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Senescence as a Factor in Latent Pathogen Infection in Eurasian Watermilfoil

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PURPOSE: This technical note describes the results of a laboratory investigation conducted to evaluate how fungal endophytes may contribute to plant senescence. This has important implications for aquatic plant control because endophytes turned latent pathogens can induce rapid declines in plant populations.

INTRODUCTION: Endophytes are microorganisms that live asymptomatically within host tissues (Petrini 1991; Wilson 1995). They are broadly defined as organisms that colonize plant tissues at some time in their life without causing harm to their host (Petrini 1991). They include microorganisms associated with the rhizosphere, the phyllosphere and vascular tissues of living plants (Arnold 2005). They may include pathogenic organisms that remain latent between infection and manifestation of symptoms. Onset of symptoms is often associated with plant stress brought on by biotic or abiotic causes (Dorworth and Callan 1996; Sinclair and Cerkauskas 1996; Shearer 2002).

Most endophytic studies have focused on terrestrial plants where endophytes appear to be ubiquitous and may range from a single species to hundreds of species within a single host (Petrini 1991; Saikkonen et al. 1998; Stone et al. 2000; Johri 2006). They have been isolated from foliage, stems, bark, roots, fruit, flowers, and seeds in alpine, temperate, and tropical regions. Some are systemic and are vertically transmitted through the seed while others are horizontally transmitted via spores that develop on senescent tissues and are carried to new hosts on air currents or water movement (Arnold 2005). Some preferentially are associated with particular host tissues. For example, the dark septate endophyte *Philocephala fortinii* C. J. K. Wang & H.E. Wilcox co-occurs with mycorrhizal fungi in root tissues but differs from them in that it never forms specialized structures (tubercles or arbuscles) within the infected roots (Addy et al. 2005).

Although a plethora of studies have examined plant tissues for the presence of endophytes, their specific role in host tissues is still poorly understood except in a few select cases where they have been shown to enhance resistance to herbivory (Clay 1996, Clay and Schardl 2002) increase drought resistance (Elmi and West 1995; Redman et al. 2002), enhance nutrient uptake (Malinowski et al. 2000), resist pathogen attack (Carroll 1991), and improve the competitive ability of the host (Marks et al. 1991; Clay et al. 1993). Apart from these specific cases it is difficult to assess in physiological terms how a host plant benefits from such a relationship when the endophyte is obligately heterotrophic and totally dependent on the host for its nutrition (Isaac 1992). Such unspecialized endophytes often have alternative lifestyles existing as coprophiles, saprophytes, or latent pathogens (Gange et al. 2007). They often produce a wide range of metabolites making them excellent candidates for pharmaceutical and industrial applications or as safe bioherbicides (Sridhar and Raviraja 1995; Schulz et al. 2002).

To date, endophyte studies of aquatic and/or wetland plants have been few but they cover a diverse assemblage of species. Lambert and Casagrande (2006) found no endophytes in leaf sheath tissues of introduced or native populations of *Phragmites australis* (Cav.) Trin. ex Steud. (common reed) in northeast United States. It seems unusual because endophytes are quite common in members of the Poaceae (Clay 1996). Examination of other host tissues or other populations would likely yield some species because *P. australis* has a diverse assemblage of phylloplane species (Van Ryckegem et al. 2007), several of which have been reported to be endophytic in other plant species. Studies in India reported endophytes in mangrove hosts (Kumaresan and Suryanarayanan 2002) and three halophytes from an estuarine mangrove forest (Suryanarayanan and Kumaresan 2000). Wang and Zhao (2006) in studies of hydrophytes in lakes and streams in China found dark septate endophytes in rooted floating *Potamogeton tepperi* A. Benn. (Tepper's pondweed) emergent *Oenanthe decumbens* (Thunb.) Kozo-Polj. (water dropwort), and *Rotala rotundifolia* (Buch.-Ham. ex Roxb.) Koehne (roundleaf toothcup) and submerged *Hydrilla verticillata* (L.f) Royle (hydrilla). *Myriophyllum spicatum* L. (Eurasian watermilfoil or milfoil) plants collected in Tennessee Valley Authority reservoirs in the 1990's were consistently infected with endophytes (Shearer 2001). The most commonly isolated species, *Mycoleptodiscus terrestris* (Gerd.) Ostazeski, occurred with high frequencies in collections from 47 different sites in 7 reservoirs (Shearer 2001). Monthly sampling at four sites on Guntersville Reservoir, Alabama in 1994 documented that *M. terrestris* was the dominant endophyte in all milfoil populations and increased in frequency in milfoil tissues from June to October (Shearer 2001). Finally three marine algal species have been shown to harbor endophytes (Stanley 1992). They differed considerably from each other in terms of seasonal occurrence and nature of the association.

