endophytic mycelia do not colonize root meristems, and roots typically lack these fungi.

Many aspects of the life histories of C-endophytes and their population dynamics remain uncertain. Notably, the origin of these asexual endophytes indicates that they may not be entirely clonal: phylogenetic analysis has demonstrated the occurrence of interspecific hybridizations (Moon *et al.*, 2002; Schardl *et al.*, 2004). This interspecies recombination has been proposed to function as a mechanism to maintain genetic variability and genome viability among endophytes that cannot undergo sexual recombination (Schardl & Moon, 2003). Alternatively, Selosse & Schardl (2007) proposed that the vertical transmission was a 'rescue mechanism' for the hybridized endophytes that were then incapable of sexual reproduction.

3. Impacts on host plant fitness: herbivore deterrence

Most clavicipitaceous endophytes enhance resistance of hosts to insect feeding (Rowan & Gaynor, 1986; Siegel *et al.*, 1989; Clay, 1990; Patterson *et al.*, 1991; Riedell *et al.*, 1991). Tintjer & Rudgers (2006) found that deterrence of insect herbivory depends on the fungal strain and growth stage of the plant. A mutational study demonstrated that the fungal metabolite peramine protects plants from herbivory (Tanaka *et al.*, 2005). Other studies have provided evidence for anti-nematode activity of Class 1 endophytes as well (Kimmons *et al.*, 1990). However, research has also shown that some Class 1 endophytes do not provide insect or nematode resistance to host plants (Saikkonen *et al.*, 1999, Faeth *et al.*, 2006), and have highlighted the importance of examining native plants under natural conditions in determining endophyte-conferred benefits. Because of several examples where endophytes do not appear to provide defensive benefits to host plants, some investigators have questioned the tendency to classify C-endophytes as defensive mutualists (e.g. Faeth, 2002).

Some endophytes have been reported to deter feeding by mammalian herbivores (White, 1987; Gentile *et al.*, 1999; Li

et al., 2004). For example, sleepygrass (*Achnatherum robustum*), common in the western USA, harbors an endophyte that produces lysergic acid amide. Horses that consume relatively small quantities of infected grass will sleep for up to 3 d and then gradually recover. Once domesticated animals experience the toxic effects of such grasses, they preferentially avoid those plants, and animals native to regions with sleepygrasses do not consume them. A recent study indicates that the endophyte infection frequency of sleepygrass varies from 50 to 100% in different geographic locations and toxic alkaloid production in infected plants varies from 0 to > 150 µg g^{-1} tissue (Faeth *et al.*, 2006). It is not yet known how this variation affects herbivory or plant fitness but the outcome of these associations appears to be influenced by host genotype, environmental conditions and coevolution between endophyte and host.

Endophytes with similar effects are seen all over the world. In South America, *Neotyphodium tembladerae* infects several species of grasses, some of which are reported to be toxic to mammals (Gentile *et al.*, 1999). In Asia, drunken horse grass (*Achnatherum inebrians*) is infected by *Neotyphodium gansuense* and similarly avoided by animals (Li *et al.*, 2004), as is the endophyte-containing dronk gras (*Melica decumbens*) of South Africa (White, 1987). Recently, Panaccione *et al.* (2006) demonstrated that even endophytes that are not acutely toxic to mammals have antifeeding effects: experiments using plants with and without endophyte-derived alkaloids demonstrated that rabbits consume less of the host plants with ergot alkaloids.

4. Effects on disease resistance and susceptibility

Relatively few studies have evaluated effects of Class 1 endophytes on disease resistance. Several C-endophytes produce compounds that inhibit the growth of other fungi *in vitro*; for example, Yue *et al.* (2000) identified several indole derivatives, a sesquiterpene, and a diacetamide from *Epichloë festucae*. It has been difficult, however, to link inhibition of pathogens *in vitro* to disease resistance exhibited *in vivo*. In the case of *E. festucae*, infected turfgrasses have shown significant resistance over uninfected turf to two major leaf spot pathogens (dollar spot disease caused by *Sclerotina homeocarpa* (Clarke *et al.*, 2006); red thread disease caused by *Laetisaria fusiformis* (Bonos *et al.*, 2005)). It is unknown whether the mechanism of enhanced disease resistance is attributable to antifungal compounds produced by the endophyte, compounds produced by the plant in response to the endophyte, trophic competition between fungi or some physical exclusion mechanism.

5. Costs of the symbiosis

There is some evidence that there is a cost to the plant host of supporting C-endophytes. One dramatic example was observed at the Rutgers University turfgrass breeding station, wherein an endophyte-containing cultivar of tall fescue grass (*F. arundinaceae*) showed enhanced crown and root rot

disease caused by *Pythium graminicola* when compared with endophyte-free plots. Numerous additional cultivars of tall fescue with and without endophytes also were examined; however, none of these showed the dramatic enhancement in disease occurrence in the presence of the endophyte. Examination of leaf sheaths of the endophyte-containing cultivar showed an unusually high number of intercellular hyphal strands. An explanation for enhanced susceptibility attributable to the endophytes may be that the abnormally high endophyte content in the grass increased the nutrient demands and stress on grass individuals, making them more susceptible to *Pythium*.

