

Clavicipitaceous endophytes: Their ability to enhance resistance of grasses to multiple stresses

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Abstract

Grasses are noted for an absence of toxic secondary metabolites. However, some grass-associated fungi are known for their production of numerous secondary compounds, which serve important functions such as plant competition and resistance to biotic and abiotic stresses. Cohabitation of certain fungi with grasses has developed into specific mutualistic associations. The fungi of concern belong to a relatively small group of species within the Clavicipitaceae. This group of fungi shares a common feature in being endophytically associated with grasses as obligate biotrophic symbionts. Specific attention is given to two related genera *Epichloë* and their anamorphic *Neotyphodium* relatives. The association of these fungi with grasses results in the accumulation of several classes of fungal metabolites that serve as relief mechanisms to biotic and abiotic stresses. These include drought tolerance, resistance to vertebrate and invertebrate pests, resistance to fungal diseases, and tolerance to poor soil conditions. Resistance to multiple stresses can occur simultaneously within the same symbiotum. The resulting resistance to multiple stresses, along with inter- and intra-specific competitive advantage suggests the potential for utilizing novel fungal endophytes new host combinations for stress resistance in naturally infected grasses as well as for resistance to specific agronomic and ecological conditions and soil remediation.

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1. Introduction

Grasses originated as understory plants in forests, and from this origin the family developed key characteristics that allowed species to reach a climax association within open habitats, creating a diversity of species. This diversity is due in part to the symbiotic relationship with grazing animals, especially ruminants, and the developments of the perennial habit along with a unique intercalary meristem. The grass family, Poaceae, is relatively large consisting of warm- and cool-season perennials and annuals comprising approximately 10,000 species worldwide, with approximately 2000 of these found in North America

(Stebbins, 1981). Grasses have contributed to the development of humankind as there are several important species that serve as essential food crops, as forage of livestock, and for recreational and conservation purposes.

Compared to dicotyledons, grasses lack the biosynthetic capacity for the production of secondary metabolites, which are useful in the long-term survival strategy of each species. However, grasses cohabit with microorganisms, which are notorious for the production of secondary metabolites. The association of grasses with microorganisms is numerous, and important to the ecological fitness and species diversity of grasses. One group of microorganisms includes the fungal, endophytes, which are defined as fungi that colonize healthy plant tissue without causing obvious symptoms in or injury to the host. Specifically, one group of fungi is perennially and systemically associ-

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ated with the aboveground portions of grasses and occasionally sedges. These fungi are further characterized as obligate biotrophic intercellular symbionts and are ascomycetes of the family Clavicipitaceae. Genera and species within this family are further characterized and distinguished on the basis of morphology of the conidial (micro- and macro-) and ascospore states (Bischoff and White, 2003; Diehl, 1950; Rykard et al., 1984; White, 1994). Further, molecular techniques are now available to distinguish species as morphological characters cannot clearly delineate several species (Moon et al., 1999; van Zijll de Jong et al., 2003; and see review of Schardl et al., 2004). Molecular techniques are also useful in distinguishing those species that exist only as anamorphs. The family includes the species *Epichloë* or its anamorphs classified in the genus *Neotyphodium* (Fig. 1a–c) as well as *Balansia* and *Myriogenospora*. A complete listing of species within this group can be found in Diehl (1950), Leuchtman (2003), Bischoff and White (2003), and of *Epichloë* species in Table 1. The *Epichloë* and *Neotyphodium* endophytes actively colonize grass tissues establishing long-term associations, and are not generally considered organ-specific. Thus, they may be isolated from leaf sheaths, stem, and from young inflorescences and seed.

Several clavicipitaceous endophytes are transmitted by seed (Tables 1 and 2), but information on infection of hosts by most are still speculative. The association of specific species with some grasses, although suggestive of a pathogenic association, is occasionally pleiotropic as individual tillers of an infected grass will produce the sexual state, manifest as choke disease (horizontal transmission), while other tillers remain symptomless, transmitting the endophyte vertically (Schardl, 2001). This pleiotropic condition is characteristic of many species of *Epichloë*, including *E. amarillans* (Fig. 1a and b), *E. brachyelytri*, *E. elymi*, and *E. festucae*. Although the group of fungi is relatively small, they colonize a large number of grass species, but because several taxa are symptomless they can only be detected either by isolating such fungi from surface-sterilized tissues placed onto agar medium, revealing the *Neotyphodium*

state or by microscopic observation of stained endophytic hyphae (Fig. 1c). However, it is these symptomless endophytes that offer the most promising benefits to technological exploitations since they do not produce signs or symptoms of infection, and they are also compatible with a wide range of agronomically important grasses (turf and grazing species). Apparently, they are genetically stable, the associations are mutualistic and persist.

Since we focus our attention on exploiting endophytes for specific purposes, we restrict this review to include those intercellular fungi that impart an ecological benefit without doing substantive harm to the grass such as the *Neotyphodium* species, referred to as symptomless endophytes. While this definition is restrictive, it conveys the essential concepts outlined in the definition of fungal endophytes (Stone et al., 2000), and will serve as the primary focus for this review that has as its specific purpose the use of endophytes as bio-control agents. A comprehensive listing of specific mutualistic interactions imparted to infected hosts by Clavicipitacean endophytes has not been established. We present here the wide assortment of known mutualistic benefits from resulting from endophyte-infected grass associations. We also discuss those benefits that are anticipated relative to future enhancement of grasses resulting from endophytic infections. Additionally, this review will consider the importance of the nature of the endophytic niche since this location is essential if we are to understand the interactions between endophytes and hosts, and to improve them so that the number of endophytic hyphae can be increased within hosts for optimum expression of the desired outcomes.

