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Relationship Between Eurasian Watermilfoil Phenology and Endophyte Presence

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PURPOSE: This technical note describes the results of a study using field-collected Eurasian watermilfoil to evaluate plant phenology as it relates to the presence of endophytes.

INTRODUCTION: Endophytic fungi are microorganisms that live asymtomatically within their host tissues (Wilson 1995). Since the term endophyte was first introduced by DeBary (1866) the definition has been much debated. Presently the definition includes a broad array of organisms that can live within plant tissues (Stone et al. 2000). What are considered "true" endophytes live benignly within a host and do not produce any symptoms that might indicate their presence (Wilson 1995). Latent pathogens, also considered to be endophytes, live asymptomatically within the host plant until it undergoes biotic or abiotic stress, which induces them to become pathogenic and visible disease symptoms appear on the host (Carroll 1988, Saikkonen et al. 2004).

Endophytic fungi are extremely common in terrestrial plants (Petrini 1991, Saikkonen et al. 1998, Arnold 2005, Arnold and Lutzoni 2007) with numbers varying from a single species per plant to hundreds of species per plant (Saikkonen et al. 1998, Stone et al. 2000). They invade all plant tissues including roots, stems, leaves, inflorescences, fruits, and seeds (Arnold 2005, Johri 2006).

The impetus for intensely studying endophytes began when a link between the presence of endophytes and toxicity to herbivores was established in section Pooideae of the grass family Poaceae (Saikkonen et al. 1998). Additional studies followed that examined ecologic, evolutionary, mycological and agronomic relationships in the grasses indicating that endophytes can have dramatic biological effects on growth and reproduction of host grasses, pathogens and herbivores of grasses, and natural enemies of herbivores (Saikkonen et al. 1998). This research also stimulated studies that examined endophytes in other groups of plants including woody plants in temperate and tropical ecosystems, herbaceous plants, mangroves, cacti, mosses, ferns, and palms (Arnold 2005, Kumaresan et al. 2002, Collado et al. 1999, Gange et al. 2007, Seena and Sridhar 2004, Sridhar and Raviraja 1995, Suryanarayanan et al. 2005, Gamboa and Bayman 2001, Stone et al. 2000).

Endophytes may play an important role in classical biological control. The enemy release hypothesis explains how plant species introduced into a new region with no natural enemies are allowed to rapidly increase in distribution and abundance (Keane and Crawley 2002). Recently, researchers cite another important factor that could be involved: the presence or absence of mutualistic endophytes (Evans 2008). There is increasing evidence that endophytes form highly specialized or co-evolved associations with their hosts and they provide protection from biotic and abiotic stresses, pests, and diseases (Evans 2008). Therefore, plants arriving without co-evolved natural enemies but with a plethora of mutualistic endophytes would have a distinct advantage over local competitors. On the

other hand, plants arriving without endophytes and without natural enemies could allocate more resources to growth and reproduction. These endophyte-depauperate alien-invasive weeds, however, would remain highly susceptible to co-evolved natural enemies and a single classical biological control agent (i.e. the "silver bullet") could bring about complete control (Evans 2008).

Little attention to date has been given to endophytes of aquatic species. Some studies have been undertaken to retrieve endophytes from plants and algae that exist in marine habitats (Jones et al. 2008, Schulz et al. 2008) yet few studies have been undertaken to look at endophytes in freshwater aquatic species. Andrews et al. (1981) reported a latent pathogen (*Acremonium curvulum* W. Gams) growing both epiphytically and endophytically in Eurasian watermilfoil (*Myriophyllum spicatum* L.) tissues in Wisconsin. The authors speculated that some declines in watermilfoil populations in the state might be attributed to the presence of the endophyte turned latent pathogen in host plant tissues. Isolations from watermilfoil populations in the Tennessee and Cumberland River systems in 1993 yielded a total of 482 fungal isolates in 18 genera (Shearer 2001). The most commonly collected isolate, *Mycoleptodiscus terrestris* (Gerd.) Ostazeski, was found in both healthy and declining watermilfoil populations. Additional laboratory studies found that *M. terrestris* could become a latent pathogen of watermilfoil if the plant was stressed by a low dose herbicide application (Shearer 2002).

