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Variation in Endophyte Symbiosis, Herbivory and Drought Tolerance of Ammophila breviligulata Populations in the Great Lakes Region

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Abstract.—Ecologists have come to appreciate that plant genotype can affect the success of restoration efforts, but the role of plant symbionts has received less attention. *Epichloë*-type endophytic fungi (EF) grow in the aboveground tissues of most plants and occur systemically in 20–30% of all grass species. Despite the potential for EF to alter plant competitive hierarchies and to be lost under improper seed storage, they have yet to be considered as an important factor in plant restoration ecology. We surveyed EF infection frequency in 42 native and restored populations of the dominant Great Lakes dune grass *Ammophila breviligulata*. We also conducted a greenhouse experiment to compare effects of herbivory and drought on an uninfected Michigan population of *A. breviligulata* and the commercially available 'Cape' variety, which is commonly planted for restoration. Surveys revealed low levels of EF infection in natural populations in the Great Lakes region. 'Cape' nursery stock was 100% infected. In the greenhouse, the Michigan plants were more sensitive to grasshopper herbivory and drought than the 'Cape' plants. Our results suggest that the variety of *A. breviligulata* used in dune restorations possibly could alter plant and insect community dynamics due to differences in EF status, though further tests under field conditions are needed.

Introduction

In recent years, restoration has shifted from a 'trial and error' practice to a scientific discipline informed by ecological theory (Montalvo *et al.*, 1997; Palmer *et al.*, 1997; Young *et al.*, 2001). For instance, an awareness of population genetics helped restoration ecologists evaluate the relative importance of gene flow and genetic diversity in restoration efforts (Montalvo *et al.*, 1997; McKay *et al.*, 2005). A community ecology perspective shifted many restoration efforts from a single species focus to one that considers interspecific interactions, even across trophic levels (Palmer *et al.*, 1997; Gratton and Denno, 2006). For example, 'target' plant species often need associated mutualisms (specifically pollinators, seed dispersers and mycorrhizal fungi) for successful growth and reproduction in restorations (Johnson *et al.*, 1997; Bever *et al.*, 2003; Young *et al.*, 2005). Organisms that form very close associations with their hosts (as parasites or mutualists), may have particularly large effects on the success of a restoration by altering host plant competitive

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ability and tolerance to the harsh biotic and abiotic conditions often found in degraded habitats. However, these interspecific relationships are often overlooked in restorations because of their cryptic nature (Clay, 2001; Bever *et al.*, 2003).

Systemic endophytic fungi (EF) are one class of symbionts whose role has gone unnoticed in restoration efforts. Systemic EF are specialized organisms that grow in intercellular spaces of aboveground plant tissue. In general, the prevalence of EF in plants is unknown due to their cryptic nature; however, *Epichloë*-type systemic EF are estimated to form relationships with 20–30% of all grass species, especially with cool season (C3) species in subfamily Pooideae (White, 1987; Clay and Schardl, 2002; Schardl *et al.*, 2004). These *Epichloë*-type endophytes (hereafter referred to simply as EF) can increase drought tolerance and nutrient uptake in some plant hosts (Malinowski and Belesky, 2000; Malinowski *et al.*, 2005), as well as confer resistance to herbivores through the production of bioactive alkaloids (Bush *et al.*, 1997; Clay and Schardl, 2002). Further, EF associations can increase competitive ability and slow succession in some systems (Rudgers *et al.*, 2005, 2007). Depending on environmental conditions, EF may also act as parasites (Cheplick *et al.*, 1989; Saikkonen *et al.*, 1998), though few experiments have manipulated EF in more than a handful of grass species (Faeth *et al.*, 2004; Saikkonen *et al.*, 2006). Based on the evidence to date, it seems probable that EF could have strong effects on plant competitive hierarchies, ecosystem processes and community assembly in restorations as well as natural plant communities (Clay and Holah, 1999; Rudgers and Clay, 2005; Rudgers *et al.*, 2007; Rudgers and Clay, *in press*).