Similarly, Wäli *et al.* (2006) found that natural subarctic populations of red fescue (*Festuca rubra*) containing the endophyte *E. festucae* sustained greater damage from winter 'snow mold' (*Typhula ishikariensis*) than endophyte-free populations. However, endophyte-containing grasses recovered more quickly in the spring as a result of enhanced vigor. Wäli *et al.* (2006) also found that, in natural populations of sheep fescue (*Festuca ovina*), seedlings containing *E. festucae* were smaller than endophyte-free seedlings. Subarctic growing conditions were particularly harsh, and light, water, and soil nutrients were restricted for long periods; accordingly, the additional nutrient demand associated with maintaining the endophyte under stressful conditions may be reflected in these studies. Similarly, White *et al.* (2001) showed that when herbivore pressure is high – for example, when Argentinian populations of *Bromus setifolius* are exposed to extensive damage by leaf-cutting ants in a desert environment – endophyte (*Neotyphodium tembladerae*) incidence is quite high (80–100% of plants sampled). By contrast, areas in which leaf-cutting ants were rare had a very low frequency of endophyte infection (0–20% of plants sampled), congruent with the hypothesis that hosting the endophyte may be costly in the absence of a major herbivore. Studies on defensive mutualisms in other systems (e.g. ant plants) have similarly suggested that the magnitude of defensive benefits of symbioses may be conditional on specific circumstances (Bronstein, 1998). A symbiosis thus may range from parasitism, where the costs of the symbiont exceed benefits, to mutualism, where benefits exceed costs (Johnson *et al.*, 1997). In the case of *Bromus setifolia* and its endophyte, in circumstances where herbivore pressure is high, the benefits of the symbiosis exceed its costs and the association is selected for; conversely, where herbivore pressure is low or other costs of the endophyte are otherwise high, the association is selected against.

6. Roles in plant ecophysiology

Despite the examples cited above, Class 1 endophytes may enhance the ecophysiology of host plants and enable plants to counter abiotic stresses such as drought (Arechavaleta *et al.*, 1989) and metal contamination (Malinowski & Belesky, 2000). Malinowski & Belesky (2000) found that *N. coenophialum* infection leads to the development of extensive root systems

that enable plants to better acquire soil moisture and absorb nutrients, resulting in drought avoidance and faster recovery from water stress. In some cases, endophytes stimulate longer root hairs and enhance exudation of 'phenolic-like compounds' into the rhizosphere, resulting in more efficient absorption of soil phosphorus and enhanced aluminum tolerance via chelation (Malinowski & Belesky, 2000).

Cheplick (2006) reviewed potential drought tolerance effects attributable to Class 1 endophytes and cited numerous studies in which endophytes did not enhance the capacity of hosts to survive drought conditions. For example, Zaurov *et al.* (2001) artificially infected several fine fescue clones with a selection of isolates of *Neotyphodium* from several different hosts. They found that some genotypic combinations had a negative effect on plant mass, some were neutral, and others increased plant mass. Similarly, some combinations enhanced tolerance to soil aluminum; others had no effect or reduced tolerance compared with endophyte-free clones (Zaurov *et al.*, 2001). This study demonstrates that genotype-specific interactions may enhance, reduce, or have no effect on plant fitness. Each of these interactions, in turn, occurs in the context of variable environmental conditions that may highlight unexpected plasticity in endophyte–plant associations. Given that endophyte–plant combinations displaying enhanced aluminum tolerance may not show enhanced drought or herbivore resistance, detecting fitness costs and benefits may be highly subject to the experimental conditions at hand.

III. Nonclavicipitaceous endophytes

More than 1000 papers have been published on NC-endophytes since 1970. The majority present data concerning the distribution and abundance of endophytes in asymptomatic leaf tissue; the isolation and analysis of bioactive compounds; their potential use as biocontrol agents; and phylogeny-based identification and systematics (Selosse *et al.*, 2004; Schulz, 2006; Arnold *et al.*, 2007; Higgins *et al.*, 2007; Kithsiri Wijeratne *et al.*, 2008). While these studies are critically important for understanding the parameters of NC-endophyte–plant associations, very few papers focus on the ecological and evolutionary significance of NC-endophytes, and even fewer demonstrate symbiotic functions *in planta*. Despite a paucity of functional studies, several NC-endophytes have been shown to establish mutualisms with plants, conferring fitness benefits such as biotic and abiotic stress tolerance, nutrient acquisition and increased growth and yields (Redman *et al.*, 2002; Arnold *et al.*, 2003; Mucciarelli *et al.*, 2003; Waller *et al.*, 2005; Schulz, 2006; Rodriguez *et al.*, 2008).

NC-endophytes traditionally have been treated as a single functional group. However, it is clear that these highly diverse fungi can be provisionally classified into at least three functional groups on the basis of differences in life history, ecological interactions, and other traits (Table 1), which we detail in the following sections.

IV. Class 2 endophytes

Class 2 endophytes comprise a diversity of species, all of which are members of the Dikarya (Ascomycota or Basidiomycota). Most belong to the Ascomycota, with a minority of Basidiomycota. Members of the former are restricted to the Pezizomycotina, wherein they represent several classes. Class 2 endophytes within the Basidiomycota include a few members of the Agaricomycotina and Pucciniomycotina.

Class 2 endophytes are distinct from the other NC-endophytes because in general they colonize roots, stems and leaves; are capable of forming extensive infections within plants; are transmitted via seed coats and/or rhizomes; have low abundance in the rhizosphere; confer habitat-adapted fitness benefits in addition to nonhabitat-adapted benefits; and typically have high infection frequencies (90–100%) in plants growing in high-stress habitats (Table 1). Although all of the examples described in this section may not have been analyzed for all of these descriptions, they conform to enough criteria to be included at this time.

1. Historical perspective

The first detailed description of a Class 2 endophyte was a *Phoma* sp. in *Calluna vulgaris* (Rayner, 1915). Although described as mycorrhizal, the fungus actually was an endophyte that colonized all parts of the plant including the seed coat, and did not form intracellular mycorrhizal structures. A recent analysis of Mediterranean plants revealed that *Phoma* spp. are common root endophytes that confer fitness benefits to plants (Macia'-Vicente *et al.*, 2008a,b). During the remainder of the 20th century, a limited number of reports appeared describing fitness benefits conferred to plants by Class 2 endophytes. One of the clearest examples was reported in the brown alga *Ascophyllum nodosum* which requires the fungus *Mycophycia ascophylli* for normal growth and development (Garbary & Macdonald, 1995).

