

## Update on Plant-Microbe Interactions

# Bioprotective Alkaloids of Grass-Fungal Endophyte Symbioses<sup>1</sup>

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Symbiotic interactions of C3 grasses with fungal endophytes, *Epichloë* species and their asexual relatives *Neotyphodium*, often provide the grass hosts with major fitness enhancements (for review, see Siegel and Bush [1994]). The endophytes protect host plants from both biotic and abiotic environmental stresses. Documented enhancements to host fitness include greater resistance to mammalian and insect herbivores, pathogens, and nematodes, as well as increased drought tolerance and competitiveness. Our understanding of the mechanisms responsible for all of these effects on host fitness is not well established. However, the antiherbivore fitness enhancements are largely attributable to the accumulation of four groups of alkaloids: lolines, peramine, ergot alkaloids, and lolitrems. The objective of this *Update* is to present the current status of knowledge on these alkaloids, focusing on their biological activities and the potential for genetically manipulating their expression in grass/endophyte symbiota.

### COORDINATED LIFE HISTORIES OF PLANT-ENDOPHYTE SYMBIOTA

Grass/endophyte symbiota are extremely intimate and perennial, and exhibit close matching of the host and symbiont life cycles (Fig. 1). Each symbiotum exhibits one of three possible life history strategies, resulting in: (a) pure vertical transmission of the endophyte, (b) pure horizontal transmission of the endophyte, or (c) a mixture of the two life cycles (Schardl et al., 1997). The asexual *Neotyphodium* species are restricted to pure vertical transmission, a highly efficient clonal propagation in flowering meristems, ovules, seeds, and, ultimately, progeny seedlings of infected mother plants. In contrast, the pure horizontal transmission strategy of some *Epichloë* species relies on the production of contagious sexual spores. These spores can only be produced on a fungal structure (stroma) surrounding the grass flagleaf sheath, but as soon as stroma are produced, the inflorescence of the affected tiller ceases

development (it seems likely that this is due to fungal products mimicking plant growth regulators). The resulting suppression of seed production is termed “choke disease.” The most highly pathogenic *Epichloë* species completely suppress host seed production, and, because seeds cannot be a vehicle for their dissemination, the species are obligately sexual and horizontally transmitted. Many *Epichloë* species use a third, remarkably balanced strategy of mixed transmission (both cycles in Fig. 1). These fungi choke some flowering tillers and produce sexual spores but leave other tillers (usually a majority) unaffected and fertile. Like the asexual endophytes they are transmitted in nearly all seeds produced by infected mother plants.

Symbiota involving asexual, vertically transmitted endophytes are analogous to individuals with maternally transmitted endophytes. However, host and symbiont can be separately propagated and experimentally manipulated. It is possible to (a) culture the fungi in the laboratory, (b) analyze fungal alkaloid expression under appropriate culture conditions, (c) transform the endophytes with recombinant DNA, (d) cure seeds of vertically transmitted fungi, and (e) artificially inoculate uninfected plants with compatible fungi (Murray et al., 1992; Schardl, 1994). Novel grass/endophyte symbiota can be generated by these methods and incorporated into grass breeding strategies.

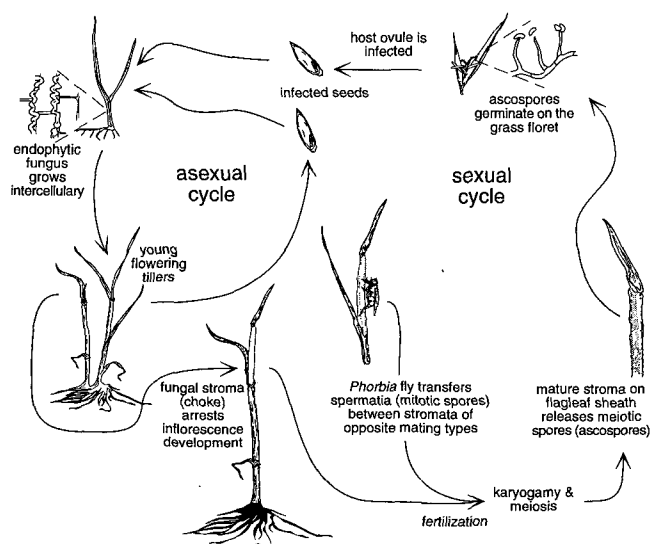
### ALKALOID STRUCTURES AND DISTRIBUTION