For this study, we surveyed EF infection in populations of *Ammophila breviligulata*, the dominant native grass of Atlantic coast and Great Lakes dunes in the U.S. Because sand dunes are subject to blow outs, mining efforts and other disturbances, restoration of dunes habitats of various sizes (from several square meters to several hectares) has become a common practice (Albert, 2000; Palmgren, 2000), and *A. breviligulata* is widely planted in restorations throughout these areas. *A. breviligulata* had variable EF infection by the endophyte *Epichloë typhina* in East Coast populations (White *et al.*, 1992), although nothing is known about EF presence in Great Lakes populations. For this study, we surveyed EF infection in *A. breviligulata* in 42 sites throughout Michigan and Indiana, as well as from six nurseries that supply plants for dune restorations. Because *A. breviligulata* is clonal and rarely reproduces by seed (Fant *et al.*, 2008), we generally expected either 100% or 0% infection of populations.

We also conducted a greenhouse experiment to compare the performance of a widely available commercial variety that was 100% infected with EF (E+) to plants propagated from a native Michigan population free of endophyte infection (E−). We manipulated both drought and herbivory, the two primary benefits thought to be conferred by endophyte symbiosis in grasses (*e.g.*, Clay and Schardl, 2002). Based on the effects of EF infection in *Lolium arundinaceum* (tall fescue), we predicted that herbivores would prefer to feed on the E− local Michigan plants. We also expected that E+ nursery plants would tolerate drought better than the local Michigan plants. Differences in the performance of local and nursery populations may have important implications for successful restoration efforts.

METHODS

GREAT LAKES DUNES SURVEY

During the summer of 2005 we conducted a survey of EF infection in *Ammophila breviligulata* from natural populations in five Michigan state parks located along the shore of Lake Michigan (*i.e.*, Petoskey, Saugatuck, Van Buren, Warren Dunes and Wilderness State Parks). In Jul. 2006, we conducted a much larger survey of 18 natural populations of *A. breviligulata* in Indiana and Michigan (Table 1, Fig. 1). As part of a larger study, we also

TABLE 1.—EF infection status of *Ammophila breviligulata* in natural and restored sites in Michigan and Indiana

Site	Natural sites (infected/total) tiller, seed	Restored sites (infected/total) tiller, seed
Indiana Dunes National Lakeshore, Site 1	0/5, n/a	0/5, n/a
Indiana Dunes National Lakeshore, Site 2	0/5, n/a	0/5, n/a
Indiana Dunes National Lakeshore, Site 3	0/5, n/a	0/5, n/a
Indiana Dunes National Lakeshore, Site 4	0/5, n/a	0/5, n/a
Indiana Dunes National Lakeshore, Site 5	0/5, n/a	0/5, 0/7
Indiana Dunes State Park, Site 1	0/5, n/a	0/5, n/a
Indiana Dunes State Park, Site 2	0/5, n/a	0/5, n/a
Sleeping Bear Dunes National Lakeshore, Site 1	0/5, 1/8	0/5, n/a
Sleeping Bear Dunes National Lakeshore, Site 2	0/5, 1/3	0/5, n/a
Sleeping Bear Dunes National Lakeshore, Site 3	0/5, 0/32	0/5, 0/36
Sleeping Bear Dunes National Lakeshore, Site 4	0/5, 0/51	0/5, 0/33
Petoskey State Park, MI	0/6, n/a	n/a
Saugatuck State Park, MI	0/9, n/a	n/a
Van Buren State Park, MI	0/8, n/a	n/a
Warren Dunes State Park, MI	0/8, n/a	n/a
Wilderness State Park, MI	1/5, n/a	n/a
Grand Mere State Park, MI	0/9, 0/9	5/20, 0/9
Pictured Rocks National Lakeshore (Grand Sable Dunes)	1/5, 0/40	0/5, 0/29
Escanaba, MI	0/5, n/a	0/5, 0/33
Marquette, MI, Site 1	1/5, 0/20	0/5, 0/35
Marquette, MI, Site 2	0/5, 0/28	0/5, 0/23
Schoolcraft County, MI	0/5, n/a	0/5, n/a
US Highway 2, MI	0/5, 0/36	0/5, 0/24
'Cape' Stock, Cape Farms (Lewes, DE)	20/20	n/a
Nursery Stock (MI genotype), Natural Garden (St. Charles, IL)	0/20	n/a
'Cape' Stock, Vans Pines Nursery (West Olive, MI)	49/49	n/a
'Vans' Stock, Vans Pines Nursery (West Olive, MI)	0/40	n/a
'Cape' Stock, Church's Nursery (Cape May, NJ)	20/20	n/a
'Cape' Stock, Peat and Sons Nursery (Westhampton, NY)	20/20	n/a

surveyed adjacent dune restoration projects where *A. breviligulata* had been planted at some point in the past 25 y. The specific planting history (plant source, exact year of planting) of most of these restorations was unknown. At each site, we collected 25 tillers from a 10m × 10m area (one every 2 m). For some sites we were also able to collect seeds from individuals, though in general flowering and seed set are very low in this species.