In addition to being required for the normal development of some plants, some Class 2 endophytes increase plant root and shoot biomass (Newsham, 1994; Gasoni & deGurfinkel, 1997; Ernst *et al.*, 2003; Mucciarelli *et al.*, 2003; Macia'-Vicente *et al.*, 2008b) and confer tolerance to a variety of biotic and abiotic stresses such as disease (Danielsen & Jensen, 1999; Narisawa *et al.*, 2002; Campanile *et al.*, 2007), drought, desiccation, heat and salinity (Redman *et al.*, 2001, 2002; Márquez *et al.*, 2007; Rodriguez *et al.*, 2008). Since the 1960s, however, there have been relatively few reports demonstrating colonization patterns, host range or host fitness benefits conferred by Class 2 endophytes.

2. Life history

The life history of Class 2 endophytes has been described for several species. These fungi, like all endophytes, colonize plants via infection structures such as appressoria or by direct

penetration of plant tissues via hyphae (Ernst *et al.*, 2003). Growth through plant tissues is dominantly intercellular with little to no impact on host cells (Rodriguez-Galvez & Mendgen, 1995; Ernst *et al.*, 2003; Gao & Mendgen, 2006). While there may be a low level of sporulation or appressorial formation in healthy plants (Freeman & Rodriguez, 1993; Newsham, 1994), these fungi rapidly emerge and sporulate during host senescence (Weber *et al.*, 2004).

There have not been sufficient ecological studies to permit full understanding of the distribution and abundance of Class 2 endophytes in the rhizosphere; however, some species (*Phoma* sp. and *Arthrobotrys* spp.; Newsham, 1994; Lopez-Llorca *et al.*, 2006) are known to occur in soils at high abundance while others (*Fusarium culmorum*, *Colletotrichum magna* and *Curvularia protuberata*; Rodriguez *et al.*, 2008) are present at very low abundance. Analysis of soil fungi typically involves making soil suspensions, dilution plating and enumerating colony-forming units; therefore, it is difficult to discern the ability of endophytes to propagate in soil versus the deposition of spores derived from senescing plants that are colonized by the fungi. Nematophagous fungi such as *Arthrobotrys* spp. can express endophytic or free-living lifestyles (Lopez-Llorca *et al.*, 2006); therefore, it is not surprising that *Arthrobotrys* spp. achieve high abundances in rhizosphere soils as nematodes are so plentiful and provide a source of nutrition. However, the high abundance of *Phoma* sp. in soil may be attributable to its emergence and sporulation in senescing plants and subsequent dissemination of spores into the surrounding soils.

Although the above characteristics highlight horizontal transmission, Class 2 endophytes often are transmitted vertically: they can be passed from maternal plants via seed coats (Redman *et al.*, 2002). Interestingly, culturable Class 2 endophytes can grow on a variety of simple media. The prevalence of these fungi in plant hosts and their abundance in soils suggest that some of them are unable to compete outside hosts while others may have multiple lifestyles (symbiotic or saprophytic). However, this remains to be evaluated.

3. Symbiotically derived benefits to endophytes

It is clear that some endophytes avoid stress through plant symbiosis. For example, *Curvularia protuberata* colonizes all nonembryonic tissues of the geothermal plant *Dichanthelium lanuginosum* (Redman *et al.*, 2002; Márquez *et al.*, 2007). When grown nonsymbiotically, neither the plant nor the fungus can tolerate temperatures above 40°C. However, the symbiosis allows both partners to tolerate temperatures up to 65°C. A similar scenario was observed with *Fusarium culmorum* which colonizes all nonembryonic tissues of coastal dunegrass (*Leymus mollis*): when grown nonsymbiotically, the host plant does not survive and the endophyte's growth is retarded when exposed to levels of salinity experienced in their native habitat (Rodriguez *et al.*, 2008). However, both partners tolerate sea water levels of salinity (300–500 mM NaCl) when grown

symbiotically. Clearly, *C. protuberata* and *F. culmorum* are able to avoid the detrimental effects of temperature and salt stress by residing in plant tissues.

Based on the observations described above, we conclude that at least some Class 2 endophytes are mutualistic, conferring positive fitness benefits to hosts while also obtaining nutrition for growth and reproduction from host tissues, and avoiding abiotic stress via symbiosis.

4. Endophyte-conferred fitness benefits and ecological adaptations of plants

It seems that most, if not all, of the Class 2 endophytes examined to date increase host shoot and/or root biomass, possibly as a result of the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi (Tudzynski & Sharon, 2002). Many Class 2 endophytes protect hosts to some extent against fungal pathogens (Danielsen & Jensen, 1999; Narisawa *et al.*, 2002; Campanile *et al.*, 2007), reflecting the production of secondary metabolites (Schulz *et al.*, 1999), fungal parasitism (Samuels *et al.*, 2000), or induction of systemic resistance (Vu *et al.*, 2006). It is also possible that symbiotically conferred disease protection may be a result of an inability of pathogens to compete with endophytes for resources or niche space. Only a few Class 2 endophytes have been examined for their direct interactions with host defenses. Endophytic isolates of *Fusarium oxysporum* and a *Cryptosporiopsis* sp. conferred disease resistance against virulent pathogens in barley (*Hordeum vulgare*) and larch (*Larix decidua*), respectively (Schulz *et al.*, 1999), and resistance was correlated to increased concentrations of phenolic metabolites. A mutant of *Colletotrichum* (isolate Path-1; Freeman & Rodriguez, 1993) expressing a mutualistic lifestyle asymptomatically colonized roots, stems and leaves of watermelon (*Citrullus lanatus*) without inducing host defense systems (Redman *et al.*, 1999). However, when Path-1-colonized plants were challenged with a virulent pathogen, host defense systems were activated very quickly to levels not achieved by nonsymbiotic plants that succumbed to disease.

