Symbiosis, 28 (2000) 291–302
Balaban, Philadelphia/Rehovot

Identification of Epiphyllous Mycelial Nets on Leaves of Grasses Infected by Clavicipitaceous Endophytes

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Received October 17, 1999; Accepted December 7, 1999

Abstract

Fungal endophytes of genus *Neotyphodium* have long been known to inhabit the internal tissues of plants without producing external reproductive structures on plants. For these endophytes, infection of the embryo within the seed has been the accepted mode of transmission. In this study, we examined several species of endophyte-infected grasses, including *Bromus setifolius*, *Festuca ovina*, *F. rubra*, and *Poa ampla*, and documented the presence of epiphyllous mycelial nets on their leaves. To evaluate whether the epiphyllous nets originated from the endophytes, we isolated the epiphyllous mycelium from the surfaces of *Poa ampla* leaves and analyzed it by DNA sequencing and microscopy. DNA sequencing demonstrated that the epiphyllous net originated from the endophyte, *Neotyphodium typhinum*. Conidial production on the epiphyllous mycelium suggests that the endophyte may have the opportunity for contagious spread to uninfected plants through epiphyllously-produced conidia. The mycelial nets produced by clavicipitaceous endophytes may also play a role in defense of host plants from potential pathogens through 'niche exclusion'.

Keywords: Clavicipitaceae, endophytes, epiphyllous, epiphytes, *Neotyphodium*

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0334-5114/2000/$05.50

1. Introduction

Endophytic fungi have been shown to be relatively common in plants (Bacon and Hill, 1985). They range from latent pathogens unable to incite disease in hosts to biotrophic mutualists that provide benefits to host plants. Some well-known and commonly studied biotrophic endophytes are classified in the genera *Epichloë* (Fr.) Tul. (anamorph *Neotyphodium* Glenn, Bacon & Hanlin) and *Balansia* Speg. (Clavicipitaceae of the family Ascomycetes). Endosymbionts of these groups have been shown to increase insect and disease resistance and drought tolerance of plants (Clay et al., 1993; West, 1994; West et al., 1990; Clarke et al., in press).

Neotyphodium endophytes grow intercellularly in leaf sheaths, culms, and seeds of host grasses. They are known to perennate through successive generations of the host by infection of the embryo in the seed (White, 1988). A small number of such endophytes have been found to produce external reproductive stromata bearing perithecia on culms of hosts (Pfender and Alderman, 1999). These endophytes are members of the teleomorphic genus *Epichloë*. When grasses begin to flower, these endophytes proliferate within inflorescence primordia. Mycelia enclose and trap both the primordia and the surrounding leaf, eventually differentiating into the stroma. The development of the stroma on plants is often referred to as 'choke disease'. Initially, conidia form on the surface of stromata. Eventually perithecia develop and upon their maturation, release ascospores.

Most *Neotyphodium* endophytes have never been shown to produce stromata on host plants. It is generally believed that these endophytes are "trapped" within the tissues of host plants, unable to exit plant tissues, and therefore unable to reproduce or spread to neighboring uninfected hosts (White, 1988; Schardl, 1992; Tsai et al., 1994). A recent study of the surfaces of leaves of endophyte-infected grasses, including *Poa rigidifolia* Steudel from South America and *Agrostis hiemalis* (Walt.) B.S.P. from North America, demonstrated the presence of an epiphyllous network of mycelium on leaves (White et al., 1996). The study reported in this paper further examines the epiphyllous 'nets' on leaves of several grasses. Through the use of DNA sequencing and fungal isolation from mycelium on leaf surfaces of big bluegrass (*Poa ampla* Merrill), we unequivocally confirmed that both the epiphyllous nets and the endophytic mycelium belong to the genus *Neotyphodium*.

2. Materials and Methods

Plant materials

Neotyphodium-infected plants of *Bromus setifolius* Presl. were collected in

the Andes Mountains in Mendoza, Argentina; tall fescue (*Festuca arundinacea* Schreb.) was represented by cultivar KY-31 collected in New Jersey; blue fescue (*Festuca ovina* var. *glauca* (Lam.) Koch.) was collected in Iceland; Chewings fescue (*F. rubra* var. *commutata* Gaudin) and creeping red fescue (*F. rubra* var. *rubra* L.) were obtained from sites in England; perennial ryegrass (*Lolium perenne* L.) was obtained from various sites in Poland; big bluegrass (*P. ampla*) was obtained from sites along the Alaska highway in Yukon, Canada. All plants were maintained in the Rutgers University Research Greenhouse Facility, in New Brunswick, New Jersey.