Milfoil is a submersed herbaceous perennial plant that is highly productive and can spread rapidly, forming dense monospecific stands (Grace and Wetzel 1978; Smith and Barko 1990). Although the plant can produce viable seed, reproduction is primarily vegetative from stem fragmentation and stolon formation (Johnson and Blossey 2004). In the southern United States, biomass allocations to milfoil plant components tend to follow seasonal patterns (Madsen 1997). For example, flowering peaks most commonly occur in June and October followed shortly thereafter by autofragmentation and senescence (Madsen 1997). Autofragmentation occurs primarily in the upper portion of the plant and is coincident with an increase in percent allocation of biomass to lower stems and root crowns. The plant forms no specialized overwintering structures (Smith and Barko 1990), but overwinters as root crowns and/or lower shoots. In preparation for overwintering, sugars and starches are stored in the lower stems and root crowns. Madsen (1997) found that in southern milfoil populations, the lower stems contained up to 15 percent starch. Additionally root crown tissues provided the main storage area for carbohydrates with starch concentrations reaching 20 percent and total nonstructural carbohydrate concentrations reaching 30 percent.

Native to Eurasia, milfoil first appeared in herbarium records in the 1940's (Couch and Nelson 1985). It was documented from widely scattered locations including Washington DC, Arizona, California, and Ohio, suggesting multiple introductions. As of 1985, it had been found in 33 states, the District of Columbia, and the Canadian provinces of British Columbia, Ontario, and Quebec (Couch and Nelson 1985). Established populations have now been documented in Alaska and every state in the continental United States except Wyoming (U.S. Geological Survey (USGS) 2009).

While Eurasian watermilfoil populations have continued to expand to new locations, in some established sites they have undergone unexplained declines. Various causes have been proposed for the declines including nutrient depletion, shading by phytoplankton and algae, attack by parasites and pathogens, long-term effects of harvesting and/or herbicides, toxins, climate, competition, and insect herbivory, but none have been adequately explained (Smith and Barko 1990). A secondary factor contributing to declines could be latent pathogen infection induced by plant stress. Greenhouse studies documented that milfoil stressed by simulated chemical runoff was susceptible to latent pathogen infection by *M. terrestris* (Shearer 2002). Four weeks following the stress-related event, shoot biomass of endophytic infected plants was reduced up to 75 percent compared to plants that were endophyte free.

In addition to plant stress, changes induced by age-dependent factors such as senescence may induce a benign endophyte to pathogenicity, potentially increasing the rate of breakdown of host tissues. In southern populations of milfoil, upper stem biomass declines in the fall in part to autofragmentation but also from onset of plant senescence (Madsen 1997). To evaluate the potential role of a latent pathogen in plant senescence and subsequent decomposition, endophyte-infected and endophyte-free plants were grown in the greenhouse for a growing season. As plants began to decline and senesce at the end of the growing season, aboveground shoot biomass was harvested to detect biomass differences between endophyte-infected and endophyte-free milfoil plants.

MATERIALS AND METHODS: Endophyte-free plants were obtained from greenhouse cultures at the Wetlands and Aquatic Ecosystem Research Center, Engineer Research and Development Center (ERDC), Environmental Laboratory, Vicksburg, MS. Endophyte-infected plants were collected from a culture pond at the Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX. To determine endophyte presence/absence in the milfoil collections, stem pieces approximately 2 cm in size were plated onto Martin's agar (dextrose, 10 g; KH_2PO_4, 0.5 g; MGS O_4, 0.5 g; K_2 HPO_4, 0.5 g; peptone, 0.5 g; yeast extract, 0.5 g; H_2O, 1 L; rose Bengal, 0.05 g; streptomycin sulfate, 0.03 g). After 7 days incubation at 28º C, the plates were visually assessed to determine presence or absence of the endophyte *M. terrestris* in the tissues.