2. The endophytic niche

2.1. Endophytic colonization

Epichloë and *Neotyphodium* species are intercellular (Bacon and Hinton, 2002; Hinton and Bacon, 1985; Siegel et al., 1987; White et al., 1997), and colonize grasses exclu-

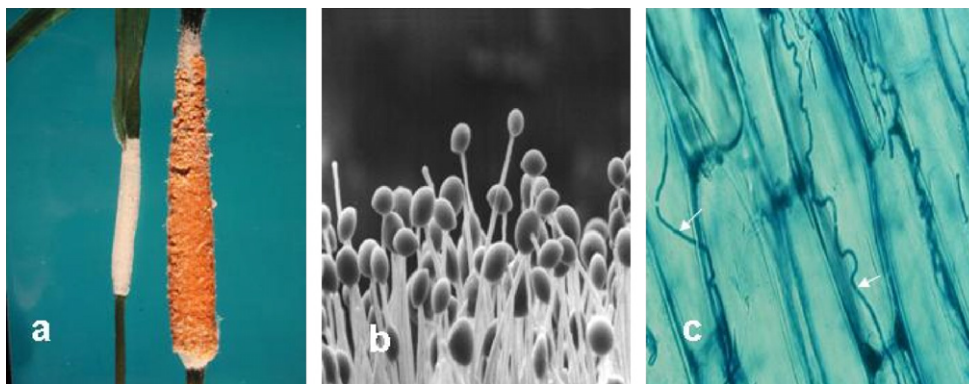


Fig. 1. Choke disease of *Epichloë amarillans* on autumn bentgrass, *Sphenopholis obtusata*. (a) Early phase of disease expression showing white-to-ivory conidiomata (left) completely covering the inflorescence of bentgrass, and at right a mature yellow-to-orange stromata bearing perithecia, around the culm, enclosing the inflorescence primordia, leaf primordia and sheath. (b) A scanning electron micrograph of the conidioma in (a) showing details of the conidiogenous cells with reniform-to-hemispherical conidia (sprematia) born at the tips of conidiophores, typical of the anamorphic state *Neotyphodium*. (c) Light microscopy (40 \times) of aniline blue stained intercellular hyphae running vertically along the intercellular spaces of the host cells.

Table 1
Species of *Epichloë* and their hosts^a

<i>Epichloë</i> species	Host species	Life cycle or transmission	Mating population
<i>E. amarillans</i>	<i>Agrostis hiemalis</i> , <i>Calamagrostis</i> , <i>Sphenopholis obtusata</i>	Vertical and horizontal	IV
<i>E. baconii</i>	<i>A. stolonifera</i> , <i>A. tenuis</i> , <i>Calamagrostis villosa</i>	Vertical	V
<i>E. brachyelytri</i>	<i>Brachyelytrum erectum</i>	Vertical and horizontal	IX
<i>E. bromicola</i>	<i>Bromus erectus</i> , <i>B. ramosus</i> , <i>Hordelymus spp.</i>	Vertical and horizontal	VI
<i>E. clarkii</i>	<i>Holcus lanatus</i>	Vertical	I
<i>E. elymi</i>	<i>Elymus canadensis</i> , <i>E. hystrix</i> , <i>E. villosus</i> , <i>E. virginicus</i>	Vertical and horizontal	III
<i>E. festucae</i>	<i>Festuca rubra</i> , <i>F. gigantea</i> , <i>F. longifolia</i> , <i>Koeleria</i>	Vertical and horizontal	II
<i>E. glyceriae</i>	<i>Glyceria striata</i>	Horizontal	VIII
<i>E. sylvatica</i>	<i>Brachypodium sylvaticum</i>	Vertical and horizontal	VII
<i>E. typhina</i>	<i>Anthoxanthum odoratum</i> , <i>Arrhenatherum</i> , <i>Brachypodium pinnatum</i> , <i>Dactylis glomerata</i> , <i>Lolium perenne</i> , <i>Phleum pratense</i> , <i>Poa</i> <i>nemoralis</i> , <i>Poa trivialis</i> , <i>Poa pratensis</i> , <i>Poa silvicola</i> , <i>Puccinellia</i>	Vertical	I
<i>E. yangzii</i>	<i>Roegneria kamoji</i>	Vertical and horizontal	

^a Clay and Schardl (2002), Li et al. (2006), Leuchtman (2003) and Moon et al. (2004).

Table 2
Neotyphodium species and their closest teleomorphic relatives

<i>Neotyphodium</i> sp. ^{a,b}	Host species	Closest nonhybrid relative
<i>Neotyphodium aotearoae</i>	<i>Echinopogon ovatus</i>	Nonhybrid species
<i>N. australiense</i>	<i>E. ovatus</i>	<i>E. festucae</i> and <i>E. typhina</i>
<i>N. chisosum</i>	<i>A. eminens</i>	<i>E. amarillans</i> , <i>E. typhina</i> , and <i>E. bromicola</i>
<i>N. coenophialum</i>	<i>Festuca arundinacea</i>	<i>E. festucae</i> , <i>E. typhina</i> , and <i>E. baconii</i>
<i>N. huerfanum</i>	<i>F. paradoxa</i> , <i>F. arizonica</i>	<i>E. typhina</i>
<i>N. gansuense</i>	<i>A. inebrians</i>	
<i>N. inebrians</i>	<i>A. inebrians</i>	Nonhybrid species
<i>N. occultans</i>	<i>Lolium multiflorum</i> , <i>L. canariense</i> , <i>L. persicum</i> , <i>L. remotum</i> , <i>L. rigidum</i> , <i>L. subulatum</i> , <i>L. temulentum</i>	<i>E. bromicola</i> and <i>E. baconii</i>
<i>N. lolii</i>	<i>L. perenne</i>	<i>E. festucae</i>
<i>N. melicicola</i>	<i>Melica decumbens</i>	<i>E. festucae</i> , <i>N. aotearoae</i>
<i>N. siegelii</i>	<i>L. pratense</i>	<i>E. festucae</i> , <i>E. bromicola</i>
<i>N. starrii</i>	<i>F. subulata</i> , <i>F. arizonica</i> , <i>Bromus anomalus</i>	Nd ^c
<i>N. tembladerae</i>	<i>P. huecu</i> , <i>F. argentina</i> , <i>F. hieronymi</i> , <i>F. arizonica</i>	<i>E. festucae</i> , <i>E. typhina</i>
<i>N. typhinum</i>	<i>P. sylvestris</i>	<i>E. typhina</i>
<i>N. typhinum</i> var. <i>canariense</i>	<i>Lolium edwardii</i>	<i>E. typhina</i>
<i>N. uncinatum</i>	<i>L. pratense</i>	<i>E. bromicola</i> , <i>E. typhina</i>

^a Clay and Schardl (2002), Li et al. (2004a,b), Moon et al. (2004) and Schardl and Moon (2003).

^b All *Neotyphodium* species are vertically transmitted.