Characterization of mycelial nets by microscopy

To visualize the epiphyllous mycelial nets on grasses, leaves were soaked in aqueous aniline blue (0.1%) for 5 minutes, washed with deionized H_2O, and air-dried for 10 minutes. White adhesive labels (MICROTUBE TOUGH-TAGS™, USA Scientific, Inc., Ocala, Florida) were applied to leaves and then removed and examined microscopically for presence of mycelium. The density (number of hyphae/ linear 10 μm) of the mycelium was determined on a video monitor.

Leaf tissue was fixed in 3% glutaraldehyde in cacodylate buffer (pH 7.2) and post-fixed with 1% osmium tetroxide followed by ethanol dehydration. Tissue was embedded in Quetol 651-NSA (Electron Microscopy Sciences, Fort Washington, Pennsylvania), stained in aqueous aniline blue (0.1%) and toluidine blue (0.1%) (Bacon and White, 1994).

Separation of fungal mycelium from phylloplane

The epiphyllous mycelium on *P. ampla* leaves was removed from leaves using Tissue Prep 2 paraffin wax (Fisher Scientific Inc., Pittsburgh, Pennsylvania). Leaves were cut from endophyte-infected plants and dipped in liquid wax (55°C) for 10 seconds. Precautions were taken to avoid dipping the razor cut ends of the leaves in the wax. The wax-coated leaves were cooled in a freezer for 20 seconds. The strips of fungus-containing wax were then peeled off the leaves and (1) grown on 1/2 strength potato dextrose agar (PDA) media (Difco Laboratories, Detroit, Michigan) over a span of 3 weeks (25°C) for growth and visual identification of the epiphyte or (2) used directly in DNA extraction for sequence identification of the epiphyte. Effectiveness of epiphyte removal by paraffin wax was determined by examining the wax peels of aniline blue-stained leaves under a compound light microscope.

The endophytic mycelium, *Neotyphodium typhinum*, was isolated from P. ampla using the surface sterilization method described by Bacon and White (1994). The surface sterilized leaf sheaths were placed on 1/2 strength potato dextrose agar (PDA) media and grown at 25°C.

Identification of epiphyllous mycelium

The wax peels made from *P. ampla* leaves were placed on PDA plates and incubated at 25°C for three weeks. As organisms developed on the PDA, they were sub-cultured onto new PDA plates. All cultures were identified to genus or lower on the basis of their morphological features. The rDNA sequences (obtained from GenBank) of each isolated fungus, or a congeneric representative, were then compared to the rDNA sequence of the known *P. ampla* endophyte, *N. typhinum* (An et al., 1992; GenBank accession # L07134) using the Gap alignment program on the Genetics Computer Group (GCG) software package (Madison, Wisconsin).

Sequence identification of epiphyllous mycelium

The isolate from endophytic mycelia of *P. ampla* was grown on cellophane discs (Flexel Corp., Covington, Indiana) on the surface of PDA. The DNA from the mycelium was subsequently extracted using the Invitrogen Easy DNA kit (Invitrogen Corp., Carlsbad, California). The DNA from epiphyllous mycelia on *P. ampla* was obtained by extracting the wax peels containing epiphyllous mycelia using the Qiagen Tissue Kit (Qiagen Inc., Santa Clarita, California).

To evaluate whether the *P. ampla* endophyte and the epiphyllous mycelium were identical, the genomic DNA extracted from both sources were PCR amplified with two sets of primers designed to amplify a 315 bp and a 284 bp region of the *N. typhinum* protease gene (Reddy et al., 1996), using the PRIME program on the Genetics Computer Group (GCG) software package. The sequences of the primers were: primer 1, 5'-ATC CTG ATG TAT GCC GAT CC – 3'; primer 2, 5' – TGA GTT GTG GAA AAA AAG GGA C – 3'; primer 3, 5' – AAT TCC ACC GTG ATA CCG – 3'; primer 4, 5' – TCA GGC TCA ATG TAG TCA AC – 3'). One µl of undiluted genomic endophytic or epiphyllous fungal DNA was amplified in a 50 µl reaction using the primers described above. Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmoles each dNTP, 25 pmole of each primer, 2 U Taq polymerase. PCR was carried out in a GeneAmp 9600 thermocycler (Perkin Elmer Corp., Foster City, California). Thermal cycling parameters for amplification consisted of an initial denaturation of 95°C for 1 min, followed by 32 cycles with a denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR amplification of DNA from endophytic cultures and epiphyllous mycelium was also performed with the universal rDNA spacer sequence primers ITS4 and ITS5 (White et al., 1990) using the same reaction conditions described above. Thermal cycling parameters for amplification consisted of an initial denaturation of 95°C for 2 min, followed by 30 cycles with a

denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min.