One attribute that appears unique to Class 2 endophytes is the ability of individual isolates to asymptomatically colonize and confer habitat-adapted (Table 1) fitness benefits on genetically distant host species representing monocots and eudicots (Rodriguez *et al.*, 2008). This phenomenon was discovered by comparing fitness benefits conferred by Class 2 endophytes in plants growing in geothermal soils (*Curvularia protuberata*), coastal beaches (*Fusarium culmorum*) and agricultural fields (*Colletotrichum* spp.). A series of laboratory studies indicated that *C. protuberata* conferred heat but not salt or disease tolerance, *F. culmorum* conferred salt but not heat or disease tolerance, and *Colletotrichum* spp. conferred disease resistance but not heat or salt tolerance (Redman *et al.*, 2001, 2002; Rodriguez *et al.*, 2008). Field studies in geothermal soils and coastal beaches confirmed laboratory results indicating that nonsymbiotic plants could not survive stresses imposed in

their natural habitats without colonization by these habitat-specific endophytes. Additional studies revealed that the ability of endophytes to confer habitat-specific stress tolerance is an adaptive process defined at the subspecies level (Rodriguez *et al.*, 2008). For example, isolates of *C. protuberata* (CpMH206) and *F. culmorum* (Fc18) from habitats devoid of heat or salt stress asymptomatically colonize plants to the same extent as isolates from habitats imposing heat and salt stress, but CpMH206 and Fc18 do not confer either heat or salt tolerance. Thus, all of these fungi establish nonpathogenic symbioses, but the fitness benefits conferred on hosts were dependent on the habitat-specific stresses. To define the symbiotic lifestyle expressed by nonstress-adapted endophytes (CpMH206 and Fc18), their abilities to confer drought tolerance and growth enhancement was assessed. All of the fungi conferred drought tolerance and growth enhancement on various host species (Rodriguez *et al.*, 2008), indicating that they were expressing mutualistic lifestyles. We have defined the ability of endophytes to confer habitat-adapted fitness benefits as habitat-adapted symbiosis and hypothesize that this allows plants to establish and survive in high-stress habitats.

The fact that individual Class 2 fungal isolates can asymptomatically colonize and confer specific stress tolerances on both monocot and eudicot hosts suggests that the symbiotic communication required for stress tolerance predates the divergence of these plant lineages between 140 and 235 Mya (Wolfe *et al.*, 1989; Yang *et al.*, 1999; Chaw *et al.*, 2004). This may not be surprising, as plant endophyte associations are represented in the fossil record at least 400 Mya (Krings *et al.*, 2007), placing endophyte symbioses in the same geological time frame as mycorrhizal symbioses (Redecker *et al.*, 2000). The ability of many symbiotic fungi, including several of the endophytes described here (e.g. *C. protuberata*, *F. culmorum*, *M. ascophylli* and *Colletotrichum* spp.), to confer drought tolerance is generally congruent with the suggestion that symbiotic fungi were involved in the movement of plants onto land (Pirozynski & Malloch, 1975). Although fungal endophytes likely arose throughout evolutionary time and differed in host range and temporal distribution, their persistence throughout geologic time and their ubiquitous distribution are a testament to their significance in plant ecology and evolution.

5. Mechanisms of stress tolerance

Class 2 endophytes commonly increase plant biomass under stressful conditions but the cellular mechanisms involved in stress tolerance and growth enhancement are poorly characterized. Physiological studies have indicated that certain biochemical processes correlate with symbiotically conferred stress tolerance. For example, upon exposure to virulent pathogens, endophyte-colonized plants activate host defenses more rapidly than nonsymbiotic plants (Redman *et al.*, 1999). In the absence of pathogen exposure, Class 2 endophyte-colonized plants do not activate host defenses.

Plant responses to abiotic stress have been intensively studied without taking symbiosis into consideration. Heat, drought and salt stress induce some similar plant responses including altered water relations, increased osmolyte production, production of signaling molecules such as abscisic acid (ABA), and the generation of reactive oxygen species (ROS) (Bohnert *et al.*, 1995; Bray, 1997; Wang *et al.*, 2003; Apel & Hirt, 2004). An analysis of Class 2 endophyte-colonized and noncolonized plants revealed no significant correlation between osmolytes or ABA and symbiotically conferred stress tolerance (Rodriguez *et al.*, 2008). However, there is a strong correlation between ROS susceptibility and stress tolerance. When plants are exposed to abiotic stress and then paraquat (an herbicide that induces ROS production and subsequent photobleaching of chlorophyll; Vaughn & Duke, 1983), nonsymbiotic plant tissues bleach white and Class 2 endophyte-colonized plant tissues remain green (Rodriguez *et al.*, 2008). ROS protection occurs in a habitat-specific manner: *F. culmorum* (confers salt but not heat tolerance) prevents photobleaching of plants exposed to salt stress but not plants exposed to heat stress, and *C. protuberata* (confers heat but not salt tolerance) prevents photobleaching of plants exposed to heat but not salt stress. The role of ROS in Class 2 endophyte associations appears to be involved in stress tolerance (Baltruschat *et al.*, 2008; Rodriguez *et al.*, 2008) and differs from the role of ROS in other fungal endophyte-plant associations. For example, *Epichloë* (Class 1 endophyte) produces ROS *in vivo* to limit its growth rate in host plants (Tanaka *et al.*, 2006). During pathogenic interactions both the hosts and the pathogens appear to produce ROS, which if produced by the plant may act to limit pathogen colonization and if produced by the pathogen may increase virulence (see Rouhier & Jacquot, 2008).