Sixteen 55-L aquaria (0.9 m tall by 0.09 m^2) were filled with a water-based culture solution recommended for aquatic plant growth (Smart and Barko 1984). Lake sediment collected from Brown's Lake at the ERDC was amended with ammonium chloride (0.5 g L^{-1} and Esmigran (1.7 g L^{-1}). Four plastic cups (0.95 L) filled three-fourths with lake sediment that contained five 20-cm apical cuttings from either endophyte-infected or endophyte-free milfoil were overlaid with silica-sand, and were placed in each aquarium. Each treatment was replicated eight times.

The plants were allowed to grow over the summer in the greenhouse. Once each month, they were amended with a water-based culture solution (Smart and Barko 1984). Observations were made on a weekly basis to assess plant health. At the end of the growing season, with the onset of senescence, the cups were harvested, and aboveground shoot biomass was collected and dried at 60º C to a constant weight.

RESULTS AND DISCUSSION: Both endophyte-infected and endophyte-free milfoil apical tips appeared green and asymptomatic of disease at the start of the experiment. Plating of the 2-cm milfoil stem pieces confirmed that milfoil collections from LAERF harbored the endophyte, *M. terrestris*, whereas the fungus was absent from greenhouse cultured milfoil. Plants remained

asymptomatic following planting and within one month had reached the water surface in the aquariums.

After an approximate 2-month period of growth, endophyte-infected plants began to show some random black spotting on the lower stems. The spots never enlarged or coalesced indicating the onset of a disease epidemic but were more typical of a phytoalexin response (Figure 1) where the plant produces antimicrobial compounds in an attempt to ward off a pathogen (Agrios 2005).

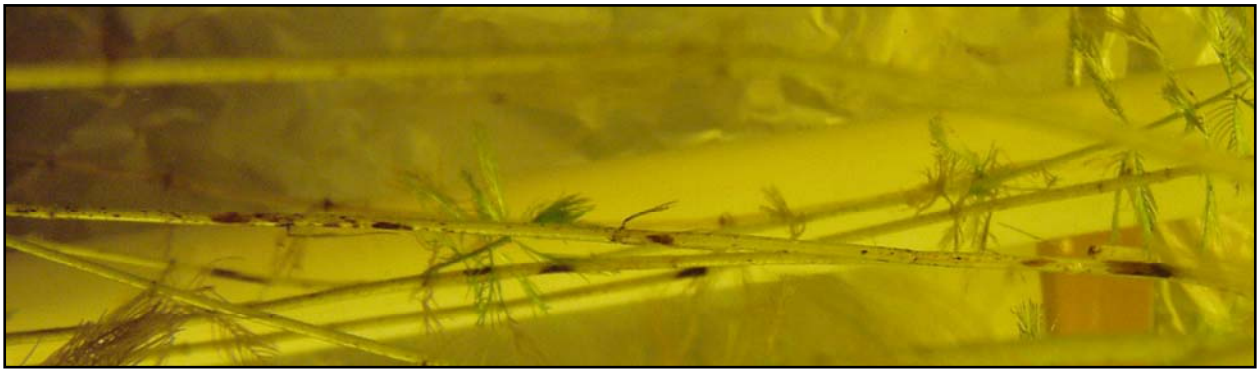

Figure 1. Black spots on Eurasian watermilfoil stems result from a reaction to the presence of the pathogen, *Mycoleptodiscus terrestris*.

The leaves in the upper canopy of both endophyte-infected and endophyte-free plants remained green and asymptomatic through most of the growing season. There was some browning and loss of leaves on the lower stems, most likely due to shading and reduction in photosynthetic capacity; however, there was no indication that the plants were diseased other than the spotting that occurred on the stems of the endophyte-infected plants.

Approximately 3 months post planting, the milfoil plants began to show symptoms of decline. Both stems and leaves of endophyte-infected plants exhibited tissue browning followed by flaccidness, symptoms that are typical of disease caused by *M. terrestris*. There was some leaf browning in the endophyte-free plants but the stems remained green and turgid. Following the visible onset of disease symptoms in the endophyte-infected plants, the canopy began to collapse over a period of 2 weeks (Figure 2) and the experiment was terminated. At this point, shoot biomass was significantly different (d.f. 1,14; $p = 0.0000$) between the treatments with an approximate fourfold higher biomass in endophyte-free compared to endophyte-infected plants (Figure 3).