PCR fragments amplified in the above reactions were resolved by electrophoresis in a 2% agarose gel using 1X TAE buffer. Successful PCR reactions resulting in single bands of expected sizes were cut out and cleaned with GeneClean (Bio 101 Inc., Vista, California). Cycle sequencing reactions were performed using an Amplitaq FS Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc Norwalk, Connecticut). Reactions were run on a 373A DNA sequencer (Applied Biosystems, Inc. Norwalk, Connecticut) following removal of unincorporated dye. Sequences were compared to the GenBank database. Alignment of sequences and adjustments to computer aligned sequences were accomplished using AutoAssembler 1.3.0 (Applied Biosystems, Inc., Norwalk, Connecticut).

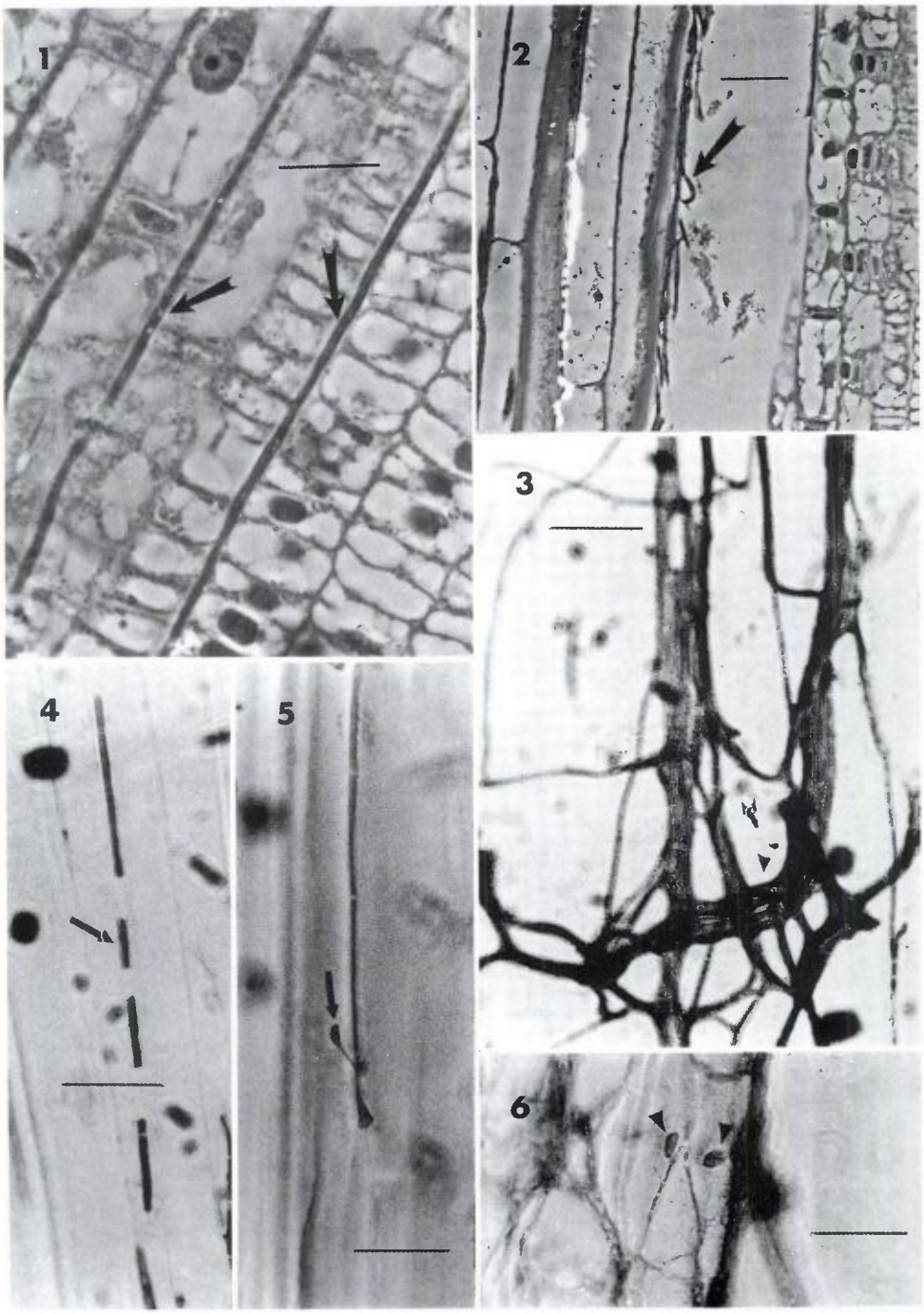
3. Results

Description of epiphyllous nets

Sections of developing leaves of endophyte-infected *P. ampla* revealed the presence of abundant, occasionally branching hyphae in the intercellular spaces of leaf mesophyll cells (Fig. 1). Hyphae were also abundant on the surfaces of immature leaves (Fig. 2). The peels of mature *P. ampla* leaves showed that the epiphyllous mycelium formed an interconnecting or overlapping network (Fig. 3). Hyphal density averaged 1.77±0.6 hyphae/ linear 10 μm on leaf surfaces. On the surfaces of young *Bromus setifolius* leaves, disconnected hyphal fragments were observed (Figs. 4 and 5). Conidiogenous cells and conidia were sometimes observed on the epiphyllous mycelial network of both *P. ampla* and *B. setifolius* (Figs. 5 and 6). Epiphyllous mycelium was also observed on endophyte-infected plants of *Festuca ovina* var. *glauca*, and *F. rubra* var. *commutata* and var. *rubra*. Where epiphyllous mycelium was observed, mycelial density ranged from 0.34 to 1.99 hyphae/ linear 10 μm (Table 1). Endophyte-infected *F. arundinacea* and *Lolium perenne*, and endophyte-free *P. ampla* were devoid of any epiphyllous mycelium.