The bioprotective alkaloids in grass/endophyte symbiota are generally grouped as pyrrolizidines, ergot alkaloids, indole diterpenes, and pyrrolopyrazines. An example of each is shown in Figure 2. Those of the pyrrolizidine class differ from most plant pyrrolizidines in that they are 1-amino derivatives of a saturated pyrrolizidine base with an oxygen bridge between carbons 2 and 7. NFL is usually the most abundant pyrrolizidine and NAL is the second. Together with loline and other derivatives, these “lolines” are potent insecticides (Dahlman et al., 1997). The ergot alkaloids are represented by many compounds with an ergolene ring system, many of which are derivatives of lysergic acid. Ergovaline and lysergic acid amide are the predominant members of this group in most symbiota in

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Abbreviations: DMAT, 4-( $\gamma,\gamma$ -dimethylallyl)Trp; NAL, *N*-acetyllooline; NFL, *N*-formyllooline.



**Figure 1.** Alternative asexual and sexual life cycles of *Epichloë festucae* in symbiosis with *Festuca* spp.

which ergot alkaloids are found (Porter, 1995). Ergovaline is toxic to grazing mammals. The indole diterpene alkaloids include tremorgenic neurotoxins commonly known as lolitrem, of which the most abundant is lolitrem B. Lolitrem is implicated in the "ryegrass staggers" disease of sheep (Rowan and Latch, 1994). The only isolated pyrrolopyrazine alkaloid is the insect deterrent peramine.

Symbioses of different grass species with different endophyte species or genotypes express various combinations and levels of alkaloids (Table I). Among the naturally occurring symbiotes analyzed to date, a majority accumulate peramine, whereas ergot alkaloids were observed in just over 50%, lolines in approximately 35%, and lolitrem in only 10% (Siegel et al., 1990). Among the most important forage grasses, tall fescue (*Festuca arundinacea* Schreb.) in symbiosis with *Neotyphodium coenophialum* Glenn, Bacon, et Hanlin (= *Acremonium coenophialum* Morgan-Jones et Gams) accumulates lolines, ergot alkaloids, and peramine; perennial ryegrass (*Lolium perenne* L.) in symbiosis with *Neotyphodium lolii* Glenn, Bacon, et Hanlin (= *Acremonium lolii* Latch, Samuels et Christensen) accumulates lolitrem, ergot alkaloids, and peramine. When the endophytes in these two grasses are switched, the alkaloid profiles qualitatively depend on the fungal species rather than on the grass species (Table I; Siegel et al., 1990). Meadow fescue (*Festuca pratensis* Huds.) in symbiosis with *Neotyphodium uncinatum* Glenn, Bacon, et Hanlin (= *Acremonium uncinatum* Gams, Petrini et Schmidt) produces only lolines and has not been implicated in any mammalian toxicosis.

### BIOLOGICAL EFFECTS

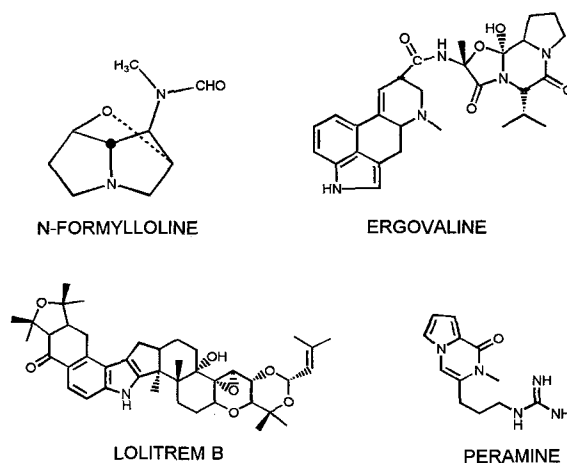
The benefits and detriments of endophytes and their alkaloids, from both the ecological and the agricultural perspective, have come to light in only the last two decades. From 1930 to 1970, millions of hectares in the temperate transition zone of the south central and southeastern

