

## No Evidence of Fungal Endophytes in Native and Exotic *Phragmites australis*

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**Abstract** - Native and exotic haplotypes of *Phragmites australis* show differential susceptibility to herbivores, but the mechanisms behind these differences are not known. Endophytic fungi are common in the grass family and confer resistance against insects through the production of toxic chemicals. We used both a common endophyte-staining technique and interference contrast microscopy to analyze leaf sheaths of native and exotic *P. australis* haplotypes from several populations in the northeastern United States to determine if the various haplotypes were infected with endophytes. No endophytes were found in any of the native and exotic haplotypes using procedures that consistently detected endophytes in infected rye grass.

### Introduction

Endophytic fungi are symbionts that grow in the tissues of higher plants and confer chemical resistance against herbivores (Carroll 1988). Endophytes infect most grass subfamilies and tribes (Clay 1989), and are most abundant in the subfamily Pooideae (Schardl 1996). *Phragmites australis* (Cav.) Trin. ex. Steudel, is a cosmopolitan perennial grass in the subfamily Arundinoideae (tribe Arundinae). The subspecies *P. australis* subsp. *americanus* Saltonstall, P.M. Peterson & Soreng, is native to North America (Saltonstall et al. 2004) and consists of 11 haplotypes of varying distribution throughout the continent (Saltonstall 2002, 2003). A haplotype is a group of alleles on a single chromosome that are inherited as a unit; they are useful as genetic markers. A non-native, European haplotype and one haplotype of unknown origin are also present in North America (Saltonstall 2002). Insect attack varies among the haplotypes, with 26 insect species attacking the European haplotype (type M) (Tewksbury et al. 2002), and only nine insect species attacking the most common native haplotype (type E) (B. Blossey, Cornell University, Ithaca, NY, pers. comm.). In this study, we analyzed native and non-native *P. australis* plants for the presence of endophytes.

The endophytes that infect grasses are members of the Family Clavicipitaceae (Ascomycetes) (Diehl 1950). They grow systemically throughout the intercellular spaces of aerial grass tissues and reproductive structures (Clay 1989, White 1987) and reach highest densities in leaf bases and sheaths (Clay 1990). Endophyte-infected (E+) grasses show no negative effects from infection (Siegel 1989), but alkaloid production by endophytes can confer resistance to herbivores (Clay 1988, 1990). Ergot alkaloids

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produced by endophytes in grasses prevent feeding by *Spodoptera frugiperda* (J.E. Smith) (fall armyworm; Clay and Cheplick 1989). Loline alkaloids produced by endophytes in *Festuca arundinacea* Schreb. (tall fescue) and *Lolium perenne* L. (perennial ryegrass) have antibiotic (Eichenseer et al. 1991) and antifeedant effects on aphids (Breen 1993, Omacini et al. 2001), *Popillia japonica* Newman (Japanese beetle) grubs, and *Cyclocephala lurida* Bland (southern masked chafer) grubs (Potter et al. 1992).

In this study, we investigated the occurrence of endophytic fungi in *P. australis* in an effort to determine the mechanism behind differences in herbivore susceptibility among native and non-native plants. Since exotic *P. australis* has a greater herbivore population than native *P. australis*, we tested the hypothesis that native populations of *P. australis* have endophytic fungi present in their tissues, and the exotic haplotype has either lower levels or no endophytes present in its tissues.

### Methods

Rhizomes from five *P. australis* populations were collected from the northeastern US and grown in a glasshouse under standardized conditions (Table 1). These populations were previously analyzed for haplotype by Saltonstall (2002) and were either native haplotype E or non-native haplotype M. Rhizomes 20 cm in length ( $\approx$  4–5 nodes) were planted 18 cm deep in a 50:50 (v/v) sand: peat mix. Soil was saturated daily throughout the experiment. After shoot emergence, plants were fertilized with Osmocote™ time-released (15N:12P:10K) fertilizer with micronutrients. Endophyte-infected (E+) *Lolium perenne* L. (perennial ryegrass) (30% perennial rye grass, 30% *Festuca rubra* spp. *commutata* Gaud. [chewing fescue], 30% *Festuca arundinacea* Schreber [tall type fescue], 10% *Poa pratensis* L.[blue grass]; endophyte infection in this grass mix is transferred through seed; Allen's Seed Store, Inc., Kingston, RI) was grown under the same conditions and used as a control.

Grass seeds and leaf sheaths are commonly used in analyses for *Acremonium* endophytes (Hignight et al. 1993, Saha et al. 1988). In this study, we used only leaf sheaths because no seeds were found in the glumes of inflorescences at the time of rhizome collection (late winter 2002). In turf grasses, the inner epidermis of leaf sheaths can be peeled off, allowing staining and visualization of endophytes in this transparent, single-celled

Table 1. Haplotypes used in endophyte analysis, place of origin of each haplotype, and sites of haplotype collection.