Identification of epiphyllous fungi on P. ampla

Microscopic examination of the morphological features of all colonies that grew off of the *P. ampla* wax peels on PDA revealed that several different species of fungi and bacteria were present on the leaf surfaces of this plant (Table 2). Isolates from wax peels included *Penicillium* sp., *Acremonium implicatum*, *Cladosporium cladosporioides*, *Verticillium* sp., *N. typhinum*, and



Figures 1-6. Hyphae or conidia on leaves of *P. ampla* or *B. setifolius*. Scale bars = 10 μm.

bacteria. Isolation frequencies from the wax peels were 20.8%, 13.2%, 20.8%, 9.4%, 18.9%, and 3.7%, respectively. The rDNA sequences of each of these epiphyllous fungi were obtained from the GenBank nucleotide sequence database and compared to the rDNA sequence of the known *P. ampla* endophyte, *N. typhinum*. With the exception of the epiphyllous *N. typhinum*, all of the isolated fungal species were less than 81% similar to the rDNA sequence of the endophyte (Table 2).

Identification of epiphyllous mycelium on P. ampla by sequence analysis

PCR amplification and sequencing of *P. ampla* epiphyllous DNA with endophyte specific primers, gave sequences that directly corresponded to the endophyte. Primers for two different regions in the *N. typhinum* protease gene gave single sequences that exactly matched (100%) the published *N. typhinum* protease sequence (Reddy et al., 1996). The universal rDNA primers ITS4 and ITS5 also gave a single sequence that matched the published ITS sequence for N. typhinum (99.9%) with only a one bp difference. The protease and ITS sequences obtained from the epiphyllous mycelium on *P. ampla* were also found to be exact matches (100%) of the sequences obtained from our endophytic isolates. Because only single, clean sequences corresponding to *N. typhinum* were obtained from the DNA isolated from the epiphyllous wax peels, the existence of any of the other *P. ampla* surface fungi (see Table 1) was never revealed.