^c Nd, not determined.

sively above ground, are associated with grasses as obligate biotrophic symbionts, and each endophytic species may colonize several grass hosts, although some endophytes are restricted to specific genera and species of grasses (Tables 1–3). *In planta*, endophytic hyphae follow a gradient with high concentrations in the basal regions to low concentrations at the apical portions. This distribution is true even within distinct organs of grasses. Thus, in the leaf, the leaf sheaths are highly infected from the basal ends and are greatly reduced at the apical ends, and only occasionally are there hyphae beyond ligules. Roots of soil-grown infected grasses are never colonized. At the plant base or lower crown, the endophytic hyphae are highly branched, but as they elongate upward along the plant axis, branching does not occur. This highly controlled growth is related to the highly compatible nature of the

association; branching hyphae are observed in the upper shoot only during the pathogenic expression and during stromata formation in the *Epichloë* species. The molecular basis for control of *in planta* branching is the accumulation in the extracellular matrix of reactive oxygen species (ROS) produced by the fungus (Tanaka et al., 2006). Strains of *Epichloë festucae* in which the *noxA* gene encoding NADPH oxidase is disrupted have decreased ROS levels and introduction of these strains into plants results in stunted plants that lose apical dominance and show an increase in hyphal branching *in planta* (Tanaka et al., 2006). The *E. festucae* gene *noxR* is a regulator of *noxA* and disruption of *noxR* results in a similar phenotype to that seen in *noxA* mutants (Takemoto et al., 2006).

The distribution of endophytes within grasses follows the natural nutrient sinks in grasses, including the seeds,

Table 3
Undescribed hybrid species of *Neotyphodium*^a

Species	Host	Closest nonhybrid relatives
FalTG-1	<i>Festuca altissima</i>	<i>E. bromicola</i> and <i>E. typhina</i>
FatG-2	<i>F. arundinacea</i>	<i>E. festucae</i> and <i>E. baconii</i>
FatG-3	<i>F. arundinacea</i>	<i>E. typhina</i> and <i>E. baconii</i>
FpaTG-1	<i>Melica ciliata</i>	<i>E. amarillans</i> and <i>E. typhina</i>
HboTG-2	<i>Hordeum bogdanii</i>	<i>E. bromicola</i> and <i>E. typhina</i>
HbrTG-2	<i>Hordeum brevisubulatum</i>	<i>E. bromicola</i> and <i>E. typhina</i>
HeuTG-2	<i>Hordelymus europaeus</i>	<i>E. bromicola</i> and <i>E. typhina</i>
LpTG-2	<i>Lolium perenne</i>	<i>N. lolii</i> (or <i>E. festucae</i>) and <i>E. typhina</i>
MciTG-1	<i>F. paradoxa</i>	<i>N. inebrians</i> and <i>E. typhina</i>
PauTG-1	<i>Poa autumnalis</i>	<i>E. elymi</i> and <i>E. typhina</i>
SroTG-1	<i>Stipa robusta</i>	<i>E. festucae</i> and <i>E. elymi</i>

^a Schardl and Moon (2003) and Moon et al. (2004).

although its absence in the roots, a nutrient-rich organ, suggests other factors are also responsible for this distribution. Hyphal growth is apical, but intercalary growth, which is not often reported in fungi, has been suggested (Christensen et al., 1997, 2000). The growth of endophytic hyphae is correlated with the host's life cycle, although hyphae located in old, matured host tissues are metabolically active (Schmid et al., 2000; Spiering et al., 2005). The distribution of *Epichloë* and *Neotyphodium* species is restricted to cool season grasses (Clement et al., 1994; Leuchtman 2003; White, 1993; White and Cole, 1985a, 1986; White et al., 1992).

2.2. Morphological details of the endophytic niche

What are the morphological characteristics of the intercellular space, and what substances are contained within it? The answers to these questions are important to understand of nature of interactions that allow endophytic fungi to exist in this niche. The intercellular spaces consist of a

series of connecting, nonliving components of the plant forming what is referred to as the apoplasm, which is distinct from the cell to cell contact referred to as the symplasm. The intercellular spaces are located in the cortical tissue of the root and in the parenchyma tissue of stems and leaves. The intercellular spaces are formed by the juxtaposition of three to four cells and the dissolution of the middle lamellae and they are significantly different in the leaves and stems as opposed to the roots. The volume occupied by intercellular spaces consists of a significant portion of the plant, and in leaves as much as 6% of the leaf tissue consists of intercellular spaces (Altus and Canny, 1985; Tetlow and Farrar, 1993). The intercellular spaces of roots in most grasses are typically schizogenous, although in grasses there also may occur larger lysigenous lacunae formed from the breakdown of cells producing two basic types (Fig. 2a and b). The internal constitution of these spaces varies and some consists only of air or dry flaky deposits (Fig. 2a), but the majority of spaces consists of fluids (Fig. 2b), which in grasses are shown to contain the inorganic ions potassium, calcium, sulphur, phosphorus, and chlorine (Canny and Huang, 1993). The intercellular spaces of the green portion of grasses also consist of gases and fluids. The intercellular spaces in leaves are predominantly located in the extrafascicular plant parenchymatous tissues and consist of oxygen and air-saturated water and fluid. The movement of fluid through the apoplastic route occurs, but the connection to the symplasm, if any, is complex and uncertain.

Fungal endophytes benefit from inhabiting the grass's interior because it is a protected niche in which there is relatively little competition from other microorganisms and because it is a reliable source of nutrition. The mechanisms through which specific nutrients within the apoplasm are made available and exchanged are not understood. However, there is evidence that fungal endophytes possess some hydrolytic enzymes, which can be used in pathogenesis and

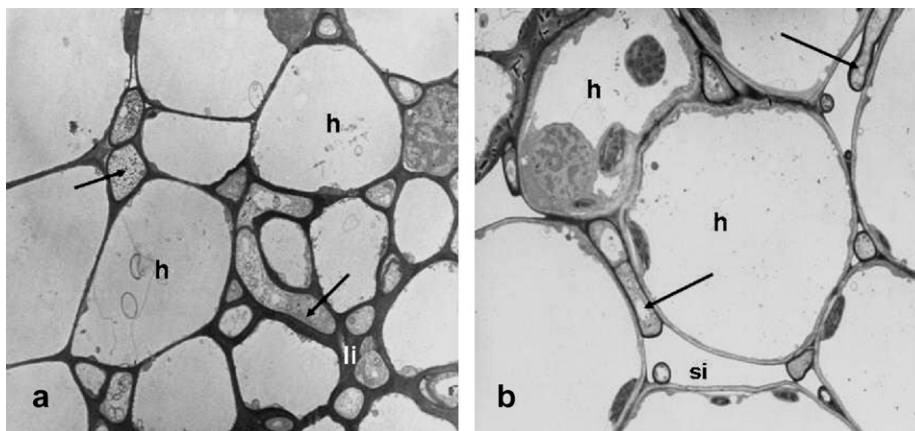


Fig. 2. Micrographs of a vertically and horizontally transmitted species of grass endophyte, *Epichloë amarillans*, in the leaf sheaths of autumn bentgrass, *Sphenopholis obtusata*, showing the two types of intercellular spaces between host cells (h). In (a), the endophytic hyphae (arrows) are embedded within a matrix filled lysigenous intercellular spaces (li). In (b), endophytic hyphae are embedded in schizogenous intercellular spaces (si), the typical habitat for endophytic hyphae in most species of grasses, especially those showing only the *Neotyphodium* state.