From 2005–2007, we purchased *Ammophila breviligulata* plants from five nurseries in New York, New Jersey, Delaware, Michigan and Illinois to test for EF infection from these suppliers. Because of the very low seed production by *A. breviligulata*, nurseries propagate this species clonally, and sell entire tillers for planting in restorations. Most of these nurseries carried the 'Cape' variety of *A. breviligulata*, which is a vigorous strain originally developed from a New Jersey population by the United States Department of Agriculture, Natural Resources Conservation Center, Cape May Plant Materials Center in Cape May, New

Jersey in the early 1970s (Soil Conservation Service, 1977). The Illinois and Michigan nurseries also carried plants that originated from natural Michigan populations.

To quantify EF infection in tillers, we took thin sections of inner leaf sheath tissue from 5–50 tillers per population (depending on the number of tillers that survived transport) and applied 0.1% aniline blue plus lactic acid stain, which allowed for visual identification of fungal hyphae present in the leaf tissue under 200× magnification (Clark *et al.*, 1983). For seeds, we soaked seeds in 5% sodium hydroxide solution for 24 h, then dissected out the aleurone layer from the seed and stained this layer with the same aniline blue plus lactic acid stain used for staining tillers (Clark *et al.*, 1983). This technique has the limitation of detecting false negatives if EF hyphal densities are low, but it has been widely used to score EF presence and is the cheapest and fastest method currently available. Also, while it is possible that detection of EF could vary with time of sampling, field sampling occurred during mid-growing season, when EF growth should be most prevalent (Ju *et al.*, 2006).

GREENHOUSE EXPERIMENT

In the summer of 2006, we established a greenhouse experiment where we filled 20 plastic pots (23 cm diameter, 30.5 cm deep) with 15:1 mixtures of screened and washed sand (Quikrete Inc., Atlanta, GA) and local field soil (collected near the W.K. Kellogg Biological Station in southwest Michigan). We planted one tiller from a Michigan population (collected from Sleeping Bear Dunes National Lakeshore, Empire MI; 44.82N, 86.06W) and one tiller of the 'Cape' variety (from Vans Pines Nursery, MI) in each pot. We measured the number of leaves and the length of each leaf for each individual, and plants were allowed to grow in the greenhouse for one week before the experiment began. We set maximum greenhouse temperatures at 37 C and provided 6 h of supplemental high pressure sodium light daily to mimic dune conditions.

Treatments were imposed in a 2 × 2 factorial design, with a drought (D+ or D−) and an herbivory treatment (H+ or H−), plus interactions of the two treatments. We constructed 61cm tall cages around each pot using wooden dowels and bridal veil (0.01cm^2 mesh). For the H+ treatment, we used *Trimerotropis maritima* (seaside grasshopper) collected from Grand Mere State Park in southwest Michigan (approximately 42.00 N, 86.50 W). Prior to collection, we observed *T. maritima* feeding on *Ammophila breviligulata* individuals along the foredune region. This species is also known to be an herbivore of *A. breviligulata* located on the Atlantic Coast (Barimo and Young, 2002). We added one grasshopper to each H+ pot, allowing them access to plants for three days at a time to prevent the grasshoppers from destroying the plants as well as from feeding on plants they normally would not choose. For the D+ treatment we watered the plants until pots were soaking wet once a week, while non drought (D−) pots were similarly watered every other day. In the H+D+ treatment, plants were exposed to both drought and herbivory. Plants in the H−D− treatment were caged as a control. Each treatment combination was replicated five times and pots were randomized and rotated on greenhouse benches.