Drought tolerance in plants often is correlated with increased osmotic potential (Bohnert *et al.*, 1995; Bray, 1997; Wang *et al.*, 2003). However, Class 2 endophyte-conferred drought tolerance does not correlate with an increase in osmotic potential (Rodriguez *et al.*, 2008). Although nonsymbiotic plants do increase osmotic potential under drought stress, they wilt much earlier (6–10 d) than symbiotic plants. Remarkably, symbiotic plants consume less water (30–50%) than nonsymbiotic plants regardless of the Class 2 endophyte, suggesting that water use efficiency may be more important for drought tolerance than osmolyte modulation.

Regardless of the mechanisms involved in symbiotically conferred stress tolerance, it is clear that Class 2 endophytes can have significant affects on the ecophysiology of plants. This may result in the rapid adaptation of plants, allowing them to establish and survive in high-stress habitats.

V. Class 3 endophytes

Class 3 endophytes are distinguished on the basis of their occurrence primarily or exclusively in above-ground tissues; horizontal transmission; the formation of highly localized

infections; the potential to confer benefits or costs on hosts that are not necessarily habitat-specific; and extremely high *in planta* biodiversity (Table 1). Class 3 endophytes include the hyperdiverse endophytic fungi associated with leaves of tropical trees (Lodge *et al.*, 1996; Fröhlich & Hyde, 1999; Arnold *et al.*, 2000; Gamboa & Bayman, 2001), as well as the highly diverse associates of above-ground tissues of nonvascular plants, seedless vascular plants, conifers, and woody and herbaceous angiosperms in biomes ranging from tropical forests to boreal and Arctic/Antarctic communities (Carroll & Carroll, 1978; Petrini, 1986; Stone, 1988; Fisher *et al.*, 1995; Davis *et al.*, 2003; Higgins *et al.*, 2007; Murali *et al.*, 2007; Davis & Shaw, 2008). In addition to occurring within photosynthetic and herbaceous tissues, Class 3 endophytes are found in flowers and fruits, as well as in asymptomatic wood and inner bark (e.g. Barengo *et al.*, 2000; Kumar & Hyde, 2004; Tejesvi *et al.*, 2005). Fungi with similar life histories to Class 3 endophytes also occur within asymptomatic lichens, and in that case are known as 'endolichenic' fungi (Arnold *et al.*, in press).

Class 3 endophytes are especially notable for their high diversity within individual host tissues, plants and populations. For example, apparently healthy leaves in lowland moist tropical forests contain numerous, independent infections, rather than systemic or otherwise extensive growth of hyphae (Lodge *et al.*, 1996; Arnold & Herre, 2003; Arnold *et al.*, 2003). The biomass resulting from any given infection is very low, such that each leaf represents a densely packed mosaic of diverse endophyte species (Lodge *et al.*, 1996). In tropical forests in central Panama, where 100% of mature leaves of diverse trees and shrubs typically contain endophytes (Arnold, 2002), individual leaves may harbor up to one isolate per 2 mm^2 of leaf tissue (Arnold *et al.*, 2000; Gamboa *et al.*, 2002) and frequently contain dozens of species. Different leaves on the same tree may have quite distinctive assemblages of endophytic fungi (Arnold *et al.*, 2003). Individual plants may harbor hundreds of species, and plant species across their native ranges may be inhabited by thousands of species.

This tremendous diversity, showcased in some tropical plants and localities, is not exclusively a tropical phenomenon: plants in temperate and boreal communities also harbor an astonishing richness of Class 3 endophytes. For example, Higgins *et al.* (2007) found that species-accumulation curves for boreal and Arctic endophytes were nonasymptotic, with > 50 species recovered among only 280 isolates examined. Similarly, Petrini & Müller (1979) recovered > 80 endophyte species from *Juniperus communis* in Switzerland, and Halmschlager *et al.* (1993) isolated 78 species from leaves and twigs of *Quercus petraea* in Austria. Although horizontally transmitted, Class 3 endophytes are typically distinct from pathogens associated with the same host species (Ganley *et al.*, 2004) and from epiphyllous fungi even on the same leaves (Santamaría & Bayman, 2005). Their distinctiveness relative to saprotrophic communities is a matter of some debate (Selosse *et al.*, 2008), but increasingly it appears that Class 3 endophytes comprise

species not known among the saprophytic decay fungi (U'Ren *et al.*, 2008, but see Promputtha *et al.*, 2007). Class 3 endophytes are rarely isolated from seeds (Arnold *et al.*, 2003; Ganley *et al.*, 2004; but see Gallery *et al.*, 2007).

The diversity of Class 3 endophytes raises several questions regarding their ecological roles. Indeed, most recent studies of Class 3 endophytes have focused on characterizing bioactive strains (e.g. Jiménez-Romero *et al.*, 2008) or enumeration of species, leaving aside the potential ecological roles of these fungi *in planta* (but see Arnold *et al.*, 2003) or their evolutionary implications for plants. Certainly it is challenging to imagine general rules given the occurrence of tens to hundreds of phylogenetically diverse endophytic fungi within the foliage of a single host.

The costs and benefits of Class 3 endophytes have been evaluated in an experimental framework only a few times, and rarely in the context of more than one environmental or biotic stressor or endophyte–plant combination. In several cases, these fungi have been implicated in enhanced disease resistance, herbivore deterrence, and changes in sensitivity to drought and other abiotic stressors (see 'Ecological roles' later in this section) – but the number of experimental and life-history studies is truly miniscule relative to the diversity of Class 3 endophytes as a whole. Experiments that take into account the natural complexity of these endophyte communities, and consider multitrophic interactions, are especially needed in years to come. Similar to the explosive growth of studies examining the diversity and ecological roles of mycorrhizal symbioses over recent decades, the study of hyperdiverse Class 3 endophytes is in an exceptionally wide-open and exciting phase.