Collection site	Latitude	Longitude	Haplotype	Origin
Holt Research Forest, Arrowsic, ME	69°46'15"	43°52'33"	E	Native
Galilee Salt Marsh, RI	71°30'11"	41°22'47"	M	Introduced
Schoolhouse Pond, Charlestown, RI	71°29'30"	41°36'49"	M	Introduced
Montezuma National Wildlife Refuge, NY	76°44'25"	42°57'55"	E	Native
Montezuma National Wildlife Refuge, NY	76°44'26"	42°57'55"	M	Introduced

layer (Saha et al. 1988). No clearing procedure is needed. However, the inner epidermis in the leaf sheaths of *P. australis* could not be peeled without damaging the tissue or removing many tissue layers. We used a turf-grass clearing technique (Hignight et al. 1993) to facilitate removal of the epidermal layer.

Individual *P. australis* stems were harvested 4–6 weeks after planting, and four leaf sheaths were removed from each of three plants from each population ( $n = 12$  samples for each location, 24 leaves for the native haplotype and 36 leaves for the exotic haplotype). One leaf sheath was removed from 12 E+ perennial ryegrass stems (other grass species originating from the seed blend were not used). Leaf sheaths of *P. australis* and perennial rye grass were placed in Carnoy's solution (6:3:1 ethyl alcohol: chloroform: 85% glacial acetic acid) for 24 h to clear chlorophyll from tissues, and then placed in 70% ethyl alcohol for 24 h to facilitate chlorophyll removal (Hignight et al. 1993). We then used a scalpel to slice the inner epidermis from the leaf sheath. Three-cm sections of epidermis were placed on a slide, and one drop of tryptan blue stain solution (500 ml glycerol, 450 ml dd. H<sub>2</sub>O, 50 ml HCl, 0.05 g tryptan blue) was applied. Tryptan blue is commonly used for staining endophytes (Hill 1999 and references therein), and mycorrhizal fungi (R. Koske, University of Rhode Island, Kingston, RI, pers. comm.). The sections were stained for 5 min., a coverslip was then added, and excess stain blotted off. The procedure was repeated using endophytic perennial ryegrass as a control. All stained sections were examined for the presence of endophytes at 100x and 400x. Additional samples of leaf sheaths from *P. australis* and E+ perennial rye grass ( $n = 12$  samples for each population, 12 rye grass leaf sheaths) were cleared of chlorophyll as above, but not stained. These samples were observed under an Olympus BX-50 compound scope equipped with Nomarski differential interference contrast.

## Results

No endophytes were found in either of the haplotypes of *P. australis*. All sections appeared to be thoroughly stained and cell walls and nuclei were visible by either the staining or interference-contrast microscopy procedures (Figs. 1 and 2). Although clearing in Carnoy's solution and ethyl alcohol aided in the removal of the single-layer inner epidermis, it was difficult to remove only one layer of cells, and often no fewer than two layers could be removed. However, both cell layers could be easily distinguished. The morphology and arrangement of the leaf-sheath cells of the two haplotypes were different from that of the perennial rye grass (Figs. 1, 2, and 3).

Although the single-layer inner epidermis of the E+ perennial ryegrass was easily removed without clearing in Carnoy's solution and ethyl alcohol, we analyzed samples using this technique to standardize the procedure for all plants. Endophytes were abundant in every leaf-sheath epidermis examined in the E+ perennial ryegrass using both the staining and microscopy