After 60 d, we again measured the number of leaves and the length of each leaf for each plant in the experiment. To quantify individual plant performance in each treatment, we subtracted final total leaf length (summed across all leaves) from the initial total leaf length. We also visually estimated herbivore damage on a 0–5 scale (0 = no herbivory, 5 = 75%+ leaf consumed) for each plant in the H+ treatments. To compare growth of the two plant types, we subtracted leaf length change of the Michigan plant from leaf length change of the 'Cape' plant in each pot (to account for any pot effects). We felt that these were more insightful measures of plant performance than final biomass, as the plants were not necessarily of equal size when planted. We also measured tillering by plants, but only four of

TABLE 2.—ANOVA results for effects of drought and herbivory on growth of the 'Cape' and Michigan plant types in the greenhouse experiment

Stress Source	df	SS	F	P
'Cape' variety:				
Herbivory	1	13,378.1	7.422	0.015
Drought	1	937.8	0.520	0.481
Herbivory × Drought	1	397.4	0.220	0.645
Error	16	28,840.9		
Michigan population:				
Herbivory	1	17,380.8	26.115	<0.001
Drought	1	8181.3	12.293	0.003
Herbivory × Drought	1	4253.8	6.391	0.022
Error	16	10,648.6		

the 40 plants in the experiment sent up an additional tiller during the 60 d of the experiment (two 'Cape' and two Michigan) and so was not insightful for comparisons across treatments.

We examined the effects of the stress treatments on each plant type using factorial ANOVA. We also planned to measure the effects of the stress treatments on differences in performance between the two grass types using factorial ANOVA. Because of our relatively low sample sizes, however, we suspected that the power to detect differences would be low, so we also conducted a confidence interval test of the null hypothesis (no difference in growth between the two plant types) (Steidl and Thomas, 2001).

RESULTS

GREAT LAKES DUNES SURVEY

The majority (18/23 sites) of *Ammophila breviligulata* populations from natural dunes sites in Michigan and Indiana were not endophyte infected (Table 1). The few populations where we did find EF (two sites at Sleeping Bear Dunes National Lakeshore, Pictured Rocks National Lakeshore, Wilderness State Park, MI and one site near Marquette, MI) had relatively low levels of infection (20–50%). In contrast, 'Cape' variety stock plants were 100% infected with EF at all four nurseries where it was sold. The two other nursery varieties ('Vans' and the Natural Garden) showed no infection by EF. We confirmed these patterns for 'Cape' and 'Vans' from Vans Pines Nursery by plating sections of tiller on corn meal malt agar (Bacon & White, 1994). All samples (15/15) from 'Cape' tissue grew cultures morphologically identifiable as *Epichloë*, whereas 'Vans' cultures did not. Given these differences between the 'Cape' and native Great Lakes populations, we expected to find high levels of endophyte in Great Lakes restoration sites. Surprisingly, however, only one restoration site had *A. breviligulata* infected with EF (Grand Mere, with 25% infection, Table 1).

GREENHOUSE EXPERIMENT

Grasshoppers, but not drought, reduced growth of the 'Cape' plants in the experiment and there were no interactions between the two stressors (Table 2, Fig. 2A). Both grasshoppers and drought reduced growth of the Michigan plants and the combination of the two stressors compounded the effect (Table 2, Fig. 2B). When comparing the growth of 'Cape' and Michigan plant types, there were no significant effects of herbivory or drought treatments in the ANOVA (H: $F_{1,20} = 0.11$, $P = 0.75$; D: $F_{1,20} = 1.49$, $P = 0.24$;