1. Life history

Like Class 2 endophytes, the majority of Class 3 endophytes are members of the Dikaryomycota (Ascomycota or Basidiomycota), with a special concentration in the Ascomycota. The Pezizomycotina are especially well represented, although some endophytic Saccharomycotina are known (Higgins *et al.*, 2007). Within the Pezizomycotina, Class 3 endophytes occur among all of the major nonlichenized clades. They are common among the Pezizomycetes, Leotiomycetes and Eurotiomycetes, and especially among the very endophyte-rich Sordariomycetes and Dothideomycetes (Arnold *et al.*, 2007; Higgins *et al.*, 2007; Arnold *et al.*, in press). Interestingly, numerous ascomycetous plant pathogens and saprotrophs are derived from these same lineages (Lutzoni *et al.*, 2004; James *et al.*, 2006). The relative prevalence of members of each class in endophyte communities differs markedly among biomes and among host lineages (Arnold & Lutzoni, 2007): for example, endophytic Leotiomycetes are especially common in conifers, and endophytic Sordariomycetes are particularly common in tropical plants.

Members of the Basidiomycota belonging to the Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina also are known as Class 3 endophytes, although they are reported much

less frequently than ascomycetous endophytes. In general, basidiomycetous endophytes are found more often in woody tissues than in foliage (Arnold, 2007). Notably, however, endophytic Basidiomycota may be more common in foliage than culture-based studies have indicated to date. For example, Arnold *et al.* (2007) reported a fourfold increase in the number of basidiomycetous species recovered from leaves of *Pinus taeda* when endophyte communities were assessed using culture-free methods (environmental PCR of surface-sterilized foliage) instead of culturing. Moreover, Pan *et al.* (2008) reported that *Ustilago maydis* is well represented among the endophyte community in maize (*Zea mays*).

Class 3 endophytes reproduce by hyphal fragmentation and/or by the production of sexual or asexual spores on dead or senescent tissue (Herre *et al.*, 2005). Spores and hyphal fragments may be released passively, by herbivores (through frass; Monk & Samuels, 1990), or by physical disturbances such as wind or rain. Some, including *Phyllosticta* sp., produce slimy spores that rely at least in part on rain for dispersal (Kirk *et al.*, 2001) and the Ingoldian fungi produce spores that depend on water for dispersal and infection (Selosse *et al.*, 2008). Although heavy wind and rain are especially effective in moving spores, even light precipitation can disperse conidia of *Colletotrichum* (Guyot *et al.*, 2005). Similarly, light wind and the currents produced by diurnal cycles of heating and cooling are significant for dispersal of dry propagules (Arnold, 2008). Some Class 3 endophytes also may travel about as spores adhering to the bodies of insects such as moths (Feldman *et al.*, 2008).

In general, seedlings raised under sterile conditions do not contain culturable Class 3 endophytes, highlighting a key difference relative to Class 2 endophytes (which may be transmitted vertically). Colonization by Class 3 endophytes proceeds rapidly given the presence of airborne inoculum and high relative humidity or wetting of leaf surfaces by dew, rain, or fog (Arnold & Herre, 2003). Field experiments using endophyte-free seedlings showed that endophytes were present in > 80% of *Theobroma cacao* leaves within 2 wk of leaf emergence during the early wet season at a tropical forest site (Arnold & Herre, 2003). Leaf toughness and leaf chemistry do not influence endophyte colonization: when placed into the field without endophytes, young and mature leaves can be colonized with equal rapidity (Arnold & Herre, 2003). Colonization generally parallels that described above for horizontal transmission of Class 2 endophytes.

Infection frequency, richness, and diversity of Class 3 endophytes generally follow a strong latitudinal gradient, with higher values of all of these measures in the tropics relative to Arctic tundra or boreal forest (Arnold & Lutzoni, 2007). However, local abiotic and land-use conditions can lead to unexpected abundances and diversities of endophytic fungi in certain regions (e.g. wet microclimates in the temperate zone), and some hosts – especially those with long-lived foliage, such as evergreens – often harbor more, and more diverse, endophytes than their latitudinal position would suggest (Arnold & Lutzoni, 2007).

2. Ecological roles

Because of their remarkable diversity within individual hosts or host tissues, the ecological roles of Class 3 endophytes are difficult to discern, and in most cases they have not been explored. However, several studies have introduced Class 3 endophytes into plants raised under sterile conditions and have evaluated the costs and benefits of infection. Importantly, plants infected by multiple Class 3 endophytes typically show no observable change in growth rate, biomass accumulation, root:shoot ratio, or other easily quantifiable characteristics following inoculation under *in vivo* conditions (Arnold, 2002). Webber (1981) highlighted the role of bark endophytes in protecting trees against Dutch elm disease. Arnold *et al.* (2003) showed that diverse assemblages of endophytes decrease lesion formation and leaf death caused by *Phytophthora* sp. in *Theobroma cacao*. Several studies indicate that Class 3 endophytes can be mutualistic, despite the fact that several aspects of their ecology (i.e. high diversity within hosts and horizontal transmission) are more frequently associated with parasitic or pathogenic lifestyles. Notably, Schulz *et al.* (1998, 1999) demonstrated that some Class 3 endophytes negatively impact plant growth, and Arnold & Engelbrecht (2007) demonstrated that some seedlings lose water more quickly under severe drought when natural assemblages of Class 3 endophytes are present. Thus the ecological roles of Class 3 endophytes appear diverse, presenting a distinctive set of research questions relative to the less diverse Class 1 and Class 2 endophytes.