United States were converted from revegetation shrubs and weeds to pastures of tall fescue. This grass was superior forage to native flora, but farmers soon realized that livestock were less productive than would have been expected based on the measured chemical nutrition of the grass. An associated complex of symptoms episodically suffered by grazing livestock was termed "tall fescue toxicosis." These problems spurred research that revealed the fungal endophyte *N. coenophialum* (Bacon, 1988). Concurrently, work in New Zealand on ryegrass staggers led to the isolation and description of *N. lolii* (Latch et al., 1984) from perennial ryegrass. Both of these endophytes were implicated in the associated livestock toxicoses, but both also greatly enhanced fitness of their hosts under biotic and abiotic stresses. For example, peramine and perhaps the lolitrem precursor paxilline protect perennial ryegrass from the Argentine stem weevil (*Listronotus bonariensis* Kuschel), a highly destructive insect pest in New Zealand (Rowan and Latch, 1994). Thus, attempts to replace endophyte-infected with endophyte-free grasses have been limited largely because the latter have inferior environmental tolerance.

### Activities of Ergot Alkaloids

Probably the main benefit of ergot alkaloids for symbiotic grasses is activity against vertebrates, although some insecticidal activity has also been shown (for review, see Siegel and Bush [1996]). Ergot alkaloids have been implicated strongly in the observed toxicity suffered by livestock consuming endophyte-infected grasses. Many of the symptoms—reduced weight gain, elevated body temperature, restricted blood flow, reduced reproduction, and reduced milk production—mimic those known for other ergot alkaloids (Strickland et al., 1996). Ergovaline and related ergopeptines have been most closely associated with tall fescue toxicosis.

The interaction of different alkaloids to produce the observed biological effects is an obvious possibility. Recent findings that some symbiotes accumulate large quantities of other ergot alkaloids lend credence to this concept. *Ach-*



**Figure 2.** Representative compounds of each alkaloid group found in grass/endophyte symbiotes.

**Table 1.** Alkaloid profiles for a variety of grass-endophyte associations<sup>a</sup>

Host Grass	Symbiont	Alkaloids <sup>b</sup>				Association
		E	L	LM	P	
<i>F. arundinacea</i>	<i>N. coenophialum</i>	0.5	1100	0	2	Natural
<i>F. arundinacea</i>	<i>N. lolii</i>	1.2	0	23	18	Artificial
<i>L. perenne</i>	<i>N. lolii</i>	1.3	0	4.7	19	Natural
<i>L. perenne</i>	<i>N. coenophialum</i>	2.5	1000	0	29	Artificial
<i>L. perenne</i>	<i>Epichloë typhina</i>	0	0	0	53	Natural
<i>L. perenne</i>	<i>N. lolii</i> × <i>E. typhina</i> <sup>c</sup>	4.8	0	0.4	22	Natural
<i>F. pratensis</i>	<i>N. uncinatum</i>	0	5600	0	0	Natural
<i>Festuca gigantea</i>	<i>E. festucae</i>	0	300	0	4	Natural
<i>Festuca longifolia</i>	<i>E. festucae</i>	0.9	0	4.0	22	Natural
<i>Festuca rubra</i> subsp. <i>rubra</i>	<i>E. festucae</i>	1.2	0	0	0	Natural

<sup>a</sup> Approximate concentrations given in  $\mu\text{g g}^{-1}$  dry wt.

<sup>b</sup> Abbreviations: E, ergovaline; L, lolines; LM, lolitrems; P, peramine.

<sup>c</sup> A naturally occurring interspecific hybrid.

*natherum inebrians* (Hance) Keng (drunken horse grass) and its endophyte accumulated up to 2500 mg kg<sup>-1</sup> ergonovine and 400 mg kg<sup>-1</sup> lysergic acid amide, substances known to have potent pharmacological activities (Miles et al., 1996).

The modes of action of the ergot alkaloids also suggest their significance to toxicosis of large herbivores. Often measured are altered neurotransmitter metabolism and reduced prolactin and melatonin levels in blood, indicating an endocrine effect and supporting a dopaminergic mechanism for toxicity. Larson et al. (1995) demonstrated that ergovaline had high affinity for D<sub>2</sub> dopamine receptors. Also, ergovaline acted as an agonist for the vasoactive intestinal peptide, stimulating cyclic AMP production in cultured cells, and this response was mediated via the reaction with the D<sub>2</sub> dopamine receptors. Lysergic acid amides are much less active or inactive in this system.

