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REGULAR ARTICLE

Fungal endophytes from seeds of invasive, non-native *Phragmites australis* and their potential role in germination and seedling growth

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Abstract
Background and aims We characterized fungal endophytes of seeds of invasive, non-native *Phragmites* from three sites in the Great Lakes region to determine if fungal symbiosis could contribute to invasiveness through their effects on seed germination and seedling growth.
Methods Field-collected seeds were surface sterilized and plated on agar to culture endophytes for ITS sequencing. Prevalence of specific endophytes from germinated and non-germinated seeds, and from seedlings, was compared.
Results One-third of 740 seeds yielded endophyte isolates. Fifteen taxa were identified with *Alternaria* sp. representing 54% of all isolates followed by *Phoma* sp. (21%) and *Penicillium corylophilum* (12%). Overall germination of seeds producing an isolate (36%) was significantly higher than seeds not producing an isolate (20%). *Penicillium* in particular was strongly associated with increased germination of seeds from one site. Sixty-three isolates and 11 taxa were also obtained from 30 seedlings where *Phoma*, *Penicillium* and *Alternaria* respectively were most prevalent. There was a significant effect of isolating an endophyte from the seed on seedling growth.
Conclusions These results suggest that many endophyte taxa are transmitted in seeds and can increase seed germination and seedling growth of invasive *Phragmites*. The role of fungal endophytes in host establishment, growth and invasiveness in nature requires further research.

Keywords *Phragmites australis* · Seed endophytes · Biological invasion · Germination · Seedling growth · Fungi

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Introduction

Endophytic fungi occur in all plant species thus far examined (U'Ren et al. 2012) where they engage in a wide variety of interactions with their plants hosts. By definition, endophytes are organisms that live inside of plants and do not cause disease symptoms on their host (Wilson 1995). Endophytes can include mutualists that enhance host plant fitness, latent pathogens that may or may not express disease symptoms at some point in the future, or saprophytes that are inactive until tissue senescence (Müller et al. 2001; Osono 2006; Kleczewski et al. 2012). Fungal endophytes also exhibit variation in their mechanisms of transmission from one host to another including strict vertical transmission from maternal plants to seeds (Rodriguez et al. 2009), contagious transmission from one host plant to another (Clay and Schardl 2002; Porras-Alfaro and Bayman 2011), or

infection by spores from environmental sources like wind, rain, soil, and leaf litter (Christian et al. 2015).

Vertically-transmitted endophytes are predicted to have a mutualistic association with their host because detrimental endophytes should be rapidly purged from the host population (Ewald 1987; Lipsitch et al. 1995). For example, many *Epichloë* endophytes infecting cool-season grasses such as tall fescue (*Lolium arundinaceum*) are only vertically transmitted through seeds and are typically mutualistic (Schardl and Clay 1997; Panaccione et al. 2014). Other endophytes infecting different groups of plants can also be vertically transmitted and are potentially mutualistic (Ernst et al. 2003; Hodgson et al. 2014), but they have not been subject to as much past research as *Epichloë* endophytes. By contrast, many fungi are seed borne (Baker and Smith 1966; Rheeder et al. 1990; Martin 1996; Donald et al. 2005) following contagious infection of developing flowers or seeds by spores. For example, plants arising from seeds infected by loose smut of barley and wheat (caused by *Ustilago* spp.) produce diseased inflorescences containing spore masses that can then contagiously infect flowers and seeds of non-diseased plants (Wunderle et al. 2012). With strict vertical transmission, every seed produced by an individual plant could be infected by a single endophyte whereas in the case of seed-borne fungi each seed produced by one plant could be independently infected by distinct fungi from different sources.

The microbiota of seeds may be an important source for colonization of the next generation of seedlings and adult plants (Hodgson et al. 2014; Klaedtke et al. 2015; Truyens et al. 2015). The seed microbiota may be especially important for invasive plant species given that there are no pre-existing conspecific populations or associated microbes present upon initial colonization of a new habitat (Newcombe et al. 2009). Vertically-transmitted, mutualistic fungi may enhance the ability of invasive host plants to colonize new habitats, compete with established species, and resist parasites and pathogens (Aschehoug et al. 2012; Saikkonen et al. 2013). For example, infection by vertically-transmitted endophytic fungi was found to be responsible for the competitive advantage of the invasive tall fescue grass (Rudgers et al. 2005; Rudgers and Clay 2008). By contrast, the absence of mutualistic fungi can limit the success of non-native species. For example, plantings of pines (*Pinus* sp.) in the southern hemisphere were initially unsuccessful until compatible ectomycorrhizal symbionts were also introduced (Pringle et al. 2009). On the other hand, fungal pathogens may limit the establishment and growth of non-native plant populations (Biotic Resistance Hypothesis) so that they never become established or reach densities high enough to displace native species (Mack 1996; Knevel et al. 2004; Parker and Gilbert 2004). However, outside of agricultural species, we have little knowledge of failed invasions due to pathogenic fungi (Scheffer 1997). Understanding the diversity and impacts of fungi associated with seeds of invasive plant species will help evaluate risks and design effective management strategies.