In considering the ecology of Class 3 endophytes, it is important to think of plant–endophyte interactions in the context of ecological complexity: plants inhabited by highly diverse endophytes also host rhizosphere and phyllosphere fungi and bacteria and they are consumed by herbivores. It is possible that Class 3 endophytes play major roles in the interaction of other organisms with plants. For example, the recovery of entomopathogenic fungi as endophytes (Bing & Lewis, 1991) opens the door to understanding symbiotic ecology that would be overlooked if the relevant herbivore–plant–endophyte combination were not examined. This example serves as a reminder of the need to gather baseline data regarding the abundance, diversity, and host specificity of all endophytes, and to build from those findings to explore their ecological importance. More generally, several studies have pointed to the diverse ecological roles and potential applications of Class 3 endophytes – a hyperdiverse group that remains woefully understudied (Schulz *et al.*, 1998; Arnold *et al.*, 2003; Arnold & Engelbrecht, 2007).

VI. Class 4 endophytes

While studying and isolating ectomycorrhizal fungi, Merlin (1922) observed a brown to blackish, pigmented fungus associated with terrestrial plant roots. He called these sterile,

root-associated fungi 'mycelium radicus astrovirens' (MRA). MRA were often found to co-exist with mycorrhizal fungi, and were referred to as 'pseudomycorrhizal' fungi (Merlin, 1922). Shortly thereafter, Peyronel reported > 135 species of angiosperms associated with dark pigmented fungi in root tissues (Peyronel, 1924). Presently, these fungi are referred to as 'dark septate endophytes' (DSE) and are grouped together as Class 4 endophytes.

In addition to the criteria listed in Table 1, Class 4 endophytes (DSE) are distinguished as a functional group based on the presence of darkly melanized septa, and their restriction to plant roots. In general, Class 4 endophytes are primarily ascomycetous fungi that are conidial or sterile and that form melanized structures such as inter- and intracellular hyphae and microsclerotia in the roots. DSE have little host or habitat specificity; they have been reported in association with *c*. 600 plants including plants that are nonmycorrhizal, from Antarctic, Arctic, alpine, sub-alpine, and temperate zones, as well as from African coastal plains and lowlands, and some tropical ecosystems (Jumpponen & Trappe, 1998; Jumpponen, 2001). DSE are often found in boreal and temperate forests associated with the fine roots of trees and shrubs, especially of conifers (Richard & Fortin, 1974). These fungi are not thought to be pathogenic, as they are observed on healthy fine roots, and in this context, will be referred to as endophytes in a broad sense in that DSE 'colonize living plant organs without any apparent, overt negative effects' (Hirsch & Braun, 1992; Jumpponen & Trappe, 1998). In short, DSE are found worldwide, are prevalent in high-stress environments, and appear to be ubiquitous in occurrence and abundant across various ecosystems. Collectively, these observations suggest that DSE may play an important role in the ecophysiology of plants. However, almost a century after their discovery, little is still known about the role of these mysterious and rather elusive fungal symbionts.

1. Phylogenetic diversity, classification, life history and potential ecological roles

It is difficult to define the diversity of DSE species and their plant host ranges until an intensive study of plant–DSE associations in habitats worldwide is undertaken. Two latitudinal studies encompassed a large number of plant species from the North to the South Pole to address this issue (Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2005). They found DSE associated with 587 plants species representing 320 genera and 114 families. Colonization studies were then conducted using five described anamorphic taxa of DSE (*Chloridium paucisporum*, *Leptodontidium orchidicola*, *Phialocephala dimorphosphora*, *Phialocephala fortinii* and *Phialophora finlandia*) under natural and experimental conditions (inoculation of root systems in pots). Collectively, these DSE species had a large host range and/or lacked host specificity: collectively, they were able to colonize 66 different

plant species, with *P. fortinii* alone able to colonize > 20 species.

DSE appear to represent a large and interesting class of endophytes that have as yet not been well defined taxonomically and/or ecologically. Therefore, classification of fungi as DSE should be approached with some caution as identification is based on rather broad and ambiguous criteria. Presently, the presence of asexual, darkly pigmented, septate endophytes in plant roots is the primary criterion for DSE designation. However, dark pigmented endophytes belonging to other classes have been mistaken as DSE. For example, the Class 2 endophyte *Curvularia protuberata* which symbiotically confers temperature tolerance to the geothermal plant *Dicanthelium lanuginosum* is a darkly pigmented, asexual septate fungus that colonizes plant roots (see 'Endophyte-conferred fitness benefits and ecological adaptations of plants' in Section IV). However, *C. protuberata* is not a true DSE: it colonizes virtually all parts of the plant (roots, crown, stem, leaves and seed coat). To avoid erroneous classification, researchers must analyze portions of plants representing all developmental tissues (roots, crown, stem, leaves and seed coat) for fungal colonization before DSE classification. Furthermore, researchers must verify the presence of fungal septa. Utilization of fungal wall/septa-specific dyes should be employed as use of more general dyes, or lack of dyes, can give erroneous results (Ormsby *et al.*, 2007; S. Kaminskyj, personal communication). Lastly, the asexual designation of DSE may need to be addressed as 'an unknown number of taxa (anamorphic and/or telomorphic) are involved, and that a considerable functional and ecological overlap might exist between soil fungi, saprophytic rhizoplane-inhabiting fungi, strictly pathogenic fungi, mycorrhizal fungi and fungal endophytes' (Jumpponen & Trappe, 1998).

Because of the presence of DSE in soils and plant roots, transmission is most likely horizontal. Mycelial fragmentation and dispersal of conidia appear to be the means of transmission as demonstrated under laboratory conditions (Jumpponen & Trappe, 1998). Although anamorph–teleomorph connections have not yet been identified for most DSE, the possibility of sexual reproduction should not be discounted. Wang & Wilcox (1985) described the asexual reproductive structures as well as the morphology of DSE-colonized roots, which were reported to resembled ectomycorrhizas, endomycorrhizas and pseudomycorrhizas.