#### Activities of Lolitrems

Ryegrass staggers is a neurotoxic disorder of livestock grazing perennial ryegrass with *N. lolii*. The causative agents have been identified as lolitrems. Structures are known for five lolitrems plus precursors in the biosynthetic pathway (Mantle and Weedon, 1994). Knowledge of their structure/activity relationships will help elucidate their interactions with target neuroreceptors. The present working hypothesis is that activity depends on a relatively planar ring structure, but if the A-ring protrudes from the planar surface onto the  $\alpha$ -face, activity is lost (Munday-Finch et al., 1996). Apparently, interference with the  $\alpha$ -face prevents effective binding to the receptor(s). What receptor(s) may be involved in the lolitrem response remains unknown. Indole diterpenes are potent inhibitors of high-conductance potassium channels, but this effect does not account for the complete symptomology of lolitrem poisoning (Knaus et al., 1994).

#### Activities of Peramine

Peramine is the only known pyrrolopyrazine alkaloid isolated from grass symbiota. Its primary activity is as a feeding deterrent to insects; it has no apparent activity against mammalian herbivores. The Argentine stem weevil

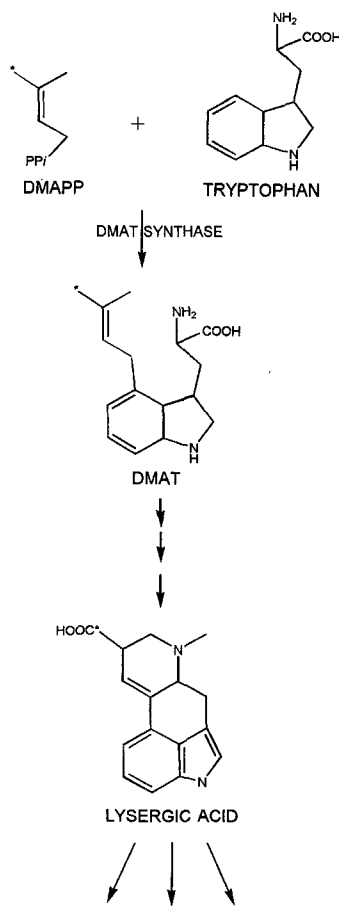
is extremely sensitive to peramine, but most other insects tested have been insensitive to this alkaloid (Rowan and Latch, 1994).

#### Activities of Lolines

Dahlman et al. (1997) have reported broad spectrum insecticidal activity of NFL as a contact and ingested substance. The naturally occurring loline derivatives with short acyl chains (1–2 carbons) and synthetic derivatives with longer acyl chains (10–14 carbons) have greater activity than the intermediate (4–9 carbons) acyl chain derivatives (Riedell et al., 1991). The LC<sub>50</sub> for NFL, NAL, and N-methyllooline is equal to nicotine sulfate for greenbug aphids (*Schizaphis graminum*). Lolines apparently act both as metabolic toxins and as feeding deterrents, depending upon the species of insect. Lolines are found in root tissue at levels that are much lower than in shoot tissue (Bush et al., 1993), but may be sufficient to help protect against some insects.

Although lolines are much less active against mammals than are ergot and indole diterpene alkaloids, activities in small mammals and mammalian tissues have been shown. Lolines may be involved in vasoconstriction and vascular thickening, and the site of action may be  $\alpha$ -2-adrenergic, D<sub>2</sub> dopamine, or serotonergic receptors (Larson et al., 1995; Strickland et al., 1996). Also, a significant immunosuppressive effect has been noted in feeding studies in mice (Dew et al., 1990). Although lolines are often highly abundant relative to other alkaloids, preliminary studies suggest that very little loline alkaloid is retained in serum following ingestion.

Some allelopathic properties that have long been associated with tall fescue may be attributable to lolines (Peters and Zam, 1981). Seed germination studies suggest that the lolines may contribute substantially to allelopathy. NFL and NAL inhibit the rate but not the amount of seed germination of several monocots and dicots (Bush et al., 1993). The amount of NFL detected in soils below tall fescue plants infected with *N. coenophialum* is four times the level required to inhibit germination of *Lolium multiflorum*.