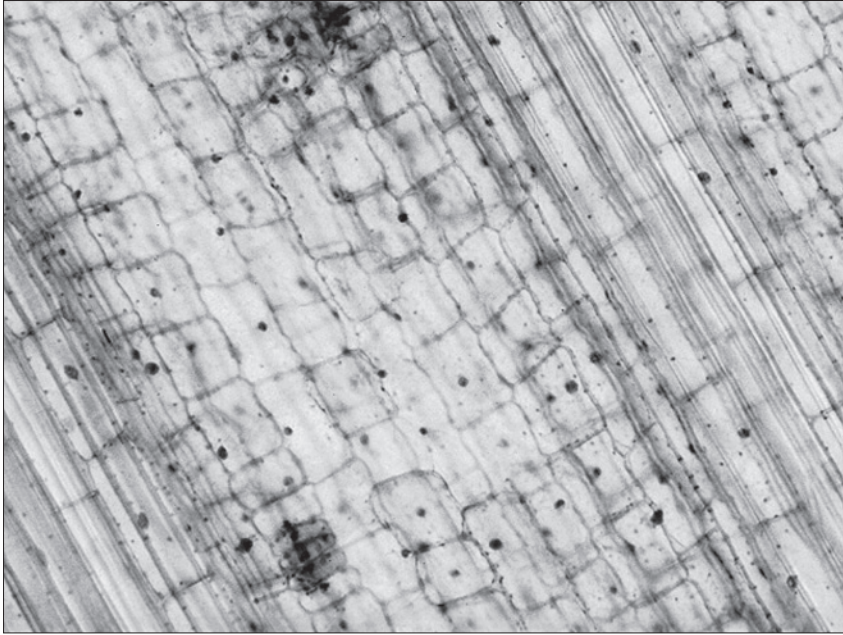


Figure 1. Inner leaf-sheath epidermis of non-native (type M) *P. australis* using Nomarsky interference microscopy. Notice the visibly stained cell walls and nuclei, suggesting that any endophytes present in this tissue would have been stained (200x).

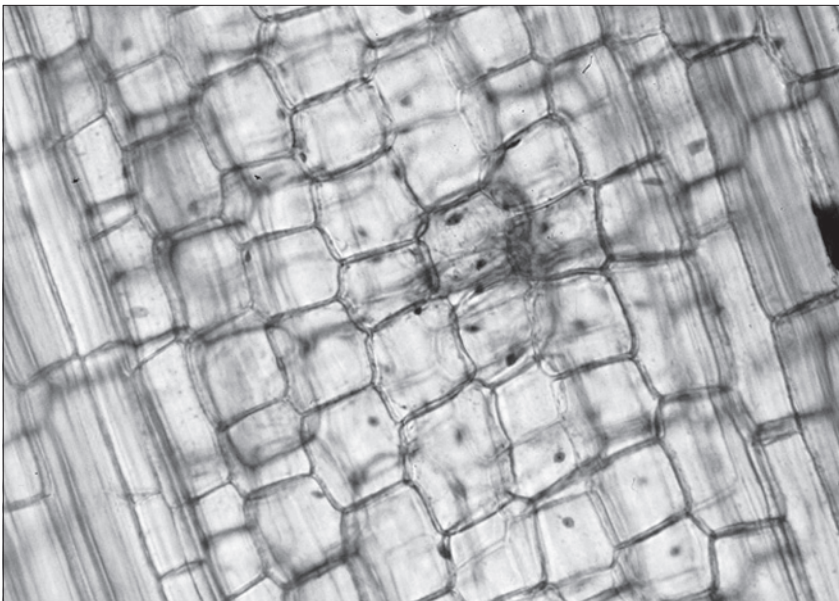


Figure 2. Inner leaf-sheath epidermis of native (type E) *P. australis* using Nomarsky interference microscopy. Notice the visibly stained cell walls and nuclei, suggesting that any endophytes present in this tissue would have been stained (200x).

procedures (Fig. 3). However, endophytes were more visible using Nomarsky interference contrast. Endophytes were present in the intercellular spaces growing as elongate, convoluted, unbranched hyphae. Infection rate averaged  $5.2 \pm 1.1$  (mean  $\pm$  SE) individual hyphae per 2–3 cm section.

### Discussion

We found no visual evidence of endophyte infection in *P. australis* leaf sheaths, suggesting that endophyte infection is not the reason for differences in herbivore populations observed on native and exotic *P. australis*. This is in contrast to other studies that have found potentially endophytic fungi in *P. australis*. Using morphological and molecular methods, Wirsel et al. (2001) found 12 genera of fungi inhabiting roots, stems, and leaves of *P. australis* in Germany. Only fungi externally culturable on agar plates were studied, and the type of plant-endophyte relationship (mutualistic or pathogenic) was not identified. Ernst et al. (2003) identified several species of *Stagonospora* fungi infecting *P. australis*, and enhancing biomass production. *Stagonospora* spp. are vectored through seed, but Ernst et al. (2003) also found that the fungi are present in soils and able to penetrate *P. australis* roots, potentially reaching and infecting developing seeds late in