Eurasian watermilfoil is an invasive submersed perennial plant found throughout North America. In winter, the plant dies back to the root crown but when water temperatures approach 15 °C in spring, new growth is initiated from overwintering shoots and roots (Smith and Barko 1990). Typically, plants grow rapidly to the water surface where they branch profusely forming a dense canopy. The shaded lower leaves often drop off the plant. Once plants reach the water surface, flowering may commence although some populations never flower (Madsen and Boylen 1989). Following flowering, plant biomass usually declines as plants undergo fragmentation of the stems (Smith and Barko 1990). Unlike northern populations, watermilfoil in southern United States follows a somewhat different pattern. In studying a watermilfoil population at the U.S. Army Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (USACE/LAERF), Lewisville, TX, Madsen (1997) found that peak biomass accumulation usually occurred in May and two flowering peaks occurred in June and October of each year. Following the flowering peaks was a period of senescence and autofragmentation (self-formed stem segments). The autofragments formed in the upper canopy with the lower stems largely unaffected. Madsen (1997) speculated that differences observed in the populations in northern and southern climates may be driven by differences in water temperatures as surface temperatures can reach 35 °C in shallow ponds in the south.

Few endophytic studies have looked at seasonality as a factor in colonization. In the marine alga, *Ascophyllum nodosum* (L.) Le Jolie, the life cycle of its obligate endophyte *Mycosphaerella ascophylli* Cotton follows that of the host (Stanley 1992). The onset and timing of reproduction is both synchronized and seasonal between endophyte and host. Examination of endophyte occurrence in the oak tree, *Quercus ilex* L, populations in Spain revealed that the geographical factor affected endophyte distribution more significantly than the seasonal factor (Collado et al. 1999). Seasonality is known to play an important role in the life cycle of watermilfoil (Madsen 1997) but it is unknown if the seasonal changes that alter host phenology in watermilfoil also impact the endophytes that live within the host tissues. In order to document any seasonality effects on the endophytes that exist in

watermilfoil tissues, plants were collected from USACE/LAERF on a monthly basis from May through October 2007 and examined for changes in the endophyte population.

MATERIALS AND METHODS: Eurasian watermilfoil plants were collected monthly from late May through late October in 2007 from culture ponds located at USACE/LAERF. Plants were collected from this location because previous studies had documented phenological changes in the plant throughout the growing season (Madsen 1997). The plants were shipped overnight in a chilled insulated cooler to the biomanagement laboratory located at the Engineer Research and Development Center (ERDC), Environmental Laboratory, Vicksburg, MS. Ten plants containing roots, root crowns, stems, and leaves were randomly selected from each shipment. Inflorescences if present were not collected because flowering is not continuous throughout a growing season nor does every plant in a population produce flowers. Each plant was processed by carefully washing it to remove sediment from the roots and any algae and debris that had become attached to the stems and finely dissected leaves. Side branches were removed leaving one main stem. Individual plants were surface sterilized for 2 minutes in a 3.5-percent sodium hypochlorite solution and rinsed in sterile water to remove any epiphytic organisms. Sterilization times were reduced from those normally used for the isolation of endophytes because even though watermilfoil has been reported to have a cuticle, it offers little if any resistance to diffusing substances (Sculthorpe 1967). Once sterilized, each plant was placed between moistened sterile paper towels to prevent dessication of plant tissues during plating.