Senescent plants have been reported more susceptible to pathogen infection than nonsenescent plants (Rejmankova 1989). Senescent plants are generally weaker and under stress, making them more susceptible to latent infection from an opportunistic pathogen that is already residing in the host tissues. It has also been demonstrated that senescence is associated with high carbohydrate levels in the plant (Parrott et al. 2005), which would provide additional food for fungal growth and expansion. Thus for biological control purposes it has been suggested that a fall application of a fungal pathogen should be seriously considered for aquatic plant control (Rejmankova 1989). The current greenhouse study would seem to support this strategy. However, plant condition in the field would also need to be taken into consideration before a fall application. At the end of a growing season, milfoil plants are often covered with algae at the water surface and submerged leaves and stems can

be encrusted with epiphytes and/or marl (carbonate deposits) (Sculthorpe 1967). For a contact pathogen like *M. terrestris* the algae, epiphytes, and/or marl may provide an impenetrable barrier to ingress by the contact pathogen.

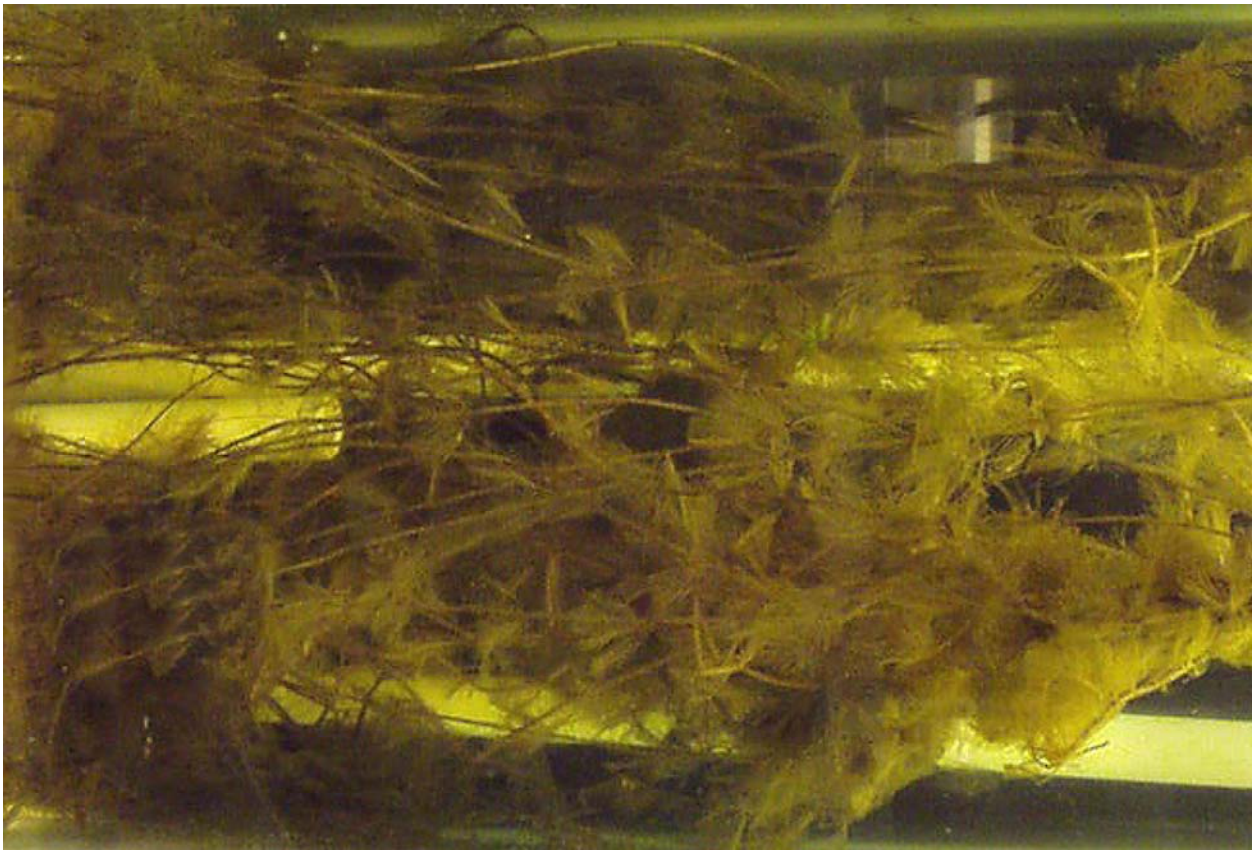

Figure 2. Disease symptoms on Eurasian watermilfoil caused by ingress of the pathogen *Mycoleptodiscus terrestris*.