in nutrient acquisition (Lam et al., 1995; Li et al., 2004a, 2004b, 2005; Lindstrom and Belanger, 1994; Lindstrom et al., 1993; Moy et al., 2002; Reddy et al., 1996). The concentrations of nutrients within the apoplasm have been suggested as being sparse, implying that intercellular inhabiting fungi might live under oligotrophic conditions. This however, has not been established as the nutritional requirements of endophytic fungi reported to date are in fact copiotrophic (Bacon, 1990; Davis et al., 1986; Langer et al., 2004), which is reinforced in the discussion below (Section 2.3) where data are presented to indicate that the intercellular spaces are in fact rich in organic and inorganic nutrients capable of supporting the intercellular concentration of hyphae observed in endophytic infected grasses.

2.3. Chemical constituents of the endophytic niche

Nutrient concentrations in both the apoplasm and symplasm are interactive with the phloem, dispelling the earlier notion that the apoplasm is relatively free of nutrients. Indeed, current research indicates that nutrient transport within grass tissues is considered to occur through an apoplastic route via the cell wall continuum but the symplastic route occurs via plasmodesmata (Canny, 1995; Dong et al., 1994; Kursanov and Brovchenko, 1970; Madore and Webb, 1981). Organic nutrients within the apoplasm consist of the several sugars and related carbohydrates derived from photosynthesis (Table 4), although several nitrogenous compounds such as nitrates, nitrites, amino acids and amine also occur, as well as key inorganic ions (Table 4). The concentrations of sugar available within the apoplasm are dependent on the nature of phloem loading either from the apoplasm or symplasm, which is both spe-

cies and individually regulated. However, plant regulation might be altered dramatically by the presence of an endophytic organism.

The sugars are considered to be compartmentalized in the apoplasm and their concentration within the apoplastic fluid is not controlled by the membranes of cells forming the spaces (Altus and Canny, 1985; Canny, 1995; Kursanov and Brovchenko, 1970; Madore and Webb, 1981; Tetlow and Farrar, 1993). Analysis of the contents of the apoplasm indicates that it consists of sucrose, glucose, and fructose in ratios close to those characteristic of surrounding cells, indicating an interaction with sugars within cells, and when sugars are removed from the intercellular spaces they are replaced within 60 min (Kursanov and Brovchenko, 1970). Thus, the intercellular space is rich in substances necessary to support the growth of fungal endophytes, which is reinforced by the large number of fungal cells observed (Fig. 2). Further, the concentrations of apoplastic sugars reported to occur in nonendophytic apoplasm are sufficient to support fungal growth (Farrar and Farrar, 1986; Huber and Moreland, 1980; Kneale and Farrar, 1985).

Detailed analysis of the apoplasm and symplasm of sugar cane indicates similarity to other compounds isolated from other plants (Tejera et al., 2006), suggesting a commonality of apoplast substances in all plants, especially grasses. However, most of the nutrients occur at a two-fold higher concentration in the symplasm than the apoplasm. Further, the dynamic nature of materials within the apoplast of grasses is also suggested from studies of pathogenic rust fungi examined early during its pathogenesis, i.e., its endophytic stage. In the brown rust of barley the concentration of nutrients within the apoplasm is dynamic as it is cycled between the host and the fungus. There is a direct

Table 4
Nutrients reported in the apoplasm of leaves and roots of plants^a

Sugars/sugar alcohols	Organic acids	Amino acids	Inorganic/organic compounds
Fructose	γ -Aminobutyric acid	Arginine	Ammonium
Galactinol	Citric acid	Aspartic acid	Calcium
Galactose	Fumaric acid	Asparagine	Chloride
Inositol	Malic acid	Alanine	Nitrate
Raffinose	Oxalic acid	Cysteine	Nitrite
Stachyose	Tartaric acid	Cystine	Phosphorus
Sucrose		Glutamic acid	Potassium
Verbascose		Glycine	Soluble protein
		Histidine	Sulphur
		Isoleucine	
		Leucine	
		Lysine	
		Methionine	
		Proline	
		Phenylalanine	
		Serine	
		Tyrosine	
		Threonine	
		Valine	

^a From: Canny and Huang (1993), Canny and McCully (1988), Kursanov and Brovchenko (1970), Madore and Webb (1981), Tetlow and Farrar (1993) and Tejera et al. (2006).

correlation between phloem loading of low concentrations of sugars and pH, which require a proton gradient across the plasma membrane of the sieve elements and companion elements (Delrot and Bonnemain, 1981; Giaquinta, 1977). Therefore, altering the pH in the apoplast can affect sugar concentration, which has been documented to occur during the biotrophic phase of the brown rust fungus on barley (Tetlow and Farrar, 1993). It was demonstrated that in host infected tissue, the pH was increased from 6.6 to 7.3, resulting in a 35–40% decrease in total soluble carbohydrate concentration. While this work concentrated on diseased tissue, it nevertheless indicates the nature of an interaction between an endophytic system and a host for sugars. It is our opinion that this concentrations gradient would favor flow of continued carbohydrates to an endophyte. By altering the pH of the apoplast, fungal endophytes can alter indirectly the activity of specific enzymes and the kinetics of sugar uptake of host cells, thus increasing the concentration of sugars in the apoplast.

Observations in the endophytic stage of pathogenic rust model, indicated an increase in the volume of the intercellular spaces in infected hosts (Tetlow and Farrar, 1993). Therefore, the extent of an endophytic microorganism's alteration on the host occurs both at the physiological and morphological levels of expression. Without doubt, the occurrence in the apoplast of protein, ammonium, nitrate, nitrite, several amino acids, organic acids, and sugars serves to regulate the biological activity of endophytic fungi and contributes to the diversity of secondary metabolites produced by specific genotypes of each endophyte species or strain.