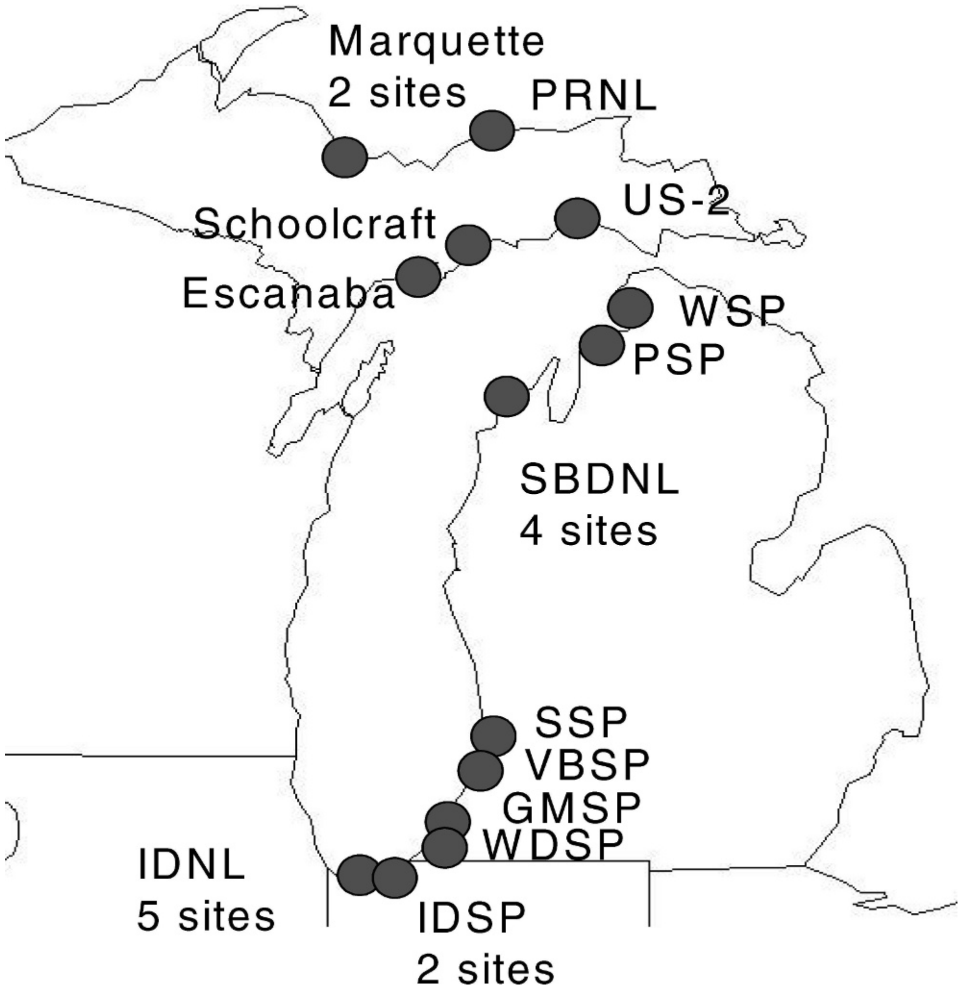

FIG. 1.—Map of sites for survey of endophyte infection in populations of *Ammophila breviligulata*. Site abbreviations correspond to those listed in Table 1. Each site included paired restored and natural dune communities

HxD: $F_{1,20} = 0.85$, $P = 0.37$), though the plant types did differ in herbivory damage (H: $F_{1,20} = 28.84$, $P < 0.001$; D: $F_{1,20} = 8.37$, $P = 0.011$; HxD: $F_{1,20} = 8.37$, $P = 0.001$, Fig. 2C). A retrospective power analysis using the program PASS (Hintze, 2007) indicated low power levels for this study (0.06–0.21 for each factor). To follow up on this analysis, we conducted a comparison of 95% confidence intervals for differences in plant growth in each treatment. This analysis showed that the herbivory (H+D−) and drought (H−D+) treatments had differences in plant growth greater than zero indicating that the 'Cape' type grew more than the Michigan type in these treatments (Fig. 2D), while the H−D− and H+D+ treatments included zero in the 95% CI, indicating no significant difference in growth between plant types.