Plant tissues were excised and systematically plated onto Martin's agar (MA) plates (H_2O, 1 L; agar, 20 g, KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; peptone, 0.5 g; dextrose, 10 g, yeast extract, 0.5 g; rose Bengal, 0.05 g; streptomycin sulfate 0.03 g). Starting with the root tip, approximate 2-cm-length root sections were cut with a sterile scalpel and inserted into slits cut in MA with a sterile forceps. The root crown was excised and plated separately. Stems were divided into 10-cm sections starting with the base of the plant and progressing up the stem. Each 10-cm section was cut into pieces approximately 2 cm in length and two to three 2-cm sections were placed on each plate. If leaves or portions of leaves were present at a node, they were carefully removed, noting their position on the stem section, and inserted between slits cut into MA in sequential nodal order from stem base upward to the apex. Each plate contained leaves from two to three nodes. The plates were incubated in the dark at room temperature. Starting approximately six days post plating, the MA plates were examined for fungal growth.

Each colony that grew from a plant segment was collected by cutting an approximate 1- x 1-mm piece from the leading edge of the colony with a sterile needle and placing it in the center of a Potato Dextrose Agar (PDA, Difco, Detroit, MI) slant. The slants were numbered sequentially. A companion log was kept denoting the plant number and the location on the plant that yielded the isolate. The slants were allowed to grow at room temperature for approximately 7-10 days, by which time the colonies had developed distinct growth patterns and coloration. Each month, the isolates from all ten plants were sorted together and enumerated into morphological "species" based on gross colony morphology. Duplicate isolates were then discarded. Following sorting, each isolate was transferred to Petri dishes containing Potato Carrot Agar (PCA) (Dhingra and Sinclair 1995) and PDA for identification. This process was repeated each month from May to October 2007.

A frequency index of endophyte occurrence throughout a growing season was calculated by totaling the number of plants infected by a particular endophyte each month divided by the total number of plants examined during the growing season. The percent frequency of colonization in a plant part (roots, root crown, stem, and leaves) by an endophyte species was calculated following Fisher and Petrini (1987) and was equal to the number of colonized segments divided by the total number of segments examined x 100.

RESULTS AND DISCUSSION: The phenology of Eurasian watermilfoil during this study followed that described by Madsen (1997) with two peaks of autofragmentation followed by senescence. However, in this study, plants appeared to undergo autofragmentation in late September/early October and by the plant collection near the end of October were definitely starting to senesce. The onset of senescence by the October collection made it difficult to find plants that were still intact from root to apex. Most of these were small plants that were lower in the canopy and were stunted. Because they were shorter (mean length 31.1 cm), the total number of segments examined was relatively low and this probably accounted for the reduced number of total isolates in the last collection of the season (Table 1). In contrast, the plants collected in July had a mean length of 78.1 cm and recorded the highest number of isolates. Of note was the peak in retrieval of isolates from the July collection (Table 1). This peak in number of isolates could have occurred because plants were stressed following a flowering event in June and subsequent onset of summer senescence. When plants are stressed they are also more susceptible to infection from both latent pathogens and opportunistic saprophytes (Carroll 1988, Saikkonen et al. 2004).

Table 1. Mean stem length, total numbers of species and isolates recovered from Eurasian watermilfoil plant tissues during six sampling periods, May through October 2007.

Variable	May	June	July	August	Sept	Oct
Mean stem length (cm)	43.9	61.6	78.1	48.9	58.7	31.1
Total species	31	34	36	23	25	30
Total number of isolates	287	260	206	211	108	143
No. of isolates from roots	59	38	75	68	33	29
No. of isolates from root crowns	14	5	16	28	7	13
No. of isolates from stems	190	223	405	261	284	194
No. of isolates from leaves	287	260	206	211	108	143

All plant tissues examined (roots, root crowns, stems, leaves) harbored endophytes throughout the growing season. The assemblage was made up primarily of ascomycetes and their anamorphs. The total numbers of root, root crown, stem, and leaf segments that were examined for endophytes were 326, 60, 1677, and 1336, respectively. As in previous studies that examined endophyte populations in Eurasian watermilfoil plants (Shearer 2001), *M. terrestris* was isolated with the highest frequency (Table 2). Other species on the list that had previously been reported in watermilfoil tissues included *Cladosporium sphaerospermum* Penz., *Plectosphaerella cucumerina* (Lindfors) W. Gams, and *Phoma* sp. (Table 2). Most of the genera not mentioned above but included in Table 2 have been identified as being endophytic in other plant species. Reports of species that cannot be identified because they do not sporulate occur throughout the literature and this study was no exception. Many are noted as unknown ascomycetes, and sterile dematiaceous or moniliaceous hyphomycetes.