**Figure 3.** Reaction catalyzed by DMAT synthase in ergot alkaloid biosynthesis. Products of lysergic acid include the simple acids and alcohols, amides, single amino acid derivatives, and complex ergopeptide derivatives such as ergovaline. DMAPP, Dimethylallyl diphosphate.

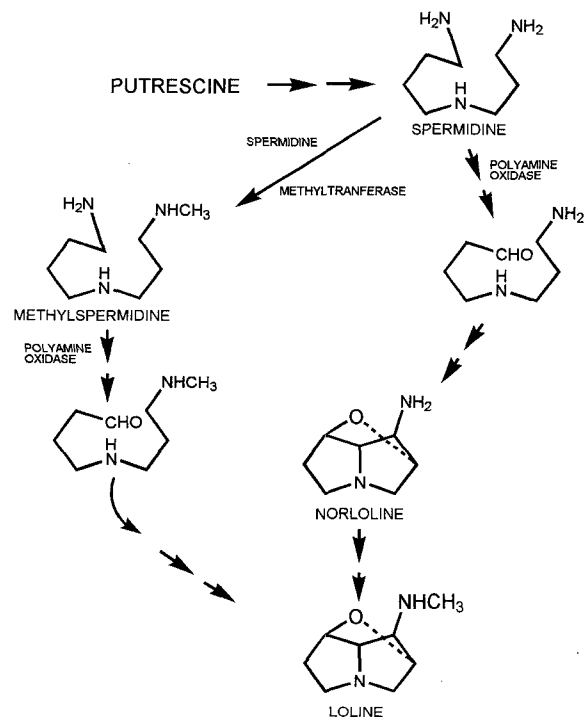
### BIOSYNTHESIS

Biosynthetic pathways for the four main groups of alkaloids found in grass/endophyte symbiots have been proposed. Biosynthesis of the ergot alkaloids is best understood because of previous research on ergot alkaloid biosynthesis in *Claviceps purpurea* (Fr.) Tul. (Reháček, 1991). The mevalonic acid derivative dimethylallyl diphosphate and Trp are the precursors for DMAT synthesis (Fig. 3). *N*-Methylation and further transformations will yield the ergolene class of alkaloids (most of the clavine alkaloids) and, subsequently, the ergolene acids, including lysergic acid and the ergolene alcohols. The simple and complex derivatives of lysergic acid are major accumulation products in several grass/endophyte symbiots. Dimethylallyl diphosphate:L-Trp dimethylallyltransferase (DMAT synthase) catalyzes the first pathway-specific and probably the limiting step in the formation of these alkaloids. This enzyme has been purified and the gene that encodes it, *dmaW*, has been cloned, sequenced, and expressed in yeast (Tsai et al., 1995). The intention is to use the cloned gene to identify the homolog(s) in *N. coenophialum*. If the *N. coenophialum* gene(s) can then be deleted, disrupted, or prevented from

being expressed (for example, by expressing antisense RNA), then little or no ergot alkaloid production is expected. This is one of several possible strategies to reduce livestock toxicosis based on the hypothesis that ergot alkaloids are the most important anti-mammalian toxins produced by *N. coenophialum* (for review, see Siegel and Bush [1996]). Another well-characterized step is the incorporation of lysergic acid into *D*-lysergyl peptide lactams, oligopeptide precursors of ergotamine, and related alkaloids. This step is carried out by a complex of two peptide synthetases recently purified from *C. purpurea* (Riederer et al., 1996). It is likely that a closely related enzyme complex is involved in ergovaline biosynthesis in *Epichloë* and *Neotyphodium* species.

Loline biosynthesis is poorly understood and the proposed biosynthetic pathway (Fig. 4; Bush et al., 1993) is based on other pyrrolizidine alkaloid biosynthetic schemes and limited direct data. Spermidine ( $\text{NH}_2\text{-}[\text{CH}_2]_4\text{-NH-}[\text{CH}_2]_3\text{-NH}_2$ ) is the likely precursor of the pyrrolizidine ring system of lolines, whereas homospermidine ( $\text{NH}_2\text{-}[\text{CH}_2]_4\text{-NH-}[\text{CH}_2]_4\text{-NH}_2$ ) may be the precursor of most plant pyrrolizidines. Enzymatic steps from spermidine are expected to include *N*-methylation, oxidative deamination, and cyclization. The order of these steps and the enzymes involved, as well as the insertion of the ether bridge, need elucidation.