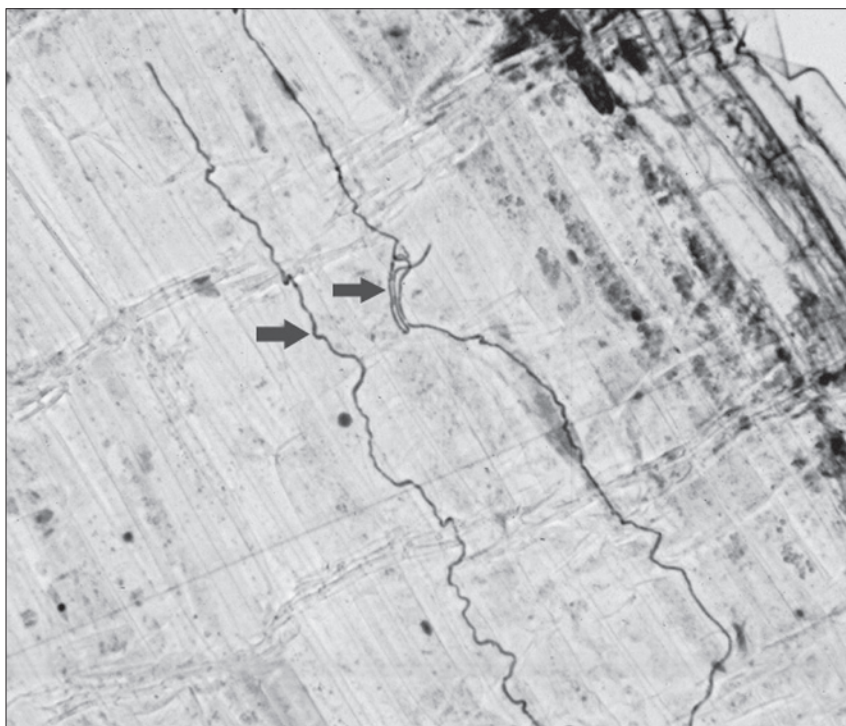


Figure 3. Perennial rye grass endophytic fungus (arrows) illuminated using Nomarsky interference microscopy (200x).

the growing season. It is possible that non-native *P. australis* populations in our collection areas were founded by propagules originating from uninfected European populations, and thus, *Stagonospora* spp. were not identified in our survey. It is important to note that endophytes are widespread in grasses (Clay 1988), but little work has been done on endophytes of the Arundineae (Clay 1990), and endophytic effects on host performance remains vague. Clay (1988) expressed a need for systematic surveys of grasses to test for ascomycete endosymbionts.

We have screened several native and exotic populations of *P. australis* and did not find any endophytes in the leaf sheaths. Our analysis was limited to several populations in the northeast US and does not reflect the diversity of *P. australis* haplotypes present throughout the United States, nor the possible fungal associations with the various haplotypes. However, our study did focus on a region where native populations are declining (Saltonstall 2003), and concern for conservation of native populations is increasing.

Although endophytes are most concentrated in the structures we studied (N. Jackson, University of Rhode Island, Kingston, RI, pers. comm.) they do grow systemically (Carroll 1988), and other tissues should be checked for endophytes, including the seeds, stems, and roots. Wirsal et al. (2001) found that fungal diversity and abundance was highest in *P. australis* roots and differed between wet and dry sites. Other methods may reveal the presence of endophytic fungi in *P. australis* in the United States and should include tests for alkaloids produced by endophytes (Clay 1988), as well as culturing plant tissues on agar plates to allow any possible endophytes to grow.

Staining techniques are commonly used to test for endophytes in grasses (Hill 1999, Marshall et al. 1999, Omacini et al. 2001, Saha et al. 1988), but some may not stain using this procedure, and other methods should be used to confirm our findings. For example, surface sterilization of plant tissues followed by cultivation on agar is another common method used to detect fungal endophytes in plants (Wirsal et al. 2001). The fungus *Discula quercina* (West.) von Arx (Coelomycetes), an endophyte of oaks, was isolated by incubating oak leaf sections on agar plates (Wilson and Carroll 1997). However, Reissinger et al. (2001) found that surface-sterilization techniques can damage epidermal tissue and may not be appropriate for detecting fungi. A few species of endophytes can also be recognized by fruiting structures on plant surfaces (Brem and Leuchtman 2002, von Aarle et al. 2002). Although we did not find endophytic reproductive structures externally on plants in this study, native *P. australis* haplotypes had an external stem fungus associated with the nodes of the stem. Preliminary identification places this fungus in the Schizothriaceae (B. Blossey, pers. comm.), possibly having a weak, pathogenic relationship with the plants (N. Jackson, pers. comm.).

Other mechanisms responsible for differential susceptibility to insect attack between native and exotic *P. australis* should be tested. For example, plant defenses can be important in mediating differences in insect

populations. Grasses have comparatively simple plant architecture and lack the secondary compounds of dicotyledons (see Bernays and Barbehenn 1987). However, they are rich in silica, an inducible compound that reduces digestibility of plant tissue, which may be a mechanism against herbivore feeding (McNaughton and Tarrant 1983). Silicate content in *P. australis* is the main deterrent to attack by *Giraudiella inclusa* (Frauenfeld) (shoot fly) (Tschamntke 1988), and differences in silicate levels among haplotypes may explain noted differences in resistance to herbivore attack.

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