Table 2. Number of plants out of 10 that were infected each month by 20 of the most commonly isolated endophytes. The percent frequency index was calculated by dividing the number of plants infected by 60, the total number of plants examined.

Species	May	June	July	Aug	Sept	Oct	Frequency index
Mycoleptodiscus terrestris	1	9	10	9	10	10	.817
Trichoderma aureoviride	6	6	9	9	5	5	.667
Plectosphaerella cucumerina	10	9	5	0	0	10	.567
Aspergillus flavus	0	0	9	10	5	6	.500
Trichoderma harzianum	7	0	9	1	4	0	.500
Cladosporium sphaerospermum	1	9	4	2	4	0	.333
Penicillium multicolor	0	3	7	9	0	0	.317
Penicillium purpurogenum	0	8	8	0	1	0	.283
Cladosporium cladosporioides	2	3	6	0	1	5	.283
Sclerotium rolfsii	2	1	1	1	4	8	.283
Fusarium redolens	0	1	6	1	3	3	.233
Dematiaceous hyphomycete	8	5	0	0	0	0	.217
Drechslera poae	5	6	1	0	0	0	.200
Curvularia pallescens	0	2	2	6	0	2	.200
Alternaria alternata	0	5	6	0	0	0	.183
Mortierella sp	0	8	3	0	0	0	.183
unknown Ascomycete sp.	10	0	0	0	0	0	.166
Phoma sp	5	0	0	1	1	3	.166
Macrophoma sp.	9	0	0	0	0	0	.150
Nodulisporium sp.	1	0	6	1	1	0	.150

The percent frequency of colonization of the 20 most commonly isolated endophytes in root tissues can be found in Table 3. Thirteen of these isolates were also among the most common isolates from watermilfoil plants (Tables 2 and 3) and 12 were also present in the most common isolates of root crowns (Table 4). Almost all the root isolates were species that are commonly found in soil, hence the high probability that they could easily enter root tissues. *Colletotrichum gloeosporioides* (Penz.) Penz and Sacc. in Penz., often isolated with high frequencies from aboveground watermilfoil tissues, was only found in root and root crown tissues and in relatively low numbers. This species was one that was examined in Europe for potential use as a biological control agent for Eurasian watermilfoil; however, it was abandoned because it was deemed only weakly pathogenic (Harvey and Evans 1997).

The percent frequency of colonization of the 20 most commonly isolated endophytes in root crowns can be found in Table 4. Twelve of these species were among those most commonly isolated from watermilfoil plants (Table 2). Fifteen of the species found in the root crowns were also present in stem tissues. Of interest is the ascomycete *Acanthophiobolus helicosporus* (Berk. and Broome) J. Walker because it is noted as a pathogen of various aquatic and/or wetland plants causing leaf lesions (BioImages 2009). It should be studied further for potential as a biological control agent for Eurasian watermilfoil.

Table 3. Percent frequency of colonization of 20 of the most commonly isolated endophytes in root tissues.

Species	No. of isolates	% frequency
Aspergillus flavus	46	14.11
Penicillium multicolor	28	8.59
Penicillium purpurogenum	25	7.67
Cladosporium sphaerospermum	15	4.60
Pythium sp	15	4.60
Trichoderma harzianum	13	2.45
Mortierella sp	10	3.07
Penicillium diversum	10	3.07
Plectosphaerella cucumerina	10	3.07
Colletotrichum gloeosporioides	8	2.45
Dematiaceous hyphomycete	7	2.15
Trichoderma aureoviride	7	2.15
Fusarium equisetii	6	1.84
Cladosporium cladosporioides	5	1.53
Mycoleptodiscus terrestris	5	1.53
Rhizopus nigricans	5	1.53
Arthrinium phaeospermum	4	1.23
Dark chlamydosporous sp	4	1.23
Phoma sp.	4	1.23
Alternaria alternata	3	0.92

Table 4. Percent frequency of colonization of 20 of the most commonly isolated endophytes in root crowns.