3. Secondary metabolites

The discovery that *N. coenophialum* can produce ergot alkaloids that were associated with toxicity of cattle and other livestock provided the first evidence that endophytic associations were responsible for antiherbivory in mammals (Bacon et al., 1977; Lyons et al., 1986). It was subsequently discovered that this class of secondary metabolites is characteristic of the fungal family, which was historically attributed to the genus *Claviceps*. Since this discovery, a primary focus for most studies of endophytic fungi is based on a fundamental principle of defensive mutualisms (Clay, 1988) that has been established as a major benefit afforded to the association. This principle has since defined several uses of fungal endophytes in agriculture.

Several alkaloids comprise the major classes of secondary metabolites that are associated with mutualistic associations in endophyte-infected tall fescue and perennial rye grasses. These include specific ergot alkaloids such as ergovaline (Lyons et al., 1986), the aminopyrrolizidine alkaloids, the pyrrolpyrazine alkaloids, the indole diterpenoid alkaloids, and recently the 11, 12-epoxy-janthitrems. These chemical classes are found variously in species of grasses infected with endophytes, but not all infected grasses will contain all classes of alkaloids. Indeed, their occurrence in grasses indicates that there are chemical ana-

logs reflective of strains of endophytes. The final quantitative amounts of these compounds are related to specific host genotypes and host–endophytes combinations (Adcock et al., 1997; Agee and Hill, 1992, 1994; Ball et al., 2006). In surveys, it was reported that the pyrrolpyrazine alkaloids, represented by peramine, was present in the majority of endophyte-infected hosts (Christensen et al., 1993; Dahlman et al., 1991; Leuchtman et al., 2000; Siegel et al., 1990). The ergot alkaloids followed next, which was followed by loline, an aminopyrrolizidine alkaloid, and lastly by lolitrem, the indole diterpenoid alkaloid.

The biological spectrum of activity expressed by these four classes of alkaloids varies from anti-feeding behavior in vertebrates and invertebrates to drought tolerance by the plant. The basic chemical structures of these alkaloids and their effects on ruminants and other animals have been presented and reviewed (Ball et al., 1995; Bush et al., 1993, 1997; Cross et al., 1995; Fletcher and Harvey, 1981; Jackson et al., 1996; Oliver et al., 1998; Schuenemann et al., 2005), and will not be presented here. One recent study however demonstrates the role of ergot alkaloids in deterring mammalian herbivory. Panaccione et al. (2006a) used perennial ryegrass infected with genetically manipulated endophytes that produced ergovaline, produced only clavine alkaloids or produced no ergot alkaloids in feeding studies with rabbits. They observed that ergovaline is responsible for reduction of appetite and that clavine alkaloids reduced the appeal of feed containing perennial ryegrass infected with these endophytes. There are other compounds, known and unknown, that should also be considered in the toxicity of endophyte-infected plants or total biological action spectrum due to endophyte infection. The known compounds include harman, norharman, and halostachine (Bond et al., 1984; Petroski et al., 1990; Porter, 1994; Powell and Petroski, 1992; Riedell et al., 1991; TePaske et al., 1993; Yates et al., 1987).

3.1. Invertebrate pest resistances

The list of invertebrate pests deterred by *Neotyphodium* and *Epichloë* infected grasses includes insects, mites, and nematodes (Table 5). Insect resistance in endophyte-infected grasses has been documented more than any other pest, and this occurs in several species of endophytes on several vastly different host species. These include approximately 45 species of insects belonging to the following families Aphididae, Chrysomelidae, Cicadidae, Curculionidae, Gryllidae, Lygaeidae, Miridae, Noctuidae, Pyralidae, Scarabaeidae, and Tenebrionidae. There is considerable evidence that specific alkaloids, especially the pyrrolpyrazine alkaloid peramine, are responsible for the toxicity observed (Ball et al., 1995; Rowan et al., 1986), although other studies suggest either synergistic or potentiating effects of ergot alkaloid ergovaline with the pyrrolizidine loline alkaloids (Siegel et al., 1990; Wilkinson et al., 2000). There is the possibility that both explanations are possible since the production of the major insect toxin, per-

Table 5
Insect and nematodes pests deterred by *Neotyphodium/Epichloë* species

Pest	Common name
<i>Abacarus hystrix</i>	Grain rust mite
<i>Agallia constricta</i>	Leafhopper
<i>Agrostis infusa</i>	Common cutworm
<i>Acheta domesticus</i>	House cricket
<i>Aphodius tasmaniae</i>	Black headed cockchafer
<i>Aploneura lentisci</i>	Gall-forming aphid
<i>Balanococcus poae</i>	Pasture mealy bug
<i>Bitylenchus dubius</i>	Nematode
<i>Bitylenchus maximus</i>	Nematode
<i>Blissus leucopterus hirtus</i>	Hairy chinch bug
<i>Chaetocnema pulicaria</i>	Corn flea beetle
<i>Costelytra zealandica</i>	Grass grub
<i>Crambus</i> spp.	Sod webworms
<i>Cyclocephala lurida</i>	Southern masked chafer
<i>Diuraphis noxia</i>	Russian wheat aphid
<i>Draeculocephala cephalae</i>	Sharpshooter
<i>Drosophila melanogaster</i>	Fruit fly
<i>Endria inimica</i>	Painted leaf hopper
<i>Exitianus exitiosus</i>	Gray lawn leafhopper
<i>Exomala orientalis</i>	Oriental beetle
<i>Graminella nigrifrons</i>	Black-faced leafhopper
<i>Heteronychus arator</i>	Black beetle
<i>Listronotus bonariensis</i>	Argentine stem weevil
<i>Locusta migratoria</i>	Locust
<i>Longidorus elongates</i>	Nematode
<i>Lygus rugulipennis</i>	European tarnished plant bug
<i>Meloidogyne marylandii</i>	Root-knot nematode
<i>Meloidogyne naasi</i>	Barley root-knot nematode
<i>Merlinius brevidens</i>	Nematode
<i>Metopolophium dirhodum</i>	Rose-grain aphid
<i>Mythimna convecta</i>	Common armyworm
<i>Oncopeltus fasciatus</i>	Large milkweed bug
<i>Ostrinia nubilalis</i>	European corn borer
<i>Oryzaephilus surinamensis</i>	Saw-toothed grain beetle
<i>Parapediasia terterrella</i>	Bluegrass webworm
<i>Parapediasia</i> spp.	Sod webworms
<i>Paratrichodorus minor</i>	Stubby-root nematode
<i>Persectantia ewingii</i>	Southern armyworm
<i>Philobota productella</i>	Pasture tunnel moth
<i>Popillia japonica</i>	Japanese beetle
<i>Pratylenchus goodeyi</i>	Nematode
<i>Pratylenchus pratensis</i>	Nematode
<i>Pratylenchus scribneri</i>	Lesion nematode
<i>Pratylenchus thornei</i>	Nematode
<i>Prosapia bicincta</i>	Two-lined spittle bug
<i>Pseudalectia unipunctata</i>	Armyworm
<i>Rhopalomyzus poae</i>	Leaf aphid
<i>Rhopalosiphum padi</i>	Bird-cherry-oat aphid
<i>Rhopalosiphum maidis</i>	Corn leaf aphid
<i>Schizaphis graminum</i>	Greenbug
<i>Sipha flava</i>	Yellow sugarcane aphid
<i>Sitobion avenae</i>	English grain aphid
<i>Sitobion fragariae</i>	Strawberry aphid
<i>Sphenophorus inaequalis</i>	Billbug
<i>Sphenophorus minimus</i>	Billbug
<i>Sphenophorus parvulus</i>	Bluegrass billbug
<i>Sphenophorus venatus vestitus</i>	Hunting billbug
<i>Spodoptera frugiperda</i>	Fall army worm
<i>Spodoptera eridania</i>	Southern armyworm
<i>Teleogryllus commodus</i>	Black field cricket
<i>Teleogryllus oceanicus</i>	Oceanic field cricket
<i>Tribolium confusum</i>	Confused flour beetle
<i>Tribolium castaneum</i>	Red flour beetle
<i>Trichodorus primitivus</i>	Nematode
<i>Trigonotylus caelestialium</i>	Rice leaf bug