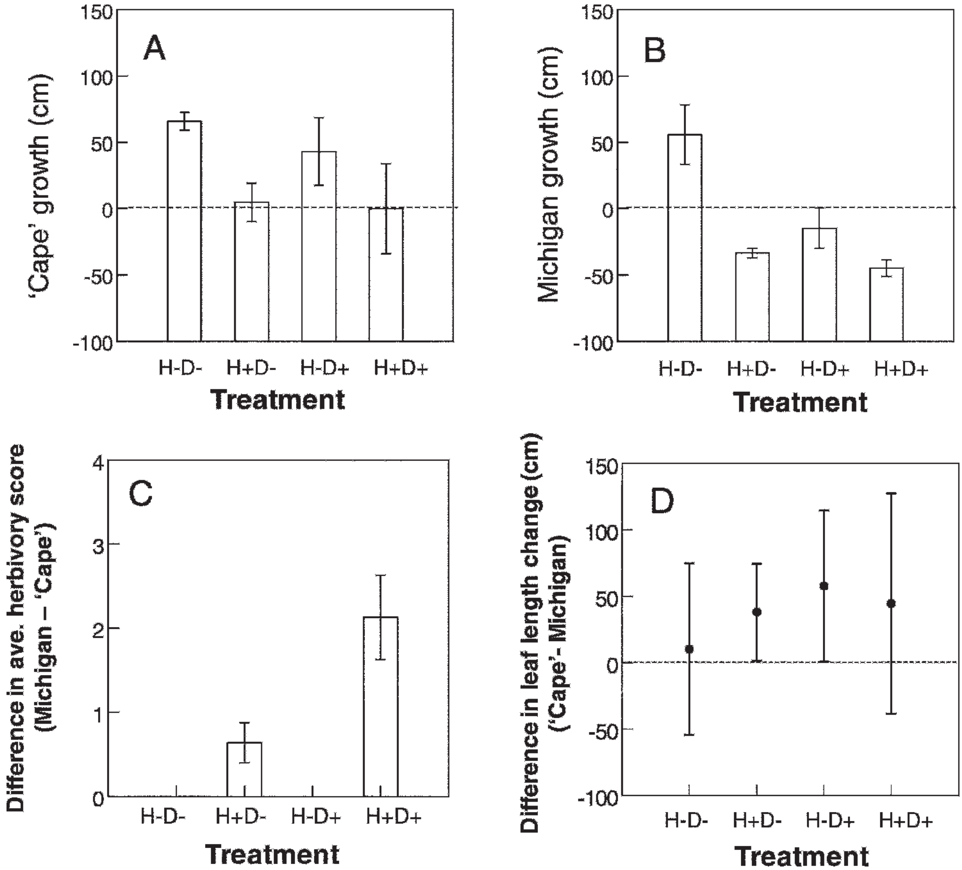

FIG. 2.—Leaf length change (growth) of 'Cape' (A) and Michigan (B) plants in each stress treatment. Positive values indicate growth over time, while negative values indicate senescence or leaf destruction (herbivory) over time. (C) Difference in visually estimated herbivore damage between Michigan and 'Cape' plants in each stress treatment (on relative scale from 0–5, where 0 = no herbivory and 5 = 75%+ leaf consumed). Positive values indicate more herbivore damage on Michigan plants than the 'Cape' plants grown with them. (D) Average differences in leaf length change (growth) between 'Cape' and Michigan plants in each stress treatment. Positive values indicate more growth in 'Cape' than Michigan, while negative values indicate more growth in Michigan than 'Cape'. A difference of zero (dashed line) indicates no difference in growth between 'Cape' and Michigan plants. Error bars represent one +/−SE (A–C) or 95% CIs (D)

DISCUSSION

GREAT LAKES AMMOPHILA AND ENDOPHYTES

We showed that many populations of *Ammophila breviligulata* sampled in Indiana and Michigan had no evidence of EF infection. However, EF are not absent from this area. At least five sites had *A. breviligulata* populations with low to moderate levels of EF infection (≤50%, Table 1). Interestingly, these five sites were all located in the northern lower peninsula and upper peninsula of Michigan. Given the potential benefits of EF for drought tolerance (Malinowski and Belesky, 2000; Malinowski *et al.*, 2005), we would have expected higher frequencies of EF in southern populations with higher rates of evapotranspiration, as has been reported for other EF infected grass species (Lewis *et al.*, 1997; Leyronas and Raynal, 2001). More extensive surveys spanning the geographic range of *A. breviligulata* would help generate hypotheses about climatic factors that could influence EF distribution within this species.

We expected that populations would typically be 100% infected with EF or 100% free of EF for several reasons. First, EF are rarely horizontally transmitted in this system (White *et al.*, 1992). Second, *Ammophila breviligulata* reproduces almost exclusively by clonal growth, resulting in very few genotypes in a given population. For example, Fant *et al.* (2008) found only 1–3 genotypes m^{-2} and 1–13 genotypes per population in Illinois and Wisconsin sites. Third, a study by White *et al.* (1992) along the New Jersey coast showed a similar infection pattern, although suffered from small sample sizes. White *et al.* (1992) also found an increase in populations with EF presence through time, possibly reflecting the increased use of the 'Cape' variety in restorations along the coast after 1971. This expected pattern does not seem to hold for Great Lakes populations, which hosted either no, or relatively low levels of infection. While it is possible our method of scoring turned up false negatives, all 'Cape' plants were 100% infected, suggesting that scoring was reliable. Temporal and spatial variation in the costs and benefits of *Epichloë* (Clay and Schardl, 2002; Morse *et al.*, 2002), as well as the potential for imperfect vertical transmission to seeds (Ravel *et al.*, 1997) could explain the maintenance of low levels of symbiosis in some natural populations.