Species	No. of isolates	% frequency
Aspergillus flavus	13	21.67
Acanthophiobolus helicosporus	13	21.67
Trichoderma harzianum	13	21.67
Pythium sp	8	13.33
Penicillium multicolor	7	11.67
Mycoleptodiscus terrestris	5	8.33
Plectosphaerella cucumerina	5	8.33
Trichoderma aureoviride	5	8.33
Penicillium purpurogenum	4	6.67
Sclerotium rolfsii	3	5.00
Penicillium diversum	2	3.33
Penicillium frequentans	2	3.33
Penicillium restrictum	2	3.33
Cladosporium cladosporioides	1	1.67
Colletotrichum gloeosporioides	1	1.67
Curvularia pallescens	1	1.67
Phoma sp.	1	1.67
Pithomyces chartarum	1	1.67
Talaromyces flavus	1	1.67
Talaromyces stipitatus	1	1.67

Fifteen of the species that were found in the stems (Table 5) were also found in leaf tissues (Table 6). *Mycoleptodiscus terrestris* was by far the most commonly isolated species overall (Table 2) and from stem pieces (Table 5). The fungus has been studied as a biological control agent for both watermilfoil and *Hydrilla verticillata* (L.f.) Royle (hydrilla) (Shearer 1993, 1996a, 1996b; Smith and Winfield 1991). It is also a latent pathogen of watermilfoil contributing to senescence and decline (Shearer 2001). The second most commonly isolated species from stem tissues (Table 6) and the most frequently isolated species from leaf tissues (Table 6), *P. cucumerina*, has also been studied as a biological control agent both for hydrilla (Smither-Kopperl et al. 1999) and for a nematode that is a pathogen of potatoes (Atkins et al. 2003).

Table 5. Percent frequency of colonization of the 20 most commonly isolated endophytes in stems.

Species	No. of isolates	% frequency
Mycoleptodiscus terrestris	651	38.82
Plectosphaerella cucumerina	148	8.83
Aspergillus flavus	97	5.78
Penicillium purpurogenum	58	3.45
Trichoderma harzianum	49	2.92
Fusarium redolens	42	2.50
Macrophoma sp.	24	1.43
Dematiaceous hyphomycete	23	1.37
Sclerotium rolfsii	22	1.31
Alternaria alternata	20	1.19
Cladosporium cladosporioides	15	0.89
Amerosporopsis gaubae	14	0.83
unknown Ascomycete sp.	12	0.72
Dark chlamydosporous sp	8	0.48
Penicillium variabile	8	0.48
Trichoderma aureoviride	8	0.48
Epicoccum purpurascens	7	0.42
Curvularia pallescens	6	0.36
Nodulisporium sp	5	0.30
Talaromyces stipitatus	5	0.30

When assessing endophyte presence in the stems of the 20 most frequently isolated species, the highest numbers were found in the 0- to 10-cm stem segment (Figure 1A). In general, the number of isolates decreased from stem base to the apex of the plant. Movement of the endophytes up the stem most likely lags behind plant growth with the newest green tissues initially lacking endophytes. Concentration of the isolates in the lower stem would be a survival mechanism for the endophytes because plants die back during the winter to lower stems or root crowns.

The low number of isolates in leaf tissues associated with the 0- to 10-cm stem segment (Figure 1B) was a consequence of three factors. As watermilfoil grows, leaves are senesced from the lower stem due to shading and age. Nodal distance is greater in the lower stems so there are fewer leaves per 10-cm stem segment. Thirdly, as leaves age they become more susceptible to ingress by opportunistic endophytes that manifest themselves as latent pathogens and these leaves tend to

dehisce from the plant. The lower numbers of endophytes in the leaves in the upper parts of the plant were most likely due to lag time between plant growth and endophyte ingression. New growth appears to harbor fewer endophytes than older tissues.