amine, is not found in all *Neotyphodium/Epichloë* species but nonperamine producing endophytic species are toxic to insects. Tanaka et al. (2005) demonstrated the role of peramine in antiherbivory by the Argentine stem weevil, *Listronotus bonariensis*, in symbiota of perennial rye grass in which an endophyte gene for peramine biosynthesis was disrupted. These results leave open the possibility that ergot alkaloids play a role in antiherbivory to invertebrates since the level of these compounds in the symbiota studied were low. Recently, Panaccione et al. (2006b) reported that ergot alkaloids are not essential for reduction of the lesion nematode, *Pratylenchus scribneri*, on roots of perennial ryegrass. In contrast, feeding of *Spodoptera frugiperda* and *Agrostis ipsilon* caterpillars on endophyte-infected perennial ryegrass provides protection to them against the parasitic nematode *Steinernema carpocapsae* through a mechanism involving ergot alkaloids (Kunkel et al., 2004; Richmond et al., 2004).

Recently, it has been documented that the janthitremes, which are found in some strains of *Neotyphodium* in perennial ryegrass also contribute to insect toxicity (Tapper and Lane, 2004). This class of compounds has a varied effect on insects, it is not specific to an insect order, and the toxicities may affect specific life stages of an insect species. Toxic effects range from mild to acute and evidence of toxicity include feeding deterrence, reduced survival, high mortality, reduced development, lower weight gain, and altered feeding behavior (Tapper and Lane, 2004). These and other specific attributes and their references, as well as a list of insects unaffected by *Acremonium/Neotyphodium*-infected grasses are contained in the comprehensive reviews by Clement et al. (1994), Breen (1994), Clement et al. (2005), Lewis and Clements (1986), Popay and Rowan (1994), Rowan and Latch (1994).

One substance that occurs in endophyte-infected tall fescue is resveratrol, and we discuss it here because of the beneficial role it plays in the life of humankind and other animals. It is usually considered a phytoalexin and is found in higher concentrations of endophyte-infected tall fescue than in noninfected tall fescue (Powell and Petroski, 1992; Riedell et al., 1991). It is not known if this increase in phytoalexin accumulation is produced by both the fungal endophyte and the plant in response to infection. Resveratrol is a polyketide-derived stilbene and has considerable chemopreventive activity in humans as it is a selective inhibitor of cyclooxygenase and peroxidases activities of COX-1 and it also has antioxidant properties (Jang et al., 1997). It is also has strong antifungal properties and is considered to prolong the life of most animals and humans. It has been shown to increase the longevity of insects, thus, suggesting a positive role for insect pests, providing *in planta* feeding removed this substance from tall fescue.

3.2. Increased plant growth

Increased plant growth is another effect observed in those endophyte-infected grasses that are well studied

such as tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*), although not necessarily in response to stress. However, an increase in growth will prevent a variety of abiotic and biotic stresses, reflecting plant vigor or persistence and is considered here as an essential component to stress resistance. An increase in the rate of growth and herbage yield may be due to physiological response of the grass from an increase in endogenous levels of plant hormones, which may be an additive effect from the fungal endophyte or from an increase in the water and nutrient content. Enhanced plant growth observed in endophyte-infected grasses is attributed to either or both production of synthetic growth hormones or phytohormones, such as IAA, which has been demonstrated to accumulate *in vitro* in cultures of *N. coenophialum* (De Battista et al., 1990) and related species (Porter et al., 1985).

The occurrence of small molecular weight indole compounds such as 3-indoleacetic acid, 3-indole ethanol, and several indole glycerols (Porter et al., 1977) suggest that they may serve as synthetic growth hormones, although most of these have also been isolated only from endophytes grown *in vitro* (Porter et al., 1985, 1977).

Finally, it has been suggested that the loline alkaloids, in addition to a role in drought tolerance that is discussed below in Section 3.3, serve as allelochemicals responsible for the phenomenon of allelopathy observed in plants, particularly rosaceous species, grown in soils planted previously with endophyte-infected tall fescue (Petroski et al., 1990). The results of such a phenomenon produce a competitive edge for infected grasses, resulting in an increase in population density.