Surprisingly, only one of the restorations we examined showed any presence of EF infection, despite a history of *Ammophila breviligulata* plantings over the past 30 y, when presumably the only commercial variety available would have been the 'Cape' variety. It is possible that *A. breviligulata* could lose its infection over time, especially if the EF are detrimental to the plants in a new habitat, or if seeds or tillers lose the infection (Clay *et al.*, 1989; Faeth, 2002; Sachs and Simms, 2006). Great Lakes dunes may be less osmotically stressful for plants than ocean dunes due to lack of salt water, and so EF may not be as beneficial in this system. However, persistent low levels of EF infection in natural populations may indicate at least occasional benefits of this symbiosis. Further, because restoration records were often poorly kept, there is a need to verify the identity of these plants before we can confidently claim that EF infections are lost in these populations.

HERBIVORY, DROUGHT AND AMMOPHILA

In the greenhouse experiment, the 'Cape' type of *Ammophila breviligulata* had less herbivory damage than the Michigan type. This could be a result of local adaptation of the grasshopper (Hanks and Denno, 1994; Mopper and Strauss, 1998), as only grasshoppers collected from Michigan were used in these experiments. However, the Michigan plants used in the greenhouse experiment were collected approximately 350km north of the site where the grasshoppers were collected, in an area where *Trimerotropis maritima* does not occur (Scholtens *et al.*, 2005). Thus, we think it unlikely that local adaptation by the herbivore was the cause of differences in herbivore resistance between the *A. breviligulata* populations.

EF infection may explain the reduced herbivore damage on 'Cape' plants, though we cannot be certain of this because of the confounding of plant type with EF infection status. However, EF infection is widely known to reduce herbivory in several agricultural grass species (*e.g.*, *Lolium perenne*, *Lolium arundinaceum*) due to the production of peramine and loline alkaloids (reviewed by Schardl *et al.*, 2004; Rudgers and Clay, 2005). While the effects of EF on insect herbivores of native grasses have received less attention and can vary with environmental conditions (Saikkonen *et al.*, 1998), EF often deter herbivores in other systems that have been rigorously tested (reviewed by Clay and Schardl, 2002; Schardl *et al.*, 2004; Rudgers and Clay, 2005).

EF have also been shown to increase drought tolerance of host plants by increasing osmotic adjustment, increasing stomatal resistance, moderating rates of photosynthesis, increasing leaf rolling and increasing growth of root hairs (Malinowski *et al.*, 1999; Bultman and Bell, 2003; Schardl *et al.*, 2004; Malinowski *et al.*, 2005). In our greenhouse experiment, 'Cape' plants maintained positive growth under drought stress, while Michigan plants lost leaf length due to leaf senescence. Again, this may be explained by differences in local adaptation to osmotic stress between the two *Ammophila breviligulata* populations, as we cannot separate plant genotype from endophyte presence in this study. Osmotic stress is expected to be greater along the Atlantic coast due to salt spray and increased soil salinity (Boyd and Barbour, 1986). However, drought and high evapotransporation rates are common in young dunes along the Great Lakes as well (Lichter, 1998).

While the 'Cape' variety was a better performer than the Michigan plants under drought and herbivory stress, the combination of both stresses eliminated significant differences between 'Cape' and Michigan plant growth. While other studies have proposed that the plant benefits of EF infection should be maximized at extreme environmental conditions (Saikkonen *et al.*, 1998; Faeth and Fagan, 2002), we know of only one study that has specifically examined the interaction between drought and herbivory on EF infected plants. Bultman and Bell (2003) found that drought generally increased EF infected plant resistance to fall armyworms (*Spodoptera frugiperda*), but not to aphids. The costs of maintaining a symbiont in high stress environments may cancel any benefits conferred by the endophyte (Johnson *et al.*, 1997)

CONCLUSIONS

Our greenhouse study demonstrates the importance of considering both plant source and symbionts in planning restoration efforts. Though we are unable to explicitly separate effects of plant type from effects of EF infection in this study, studies in other systems have shown that EF infected plants can slow successional dynamics (Rudgers *et al.*, 2007). Nursery stock plants of 'Cape' are 100% infected with EF, and tolerate drought and herbivory better than native Michigan plants. While the 'Cape' variety is widely available for restoration efforts throughout the Eastern US, managers should consider the possible effects of cryptic symbionts such as endophytes, as well as plant genotype, when selecting stock populations for restorations. Further work is needed to confirm field performance of this variety, to understand the implications of altering EF infection frequencies in dune restorations, as well as to add to our knowledge of mechanisms regulating biodiversity of dune systems.

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