Table 6. Percent frequency of colonization of the 20 most commonly isolated endophytes isolated in leaves.

Species	No. of isolates	% frequency
Plectosphaerella cucumerina	203	15.91
Mycoleptodiscus terrestris	129	9.66
Macrophoma sp.	119	8.91
Aspergillus flavus	112	8.38
Penicillium multicolor	87	6.51
Trichoderma harzianum	52	3.89
Penicillium purpurogenum	45	3.37
Alternaria alternata	32	2.40
Cladosporium sphaerospermum	32	2.40
Arthrinium phaeospermum	25	1.87
Curvularia pallescens	25	1.87
unknown Ascomycete	21	1.57
Fusarium redolens	18	1.35
Penicillium variabile	18	1.35
Cladosporium cladosporioides	15	1.12
Curvularia lunata	15	1.12
Sclerotium rolfsii	15	1.12
Nodulisporium sp.	14	1.05
Amerosporiopsis gaubae	13	0.97
Dematiaceous hyphomycete	11	0.82

Six species, *M. terrestris*, *Aspergillus flavus* Link:Fr., *Cladosporium cladosporioides* (Fresen.) G.A. DeVries, *Penicillium purpurogenum* O. Stoll, *Trichoderma harzianum* Rifai, and *P. cucumerina*, were found in all plant tissues examined. The study of endophytes offers great potential to find new biological control agents and agents that can colonize all plant tissues would be more effective than those that can only attack specific tissues. As mentioned above, *M. terrestris* and *P. cucumerina* have been studied as potential biological control agents against invasive species. The other four species that inhabited all plant tissues are widespread in nature and are considered weakly pathogenic and thus would not be good candidates for biological control purposes. All six species do possess certain characteristics that make them effective endophytes. One or more of them are capable of producing an array of extra-cellular enzymes, antibiotics, secondary metabolites, or various toxins (Agrios 2005; Joye and Paul 1991; Webster and Webster 2007). It should be noted that there was no apparent antagonism between the endophytes because the presence of one of them in host tissues did not exclude any of the others.