3.3. Tolerance to abiotic stressors

Enhanced drought tolerance is documented in several studies of endophyte-infected species (Arechavaleta et al., 1989; Lewis et al., 1997; Lewis, 2004; Malinowski et al., 1998a; Malinowski and Belesky, 1997; West et al., 1993; West, 1994). Endophyte-infected grasses also respond to poor soil nutrition by an increased growth rate (Arechavaleta et al., 1992; Lewis et al., 1996; Lewis, 2004; Malinowski and Belesky, 1997; Malinowski et al., 2000; Rice et al., 1990). More recently, specific fungal secondary metabolites have been implicated in growth response and drought-tolerance mechanisms, such as the production of loline alkaloids that affect osmotic potential that reduces the effects of drought stress (Bush et al., 1997). This suggests that the loline alkaloids have a dual role in grass protection, from insect pests and drought stress. There is an association of endophyte status with dehydrins, a group of intrinsically unstructured proteins abundant during late embryogenesis (Carson et al., 2004), that is associated with protection from drought and temperature stresses in several grasses, including tall fescue. Endophyte-infected grasses also show an increase in the rate and length of root growth (Richardson et al., 1990, 1993), which is certainly

expected to play a role in drought protection and or nutrient acquisition.

In addition to the above-mentioned endophyte-mediated stress protection that is associated with secondary metabolites, resistance to stresses in endophyte-infected grasses that have no known metabolic association is also observed. These include tolerance to low soil pH and low levels of soil phosphorus, favorable growth in soils under high and low mineral stresses, and relief from soil aluminum toxicity (Belesky and Fedders, 1995; Malinowski et al., 2000, 2004) as well other abiotic soil stresses (Malinowski and Belesky, 1997, 1999a Malinowski et al., 1998c, 2000, 2004). It is our contention that tolerance to these abiotic soil stresses might be related to chemical modification and as discussed below, may well be a remediating property of endophytic fungi.

3.4. Grass disease resistance

Biological control of diseases is another effect observed on grasses infected by endophytic fungi although there are mixed results. *In vitro* suppression of plant pathogens by endophytic fungi is demonstrated (Siegel and Latch, 1991; White and Cole, 1985b), which may not be the mechanism expressed under natural conditions, but it is this observation that created a potential use of endophytic fungi. Field data support the suppression of various diseases of grasses, which correlates with most of the *in vitro* studies that drive the interests in fungal endophytes. Several studies indicate that species of fungal endophytes produce degradative enzymes and antibiotics in culture that can suppress the growth of several pathogens (Siegel and Latch, 1991; White and Cole, 1985b). The association of disease resistance to specific chemical components has not been established *in planta*, and fungal disease suppression in endophyte-infected grasses is not always clear. There is resistance to *Sclerotinia homeocarpa* (dollar spot), in chewing fescue, hard fescue, and strong creeping red fescue, but there was an increase in disease incidence to *Pythium* blight in *N. coenophialum*-infected tall fescue (Clarke et al., 2006; Blank, 1992). A similar increase in symptoms from *Drechslera* spp. was observed in *N. uncinatum*-infected meadow fescue (Panka et al., 2004). On the other hand, using identical grass materials it was also shown that there was a decrease in disease from *Puccinia coronata* (Panka et al., 2004). However, these experiments were not rigorous enough to distinguish if the strain used was resistant in order to distinguish endophyte-enhanced resistance within endophyte-free cultivars. Specific chemicals, such as several indole compounds, a sesquiterpene and a diacetamide, as well as some unidentified volatile compounds (Yue et al., 2000, 2001) have been associated with *Epichloë* species for resistance to leaf spot, *Cladosporium phlei* and stem rust, *Puccinia graminis*, on infected *Phleum pratense* (Koshino et al., 1988, 1989; Yoshihara et al., 1985). Later studies confirmed mitigation of these two dis-

eases by endophytes under field conditions (Greulich et al., 1999; Welty et al., 1993).

A recently proposed mechanism of disease control by microbial endophytes in plants is induced systemic resistance (Chen et al., 1995; Kloepper and Beauchamp, 1992), which has only been partially examined in endophyte-infected grasses (Kunkel et al., 2004; Roberts et al., 1992). The reduction in the numbers of *Alternaria*, *Cladosporium*, and *Fusarium* species on leaves of endophyte-infected *Agropyron cristatum*, *Elymus cylindricus*, and *F. rubra* compared to noninfected grasses (Nan and Li, 2000) is suggestive of induced systemic resistance. Alternatively, epiphyllous mycelial nets observed in some endophyte-grass associations may play a role in defense against pathogens by niche exclusion (Moy et al., 2000).

Regardless of the mechanism of action, there are several fungal pathogens that are controlled to some level by endophyte infection: *Alternaria triticina*, *Cercospora*, *Cryphonectria parasitica* (*in vitro*), *Cladosporium phlei*, *Laetisaria fuciformis*, *Sclerotinia homeocarpa*, *Puccinia coronata*, *Rhizoctonia zea* (Bonos et al., 2005; Gwinn and Gavin, 1992; Koshino et al., 1988, 1989; Yoshihara et al., 1985; Yue et al., 2000). Additionally, barley yellow dwarf virus is significantly reduced, which is due to an indirect effect caused by reduction of virus spread by controlling the aphid vector (Mahmood et al., 1993).

4. Prospects for utilization in biological control and plant improvement

Historically, endophytic microorganisms that did not produce diseases were described well over a century ago (Guérin, 1898; Hollis, 1951; Tervet and Hollis, 1948; Vogel, 1898; Voorhees, 1934) but the impact of these and symptomless fungal endophytes on plant disease resistance and their potential use as biocontrol agents are relatively recent (Bacon et al., 1986; Chanway, 1996, 1998; Clay, 1990; Clay and Schardl, 2002; Schardl, 2001; Schardl et al., 2004; Schardl and Phillips, 1997; Sturz et al., 2000). Further,

endophytic microorganisms in general are rapidly becoming a distinct group of biocontrol agents as indicated by the recent increase in publications, which reflects an interest in their potential benefits to agriculture (Table 6) (Bacon et al., 1997; Campbell, 1989; Chanway, 1996; Clay and Schardl, 2002; Hallmann et al., 1997; Headrick and Goeden, 2001; Hemming, 1990; Paulitz and Bélanger, 2001; Scott and Schardl, 1993; Sturz et al., 2000).

We have described the intercellular space as the endophytic niche that offers a unique habitat from which biotic and abiotic stresses can be controlled. An additional characteristic of the intercellular space relates to the nature of the basic biology of the *Epichloë* and *Neotyphodium* species occupying it. The endophyte is contained and is not subject to the direct influence of the environment and will multiply within the intercellular spaces as the grass grows, thereby colonizing the plant axis according to each endophyte's potential for habitat colonization. The endophytic features are due to the reproductive mode used by each endophytic species, especially the sexual species that are both vertically and horizontally transmitted (Table 1). Extensive discussions of this characteristic of fungal endophytes are recent reviews by Schardl (2001), Schardl et al. (2004), and Clay and Schardl (2002).