FUTURE WORK: Since milfoil senescence appears to increase susceptibility to latent pathogen infection, a field application at the end of the growing season should be attempted. Following overwintering, treated and untreated milfoil populations would be assessed for ability to survive and regrow. Since few biocontrol techniques are available for milfoil control, a timely application of a virulent pathogen alone or in an integrated pest management approach may offer an additional management technique.

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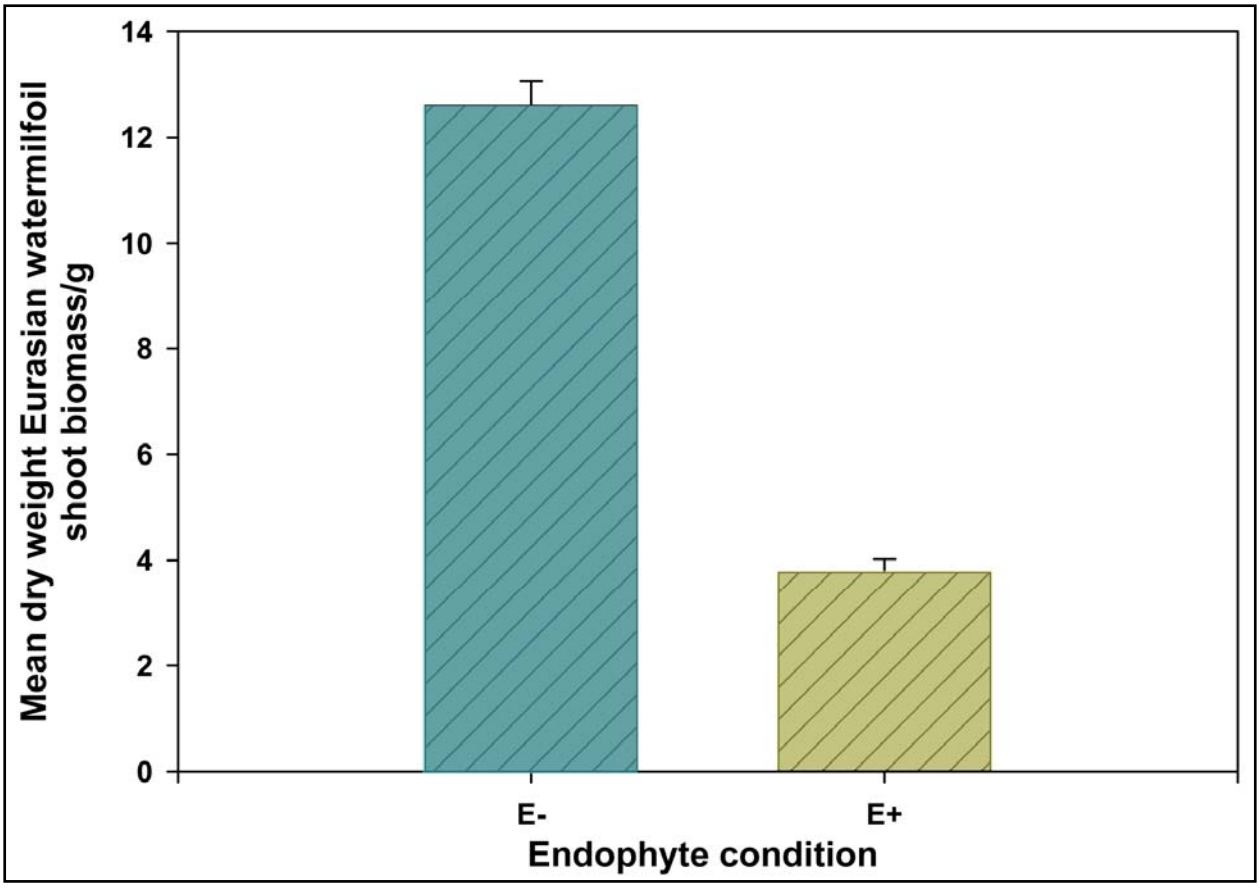

Figure 3. Mean dry weight Eurasian watermilfoil shoot biomass of endophyte free (E-) and endophyte infected (E+) Eurasian watermilfoil following senescence.

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