Root colonization by *P. fortinii* is described by several authors as a classic representative of DSE (Currah & Van Dyk, 1986). Colonization begins with superficial and/or runner hyphae that form a loose network of hyphae on the root surface. Individual hyphae then grow along the main axis of the root and can grow between cortical cells and also within the depressions between epidermal cells (O'Dell *et al.*, 1993). Colonization may also be intracellular without causing any distortion to host roots but the endophyte may form clusters of closely packed thick-walled cells within the cortical cells collectively referred to as 'thick pseudoparenchymatic mass,

sclerotia, microsclerotia or sclerotial bodies' (Wang & Wilcox, 1985; Jumpponen & Trappe, 1998). In addition, some DSE form a Hartig-like net and/or labyrinthine tissue, and in some instances, colonization of the root cortical layer results in the formation of chlamydospore-like, rounded cells within the cortical cells (O'Dell *et al.*, 1993).

Although there is a plethora of knowledge regarding the ecological role and symbiotic functionality of Class 1 endophytes, and a growing body of knowledge regarding Class 2 and 3 endophytes, little is known about the large group of fungi that constitute the Class 4 endophytes. The taxonomic identification of DSE and their host counterparts is indeed a required first step. However, the time has come to ask questions regarding the evolutionary history, symbiotic functionality, phylogenetic relationships, and finally, the ecological role of these largely neglected fungi. Mandyam & Jumpponen (2005) propose that DSE symbioses, like mycorrhizas, are multifunctional and not limited to nutritional acquisition and host growth response. They propose that DSE colonization may play a role in deterring pathogens by minimizing available carbon in the rhizosphere, and that high melanin levels may potentially be involved in the production of secondary metabolites toxic to herbivores, both factors giving Class 4 symbiotic plants a competitive edge. Mandyam & Jumpponen (2005) emphasize the need for simple experiments to determine the basis of biological functions of DSE and urge the scientific community to do so, with which we agree whole-heartedly. In summary, although the role of Class 4 endophytes is still unknown, because of the apparent abundant associations with roots of diverse plants from different habitats, the biological and ecological roles of DSE must indeed be important in plant ecophysiology.

VII. Conclusions

Fungal endophytes comprise a diverse group of species that vary in symbiotic and ecological functions. Here, we used host range, colonization and transmission patterns, tissue specificity, and symbiotically conferred fitness benefits to differentiate endophytes into four functional classes. While the impact of each class on plants may differ significantly, the vast majority of endophytes have yet to be adequately characterized. Regardless, it is clear that these fungi can have profound impacts on the survival and fitness of plants in all terrestrial ecosystems, and therefore likely play a significant role in plant biogeography, evolution and community structure. As more researchers perform Koch's postulates to assess host range, colonization and transmission patterns, and symbiotic/ecological function, our perspective on endophytes will change. In addition, several new and emerging molecular (e.g. denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP)) and biochemical (e.g. stable isotope profiling (SIP) and metabolic incorporation of nucleotide analogs such as bromodeoxyuridine (BrdU))

technologies are now being applied to better characterize endophytes and their roles in plant ecophysiology (see review by Johansson *et al.*, 2004; Vandenkoornhuyse *et al.*, 2007). For example, molecular techniques can be used to assess community structure of endophytes across landscapes (e.g. Arnold & Lutzoni, 2007; Macia'-Vicente *et al.*, 2008a), and biochemical techniques can indicate which members of the community are metabolically active. These data can feed directly into studies of the systematics and taxonomy of these little-known fungi. In addition, as we learn more about the contribution of endophytes to plant gene expression, it will be possible to profile gene expression patterns to assess the symbiotic status of plant communities. Ultimately it should be possible to determine the community structure and metabolic activity of all fungal symbionts associated with plants across landscapes. This may allow the development of new tools to assess changes in ecosystems resulting from natural fluctuations, climate change, and other anthropogenic features of environmental modification.

More extensive characterization of different endophyte–plant associations may also provide greater insight into the evolution of mutualisms. For example, the mode of endophyte transmission (vertical or horizontal) is thought to significantly influence the evolution and sustainability of mutualisms (Sachs *et al.*, 2004). For vertically transmitted endophytes, the fitnesses of the two partners are linked, the outcome of the association is predictable and mutualism is strongly selected. Alternatively, the nature of horizontal transmission provides opportunities for plant colonization by a variety of fungi that may express different symbiotic lifestyles. Therefore, horizontal transmission may disrupt fitness linkages between specific species. Among the endophyte classes, Class 1 and 2 endophytes are transmitted either vertically or horizontally while Class 3 and 4 endophytes are transmitted only horizontally. Yet, mutualisms have been described in all four classes. Clearly, the mode of endophyte transmission alone does not dictate the outcome of symbioses but it may well influence the fitness benefits conferred by endophytes, the longevity of symbiotic associations, and the vulnerability of hosts to nonmutualistic endophytes.

Over the next several decades researchers will begin to address some fundamental questions in endophyte biology, such as:
• How do endophytes communicate with hosts to confer fitness benefits?
• What is the distribution of these functional classes across environmental gradients?
• Do endophytes provide a mechanism to explain plant biogeographic patterns?
• What are the evolutionary dynamics of habitat-adapted symbiosis?
• What are the evolutionary origins of these diverse symbiotic fungi?
• What are the genomic differences among functional classes, and can they be used to predict the outcome of plant–fungal interactions?

Our limited understanding of such important microorganisms is a testament to the fact that the 'age of discovery' is just beginning. Until we understand more about the significance of endophytes in plant biology, our understanding of plant community dynamics and ecosystem function will be limited. It is important to point out that individual plants comprise communities of microorganisms including fungi, bacteria, viruses and sometimes algae. Yet, scientists typically take fairly reductionist approaches to studying plant–microbe interactions. We are now poised to begin taking a systems approach to plant symbiosis by studying multiple symbionts within individual host plants, and multiple hosts within a single habitat or across landscapes.

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