Postulated biosynthetic schemes for indole diterpenes (Miles et al., 1992; Mantle and Weedon, 1994) are based on radiolabel feeding studies and identification of likely intermediates from *N. lolii* and other indole-diterpene-producing fungi such as *Penicillium paxilli*. Geranylgeranylpyrophosphate and Trp are logical precursors to paxilline via emindol



**Figure 4.** Proposed pathway for loline biosynthesis.

SB, paspaline, and dehydroxypaxilline. Intermediates in the conversion of paxilline to lolitrem B likely include  $\alpha$ -paxitriol, lolitriol, and lolitrem E.

Peramine is the simplest of the alkaloids in these symbiotes, and a scheme involving Pro, Arg, and a methyl donor is readily visualized as the biosynthetic route. Such a scheme is consistent with the possibility that a multi-domain peptide synthetase is involved.

### GENETIC ANALYSES

Because of the rich biochemical diversity and ability of endophytes to produce alkaloids, some of which have no close counterparts from plants or other fungi, it is highly desirable to apply genetic technologies to these systems. Mendelian and molecular genetic techniques have been adapted to the endophytes with the objective of identifying and manipulating genes involved in the alkaloid biosynthetic pathways. DNA-mediated transformation has been accomplished with *N. coenophialum*, *E. festucae* Leuchtmann, Schardl et Siegel, *E. typhina* (Fr.:Pers.) Tul., and a *N. lolii* × *E. typhina* hybrid from perennial ryegrass (Murray et al., 1992; Schardl, 1994). These techniques are essential for disrupting genes such as *dmaW* to ameliorate livestock toxicosis and to determine the role of alkaloids in plant fitness.

It is not only important to eliminate or reduce the alkaloids thought responsible for livestock toxicoses, but also to understand the roles and biosynthesis of the other major alkaloids such as the anti-insect lolines and peramines. A most promising strategy is genetic analysis of loline and peramine expression using a classical genetic Mendelian approach and map-based cloning of genes that control expression of the alkaloids. An appropriate species to choose for this work would be *E. festucae* (Leuchtmann et al., 1994). Within this species there is considerable variation in alkaloid profiles. Isolates of *E. festucae* that, in symbioses with their natural hosts, express each of the four alkaloid types have been found (Table I; Siegel et al., 1990).

Whether or not variation in alkaloid profiles is genetically controlled by fungus is presently under investigation. By mating strains that are of opposite mating types and also vary qualitatively in the expression of the alkaloid of interest, the pattern of segregation (alkaloid+ versus alkaloid-) in the progeny can be analyzed and the heritability of the alkaloid profiles can be determined. If the profiles are heritable, and if the genetics of expression are fairly simple, then genes controlling alkaloid expression should be amenable to map-based cloning. Because *Epichloë* species are haploid, 1:1 segregation among progeny in a parental cross and appropriate backcrosses would imply that a single locus is involved; 3:1 segregation would imply two loci, and so forth. Preliminary results suggest that a single *E. festucae* locus segregates with expression of lolines (H.H. Wilkinson, C.L. Schardl, and L.P. Bush, unpublished data), and that there is a single *E. typhina* locus controlling peramine expression (K.-R. Chung and C.L. Schardl, unpublished data). Fungal biosynthetic pathways often involve gene clusters. Therefore, by identifying one gene that seg-

regates with an alkaloid expression phenotype, there is a high probability of identifying an entire gene cluster for the biosynthetic pathway.

### EVOLUTION OF GRASS/ENDOPHYTE SYSTEMS

What is remarkable about the life history of some *Epichloë* species is their ability to transmit contagiously via sexual spores or vertically by systemic infection of developing seeds (Fig. 1). This is particularly surprising because there is a direct antagonism between these two modes of transmission. The contagious state of the fungus directly suppresses seed production and, therefore, precludes vertical transmission. However, different flowering tillers of the same plant may or may not exhibit choke disease, and those that do not will produce *Epichloë*-infected seeds. An evolutionary study suggests that this exquisite developmental balance between host and symbiont is associated with a long history of cospeciation of the symbiotic partners (Schardl et al., 1997). Conversely, cospeciation is not indicated where only vertical or only horizontal transmission occurs. In both cases quite a different evolutionary process, interspecific hybridization of the fungal symbionts, is sometimes involved. In fact, phylogenetic analysis of asexual descendants of *Epichloë* species, namely the *Neotyphodium* species, provided the first genetic documentation of fungal evolution by interspecific hybridization (Schardl et al., 1994; Tsai et al., 1994).