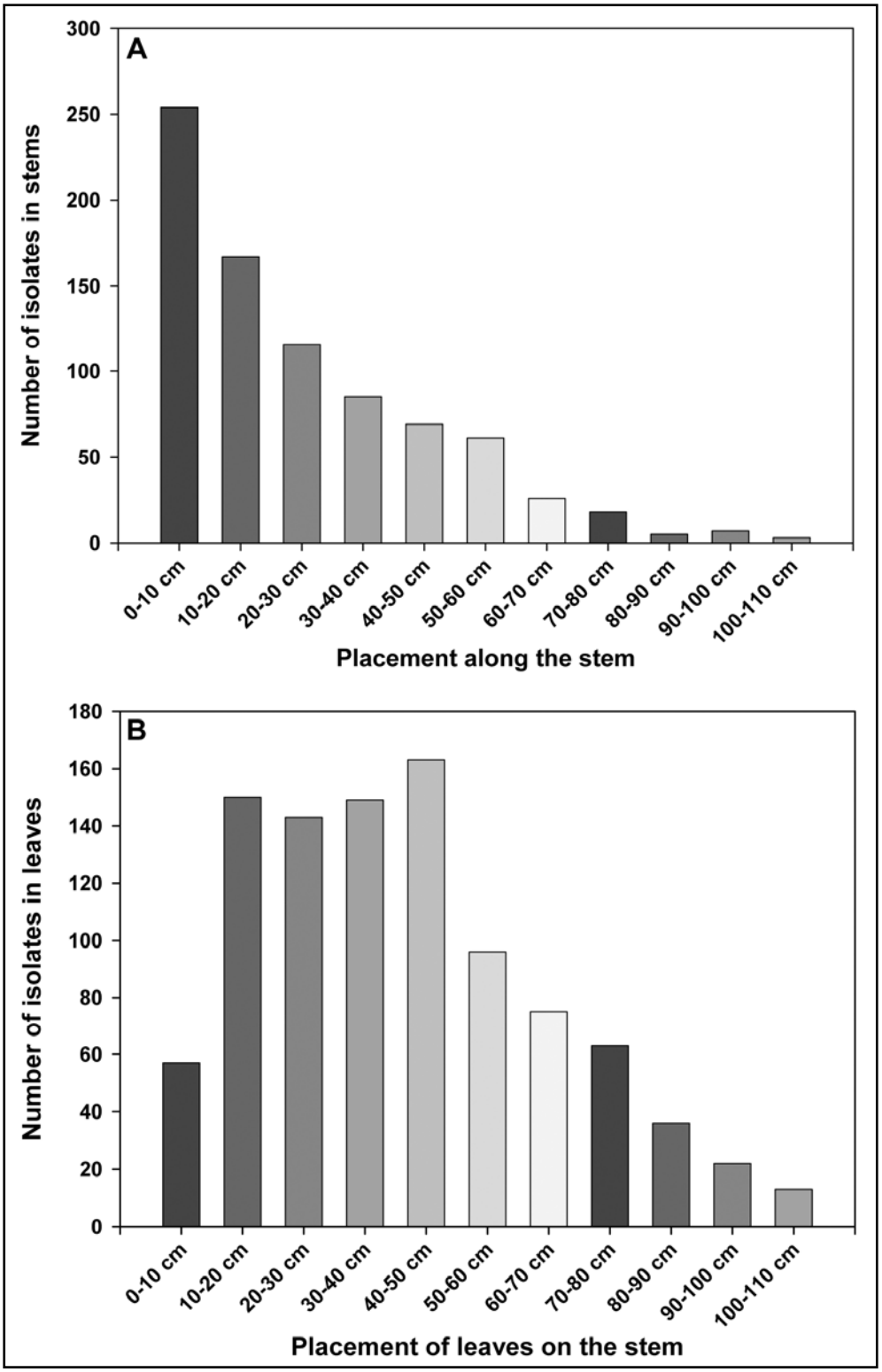

Figure 1. Total number of isolates of the 20 most common endophytes in stem (A) and leaf (B) tissues of Eurasian watermilfoil collected monthly from May to October 2007.

Host phenology did not appear to have a dramatic impact on presence/absence of endophytes in host tissues except for the mid-summer collection in July. Following flowering and the onset of autofragmentation and summer senescence, there was an increase in isolates from roots, root crowns, and stems and a decrease in isolates from leaves. Two contributory factors could account for these conditions. One, it has been demonstrated that senescence is associated with high carbohydrate levels in the plant (Parrott et al. 2005) potentially increasing available food to the microorganisms. Two, autofragmentation occurs primarily in the upper portion of the plant and is co-incident with an increase in percent allocation of biomass to lower stems and root crowns (Madsen 1997). Thus these two factors could have contributed to increased number of isolates from basal areas of the plant and a decrease in isolates from leaves in the upper part of the plant. Senescent plants are also generally weaker and, being under stress, they are generally more susceptible to latent infection from opportunistic microorganisms that are already residing in the host tissues (Rejmankova 1989).

FUTURE WORK: Future work will focus on the potential role of endophytes in invasion biology of introduced weeds. There is increasing evidence that endophytes are in coevolved mutually beneficial associations with their hosts and their presence provides the host with protection against biotic and abiotic stresses including pests and diseases. Invasive plants that arrive without their cadre of endophytes may be highly susceptible to natural enemies (classical biological control agents) resulting in highly successful biological control projects. In contrast, endophyte-enriched invasive weeds that also form mutualistic associations with indigenous endophytes may help to explain inconsistencies in agent performance in classical biological control programs.

Work is also planned to examine potential interactions of endophytes and the Eurasian watermilfoil weevil, *Euhrychiopsis lecontei* (Dietz). Because endophytes can become latent pathogens when plants are under stress, the weevil could induce pathogenicity in some endophytes by foraging on Eurasian watermilfoil plants. The presence of both weevils and latent pathogens could potentially increase rapidity of decline.

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