The asexual species offer considerably more in terms of biocontrol agents although similar benefits indicated above can be achieved by a careful selection of sexual species. Within the stable intercellular environment there is very little likelihood for genetic modifications of a strain during the perennial growth of grasses, which indicates that the original combination should remain as introduced. Once established, the endophytic associations are long-term and are metabolically active after two or more years (Herd et al., 1997; Schmid et al., 2000). Thus, fungal endophytes will likely provide many and perhaps all mutualistic effects on a long-term basis, providing additional impetus for technological exploitations evidenced by wild populations of infected grasses as summarized in Table 6.

Table 6
Summary of endophyte mediated resistances to multiple biotic and abiotic stresses

Stress	Endophyte-mediated response	Endophyte
Drought tolerance	Increased root growth; altered stomatal behavior and osmotic adjustments, altered stomatal behavior	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Nematode resistance	Unknown	<i>Neotyphodium</i> spp.
Growth responses	Phytohormones and synthetic growth hormones, increased phosphorus and mineral uptake on, growth tolerance to low soil pH or high aluminum concentration, nitrogen efficiency	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Interspecific competition	Increased clonal growth and lateral spread; production of alleochemicals; increased seedling vigor and seed yield	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Disease resistance	Unknown	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Insect resistance	Peramine, lolines, ergot alkaloids	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Anti-herbivory of mammals	Ergot alkaloids; and lolitrems	<i>Epichloë</i> spp. and <i>Neotyphodium</i> spp.
Tolerances to heat and low light intensity	Unknown	<i>Neotyphodium</i>

Broad groups of grass species are associated with each species of *Epichloë* and *Neotyphodium*, but there are some species, including other species within the Balansieae that apparently have evolved along with grasses and have developed some degree of host specificity (Clay and Schardl, 2002; Leuchtman and Clay, 1989). Usually an individual clump of a grass species is infected with only one endophytic species, although there are exceptions. For example, *Lolium perenne* has been reported as being infected by several endophytes including *E. typhina*, *N. lolii*, and LpTG-2 (Table 3), a heteroploid originating from a hybridization of the two other species (Schardl et al., 1994). Similarly, several species have been reported to occur within one population of grasses (Christensen and Latch, 1991), which may reflect multiple infections within a tiller or multiple infections within individuals within a population. This suggests that the intercellular space of most plants is perhaps compatible with most endophytes, and may not require considerable genetic modifications to accommodate any and all endophytic species, i.e., compatibility may not be a major problem. This is particularly important since there are current attempts at modifying, qualitatively and quantitatively, the intercellular space of crop species by conventional plant breeding to facilitate the greater uses of endophytes.

The use of grass endophytes for biocontrol holds much promise for the reasons discussed herein. However there are challenges due to the complexity and numbers of grass-endophyte associations. It is clear that our current understanding limits our ability harness the advantages of these associations. There are numerous examples to illustrate this including the following. Guo et al. (1992) reported that endophyte infected tall fescue associations reduced root colonization by two species of mycorrhizal *Glomus* compared to those without endophytes. While much of the literature on this topic focuses on agronomic species the diversity of endophytes is in the wild and it is not a given that all of these associations are mutualistic. Faeth and Sullivan (2003) report that associations between Arizona fescue (*Festuca arizonica*) and *Neotyphodium* are most often parasitic and of no advantage to the plant according to their observations. Similarly, Wäli et al. (2006) reported that meadow fescue plants with *Neotyphodium* were more susceptible to snow mold disease than endophyte free types. This group also noted however that endophyte infected lines had better regrowth after winter than uninfected ones that may offset the impact of snow mold disease.

Future plans for endophytes, beyond those for plant protection, include the introduction of native and novel or transformed endophytes into grasses for a variety of uses. Of particular use are transformed endophytes with specific desirable characteristics intended for grass improvements (Murray et al., 1992; Scott and Schardl, 1993). Most of the uses of surrogate transformed grasses are for the delivery of pesticides, delivery of genes for enhanced biotic and abiotic stress resistance, accelerated seedling emergence

and subsequent plant development, increased or improved nutritional qualities and increased herbage yield. Endophytic fungi have the potential for use as vectors for transformation of useful products that can be expressed *in planta*, augmenting either the natural nutritional qualities of the food or producing value added components of food crops, as well as delivering a natural pesticide. Such surrogate transformed grasses might have greater value and public acceptability over traditional transgenic plants.

Plants have been shown to have the potential to remediate polluted soils (Salt et al., 1999), and concurrently it has been shown that endophyte-infected grasses tolerate polluted soil types (Belesky and Fedders, 1995; Malinowski et al., 1998b, 2004, 2005; Malinowski and Belesky, 1999a, 1999b). The interpretations for soil tolerance have not been determined. One suggestion that we favor is that fungal endophyte-grass symbiote have the potential for remediating polluted soils. Soil remediation by bacterial endophytes has been established (Siciliano et al., 2001). The range of uses for phytoremediation varies from reducing petroleum hydrocarbon contaminations in soils to reducing heavy metals levels in soil, including water soluble and volatile organic xenobiotics. In the instance of bacterial endophyte, a more efficient job of remediating soils is accomplished simply because plants do not degrade many pollutants completely, a similar biochemical attribute might be true for fungal endophytes. Again, transformed endophytes may be used alone or in combination with grass species with naturally high tolerance to polluted soils for a more efficient degradation of soil pollutants.

Endophyte-grass symbiote hold great promise for utilization in agriculture, turf, and remediation projects but incomplete understanding the basic biology of these relationships present challenges to their full utilization. There is ample evidence that many of these fungus-grass relationships show specificity, yet understanding of the spectrum and mechanisms of this specificity is at an early stage (for example, see: Brem and Leuchtman, 2003). New host-fungus combinations may result in plant disease, not produce the desired benefit to the plant, or simply not establish in a reliable fashion. The phenotypic expression of the symbiote is highly influenced by environmental factors as described herein and by others adding additional factors that must be considered to realize successfully targeted utilization of these relationships (for example, see: Cheplick, 2004). In addition, there likely remains much undescribed endophyte diversity as suggested by recently published papers from under-surveyed parts of the globe (Gentile et al., 2005; Li et al., 2004a, 2004b; Mirholi et al., 2006). Knowledge of this diversity is needed to realize the full potential of the grass endophytes in biocontrol and other arenas.

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