Asexual endophytes produce extraordinarily high levels of antiherbivore alkaloids relative to what is observed in *Epichloë*-grass symbiotes (Siegel et al., 1990). For example, judging by alkaloid levels and estimates of endophyte biomass in symbiotes (Bush et al., 1993), it is reasonable to estimate that levels of loline alkaloids produced in tall fescue and meadow fescue and of ergot alkaloids produced in *A. inebrians* approximately equal or exceed total endophyte biomass (even though these data are not all from the same species). Though surprising, this makes evolutionary sense because the endophytes and their hosts share the same diaspores (vehicles for propagation and dissemination). Thus, the *Neotyphodium* species are essentially maternally inherited components of the symbiote. The fitness of a symbiote, and thus the fitness of the grass host, directly determines the survivability of the symbiont. If the symbiont's metabolism is largely directed to products that enhance host survival (while maintaining a compatible interaction), the symbiote will be favored by natural selection.

The problem with the asexual endophyte (*Neotyphodium*) species is that they may be evolutionary dead ends (Schardl, 1996). This is because sex may be needed to purge genomes of accumulated deleterious mutations. If a plant species such as tall fescue is dependent on an asexual symbiont to confer protection and stress tolerance, then the plant species may eventually lose fitness or even go extinct with the extinction of its symbiont. However, asexual fungi can sometimes recombine genetic material in "parasexual" ways, i.e. they can fuse somatic cells (hyphae) and sometimes nuclei. Although parasexual genetic exchange is commonly between conspecific fungi, many *Neotyphodium*

species have apparently undergone hyphal fusion and nuclear fusion with *Epichloë* species (Scharidl et al., 1994; Tsai et al., 1994). Presumably, this occurs after a grass-*Neotyphodium* symbiotum becomes infected with the *Epichloë* species so that the two fungi coexist in one plant. Because the effect is an infusion of the genome from a sexual species into the asexual endophyte, this would generate new endophyte species within host plants. Endophytes of tall fescue have diversified considerably by this process (Tsai et al., 1994). Considering the diversity of their alkaloid profiles, one may speculate that hybridization directly affects the regulation of biosynthesis. In fact, the genetic diversity of hybrid endophytes in tall fescue and perennial ryegrass reflects the diversity of their alkaloid profiles (Christensen et al., 1993).

### CONCLUSIONS

Because grasses symbiotic with fungal endophytes frequently show increased fitness relative to uninfected conspecifics, these associations are important to agriculture and amenity grasses and are models for the ecology and evolution of protective symbioses. Four alkaloid classes have been identified as metabolites within these associations. The antiherbivore effects of these chemicals have been clearly demonstrated in studies correlating qualitative variation in alkaloid profiles and in studies introducing purified alkaloid extracts to herbivores. Because of their importance to medicine and agriculture, the biosynthetic pathway of the ergot alkaloids is the only one that has been elucidated thus far. Genetic analysis of alkaloid expression has been facilitated both by Mendelian analysis of the closely related sexual fungal endophytes (*Epichloë* species) and by molecular techniques such as DNA-mediated transformation. Levels of alkaloids expressed in each symbiotic association may be related in part to the coordinated grass and fungal life history strategies. For instance, relatively high levels of alkaloid expression have evolved in symbiots containing endophytes with pure vertical transmission (i.e. the asexual *Neotyphodium* species). This makes intuitive sense because these symbiots are most analogous to physiologically distinct individuals. The fungus continuously associates with that host lineage with no possibility of spread to different hosts, so selection ought to favor increased specialization and efficiency in these associations. Furthermore, because any benefit that the host receives from the fungus also benefits the endophyte directly, there is presumably less selection pressure on these asexual fungi to limit the costs of alkaloid production. With high-level production of alkaloids the cost to the endophytes must be significant. As we gain information about their biosynthesis and the capability to manipulate genetically the biosynthetic pathways, we will be able to determine to what extent the benefits of the alkaloids outweigh the costs of their production.

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