4. Discussion

Identification of epiphyllous mycelium

Amplification of the protease and ITS rDNA sequences of the epiphyllous and endophytic mycelium on *P. ampla* yielded identical sequences corresponding to the endophyte. This demonstrated that the endophyte,

Figure 1. Hyphae (arrows) present in the *P. ampla* intercellular spaces of leaf mesophyll tissue.
Figure 2. Hyphae (arrow) present on the surfaces on immature *P. ampla* leaves.
Figure 3. A network of interconnecting and overlapping epiphyllous mycelium (arrow) present on the surfaces of mature *P. ampla* leaves.
Figure 4. Disconnected hyphal fragments (arrow) present on the surfaces of immature *B. setifolius* leaves.
Figure 5. Conidia and condiogenous cells (arrow) on the surfaces of *B. setifolius* leaves.
Figure 6. Conidia and condiogenous cells (arrows) on the surfaces of *P. ampla* leaves.

Table 1. Epiphyllous hyphal densities of endophyte-infected grasses

Grass species	Number of plants examined	Endophyte species	Epiphyllous hyphal density (hyphae/ linear 10 μm)[1]
Bromus setifolius	4	*Neotyphodium typhinum*	0.34±0.22
Festuca arundinacea	4	*N. coenophialum*	0
F. ovina var. *glauca*	4	*Epichloë festucae*	0.82±0.73
F. rubra var. *commutata*	5	*E. festucae*	1.01±0.43
F. rubra var. *rubra*	5	*E. festucae*	1.99±1.00
Lolium perenne	10	*N. lolii*	0
P. ampla	5	*N. typhinum*	1.77±0.06

[1]Hyphal density reported as mean ± standard deviation.

Table 2. Fungi and bacteria isolated from *P. ampla* wax peels: isolation frequencies and comparisons to the endophyte rDNA sequence[1]

Epiphyllous isolate	Isolation frequency (%)	Similarity to endophyte sequence[2] (%)	GenBank ITS accession number
Penicillium sp.	20.8	68.5, 68.7, 68.7	AF034857, AF034450, AF034449
Acremonium implicatum	13.2	77.4, 76.9	AF081444, U57671
Cladosporium cladosporioides	20.8	70.9	AJ244241
Verticillium sp.	9.4	80.4	AF110529
Neotyphodium typhinum	18.9	99.9	L07134
Bacteria	3.7	na	na

[1]Comparisons were made among rDNA sequences of each of the isolates to *N. typhinum*. Each of the rDNA sequences contained ITS 1, 5.8s, and ITS2 regions. [2]For similarity to *Penicillium*, the sequences of *P. chrysogenum* was used; for similarity to *Verticillium* sp., the sequence of *V. balanoides* was used.

Neotyphodium typhinum, could form a stable external mycelial network in addition to an internal network. The use of universal PCR primers for the rDNA ITS region would have revealed presence of other fungal species if they were major components of the epiphyllous mycelial network. Since we were only able to amplify the ITS sequence of *N. typhinum*, it is reasonable to conclude that *N. typhinum*'s ITS sequence is the predominant ITS moiety, and

more than likely it is that species that produced the epiphyllous mycelial network.

Because we were able to isolate *N. typhinum* from some of the wax peels, it is evident that the epiphyllous mycelium or conidia were viable and thus may represent a living and growing stage of development for the fungus. However, because we were able to isolate several other fungi from the wax peels, it was not clear how much of the epiphyllous mycelium was contaminating leaf surface fungi. Data that impact on this question include the fact that endophyte-free plants were found to be free of the epiphyllous mycelial network. This circumstantial evidence suggests that it is the endophyte that is responsible for production of the epiphyllous network of mycelium rather than other fungi landing on leaf surfaces and growing on the phylloplane. It is notable in this respect that some of the fungi (e.g. *Cladosporium* spp.) that we encountered on leaves are well-known phylloplane inhabitants and have not been reported to establish epiphyllous mycelial networks (Webster and Dix, 1960) comparable to what we observed. Some fungi isolated from leaf surfaces (e.g. *Penicillium* spp.) may occur there merely as dormant spores on the phylloplane. *Penicillium* spp. are often saprophytes, and in some cases pathogens, but are not frequently reported as inhabitants of phylloplanes. Species of *Acremonium* and *Verticillium* have been reported as endophytes of plants and might be expected to grow on the phylloplanes of plants as well (Bacon and Hill, 1985). However, histological studies are needed to provide evidence for the presence of an epiphyllous stage in these species.

Differences in density of epiphyllous mycelium among grasses

We were unable to find epiphyllous mycelium on leaves of *Neotyphodium*-infected tall fescue and perennial ryegrass. It may be that some species, such as these, never or infrequently produce epiphyllous stages or that epiphyllous stages in these plants may be relegated to specific stages in development of plants or to specific seasons of the year. It is noteworthy that grass species were seen to differ in the amount of epiphyllous mycelium on leaves. For example, the leaves of *P. ampla* had 5 to 6 times the mycelial density on leaf surfaces than was measured on leaves of *B. setifolius* (Table 1).

Potential for contagious spread of endophytes

It is commonly believed that Clavicipitaceous endophytes are confined to host plants unless they form stromata on culms of grasses. Experiments involving ascospores from stromata have suggested the capacity of this stage to infect ovaries of uninfected plants (Chung and Schardl, 1997). However, many

Neotyphodium endophytes do not produce stromata. Consequently, it has been suggested that *Neotyphodium* endophytes are trapped within plants. Without the capability of spreading from one plant to another, they have had to rely on seed transmission through successive generations of the host (White, 1988; Chung and Schardl, 1997). The production of conidia on leaf surfaces (Figs. 5 and 6, arrows) suggests the possibility that some endophytes may have the ability to spread even though they do not form stromata. However, experimental evidence of horizontal spread of endophytes involving epiphyllously-produced conidia is at present lacking. Based on years of field observations on pastures of tall fescue and perennial ryegrass, it is frequently suggested that lateral transmission occurs only infrequently or not at all (Siegel et al., 1987). Our data suggest that tall fescue and perennial ryegrass do not show an epiphyllous stage and thus may not be representative of all *Neotyphodium* endophytes. Dissemination studies of *Neotyphodium*-infected grasses that possess a well-developed epiphyllous stage are needed to evaluate whether such endophytes may spread using conidia produced in the phylloplanes.

Epiphyllous mycelia as 'defensive nets'

Several endophytes and epibionts of the family Clavicipitaceae have been shown to increase resistance of infected hosts to infection by fungal leaf pathogens. Stovall and Clay (1991) found that plants of purple nutsedge infected by the epibiotic fungus *Balansia cyperi* had increased resistance to infection by *Alternaria* and *Rhizoctonia* species. Clarke et al. (in press) similarly demonstrated increased resistance to 'dollarspot' disease caused by fungus *Sclerotinia homeocarpa* in fine fescues when plants were infected by *Epichloë festucae*. *Epichloë*-infected plants of the grass *Phleum pratense* were shown to be more resistant to *Cladosporium* 'eyespot' caused by *Cladosporium phlei* than plants without the endophyte (Tajimi, 1990). In every study of enhanced resistance to fungi, an explanation for increased fungus disease resistance has been proposed to involve antifungal compounds produced by the fungal symbiont. An alternate mechanism for increased resistance to leaf diseases may involve niche exclusion. Many leaf pathogens must first colonize the surfaces of leaves before penetrating leaves to cause disease. Epiphyllous mycelia of species of Clavicipitaceae may form a 'defensive net' on leaves that prevents initial colonization of leaf surfaces from occurring. The superficial network of mycelium on leaves may occupy spaces and absorb nutrients. Thus, potential pathogens cannot then obtain these nutrients, effectively excluding them from the phylloplane. It is interesting to speculate that one or some of the compounds that endophytes produce, including indole derivatives such as

terpenoids, and alkaloids, may act to modify the surface of leaves to effectively camouflage them from potential leaf pathogens. Alternatively these compounds could act directly on spores of potential pathogens to inhibit their germination or growth. Further research is warranted to evaluate the 'defensive net' concept.

Defensive mutualism

Clay (1988) proposed that the relationship between *Neotyphodium* and its grass host is a 'defensive mutualism'. Here the fungus defends its plant host through the production of alkaloids or other chemicals that deter feeding by insects and perhaps other predators of plants in return for nutrients and protection within tissues of the host. Observations that increased fungus disease resistance may result from endophyte infection are consistent with the 'defensive mutualism' hypothesis. However, our proposed mechanism alters the concept slightly; defense from fungal predators of leaves may not simply be the result of defensive compounds produced by endophytes but instead may stem from niche exclusion.

Acknowledgements

This research was supported by a grant from the National Science Foundation (DEB-96-96041) and the Rutgers Center for Turfgrass Research.

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