Phragmites australis (Cav.) Trin. ex Steud.) (hereafter *Phragmites*) is a clonal wetland grass that is distributed worldwide in temperate and subtropical regions (Haslam 1972; Kirk et al. 2011; Guo et al. 2013). Haplotype M is an aggressive invader that is widespread in the United States (Saltonstall 2002, 2003). Invasive, non-native *Phragmites* (hereafter invasive *Phragmites*) can result in the displacement of native plant and animal species (Keller 2000; Holdredge and Bertness 2011; Kessler et al. 2011; Price et al. 2013), and alter wetland hydrology and soils (Windham and Lathrop 1999). Several mechanisms have been proposed to explain the success of invasive *Phragmites,* including its potential association with mutualistic fungal endophytes (Kowalski et al. 2015; Clay et al. 2016). In a recent study, fungal endophytes associated with leaves, stems, and rhizomes of invasive *Phragmites* were isolated and sequenced to reveal significant differences in endophyte community structure among tissue types and populations (Clay et al. 2016). The functional role of these endophyte associations is not known, but one commonly isolated fungal genus in the Great Lakes region (*Stagonospora,* Clay et al. 2016) has been reported to be vertically transmitted through seeds and to enhance *Phragmites* growth in Europe (Ernst et al. 2003).

The goals of this study were to isolate and identify fungal endophytes from seeds and seedlings of invasive *Phragmites* from the Great Lakes region of the United States and to explore their potential impacts on seed germination and seedling growth. While our results are derived from a limited number of sites and an unknown number of established *Phragmites* genotypes, they represent the first critical analysis of fungal endophytes in the seeds of this important invasive species. Further, because of previous results based on endophyte isolations from leaf, stem, and rhizome tissues of adult *Phragmites*, a general comparison of endophyte

diversity in seeds and seedlings with adult plants could potentially provide insights into the ability of endophytes to be transmitted in seeds and colonize ensuing plants. In particular, we addressed the following questions:

Do fungal endophytes colonize *Phragmites* seeds and if so, what fungi?
Do fungal endophytes in seeds colonize *Phragmites* seedlings and if so, what fungi?
Does endophyte colonization affect the germination of seeds or growth of seedlings?
Are there differences in fungal endophyte communities among sites, between seeds and seedlings, or between seeds and adult plants (with data from Clay et al. 2016)?

Our results may help to identify potential microbial mechanisms of invasiveness and biocontrol for *Phragmites*, and provide insights into the success of other invasive plant species.

Methods

Site description and seed collection

Seeds were collected in September and October 2014 from three sites across southeast Michigan and northwest Ohio (Supplemental Fig. 1). At all sites, *Phragmites* was identified as the non-native, invasive haplotype based on morphology (Saltonstall et al. 2005). The Sandusky State Game Area (SAN) is an approximately 600 ha former peat bog located in Sandusky, MI (43°24′ 04″N, 82°47′ 04″W). After commercial harvest ended, a thin layer of dead peat material (~30 cm) was spread over the surface and the majority of the disturbed surface was then colonized by *Phragmites*. The Turtle Creek public access site (TC) is located in Oak Harbor, OH (41° 36′ 17″N, 83° 08′ 43″ W) on the western end of Lake Erie along the banks of Turtle Creek. We also collected seeds from *Phragmites* along nearby Crane Creek (CC, 6 km from the TC site) within the U.S. Fish and Wildlife Service Ottawa National Wildlife Refuge near the mouth of Crane Creek to Lake Erie (41° 37′ 17″N, 83° 12′ 28″W).

Seeds were collected at each site by clipping the entire inflorescence from 5 to 10 shoots within a single patch of *Phragmites* from multiple patches within each site. It is likely that different patches represented different genotypes (or clones), but we did not do any diagnostic tests to identify distinct *Phragmites* genotypes. Seed-borne endophytes or those from environmental sources could be unrelated to plant genotype whereas seed-transmitted endophytes could be more specific to individual genotypes. Upon return to the lab, a subset of florets were dissected to verify viable seed was present. Inflorescences were then wrapped in filter paper, placed into trays, and buried below a thin layer of moist topsoil. Trays were kept at approximately 4 °C for six weeks for cold-stratification (Kettenring and Whigham 2009). After the stratification period, florets were removed from inflorescences by manually rubbing seeds with attached glumes across a 1.7 mm square wire mesh, placed into envelopes labeled by site, shipped to Indiana University, and stored at room temperature. Each envelope contained >10,000 seeds (based on weight). Given that our goal was to obtain a broad sampling of seeds from each site, and that the genetic identities of *Phragmites* patches and the source of endophyte colonization was not known, all seeds from different patches and inflorescences collected at a single site were bulked.

Seed sterilization and plating

Three hundred undamaged seeds per site (900 total) were randomly selected and inspected using a dissecting scope to ensure that they were undamaged by the separation process. Seeds were then placed on the membrane of a 0.22 μm vacuum filtration apparatus inside a laminar flow hood and were surface sterilized using a modified method described by Schulz et al. (1993). Seeds were first submerged in 70% ethanol for 60 s, then 0.5% hypochlorite for 30 s, then 95% ethanol for 60 s, and sterile water for 120 s. Between each soaking step, the previous liquid was vacuumed off the seeds rapidly to prevent excessive damage to the seeds from the sterilizing agents. We plated 250 sterilized seeds from each site individually on 60 × 15 mm water agar (WA) petri plates to assess germination and to isolate endophytes growing from the seeds. 10 mL of sterile water was applied to the seed on each plate to promote germination. We also plated 50 sterilized seeds from each site on corn meal agar (CMA) to determine if germination and endophyte isolation differed based on media. However, endophyte isolates grew rapidly on CMA plates and engulfed the seed, making it impossible to assess germination status.

To test the effectiveness of our surface sterilization technique, two seeds from each site and batch of 40–50 seeds processed were placed on CMA plates for 5 min following seed sterilization before they were removed (a total 12–15 seeds per site). The absence of subsequent fungal growth on any of these control plates indicated that our surface sterilization procedures were effective. All plates were sealed with Parafilm and then incubated at 24 C under 40 watt cool white fluorescent bulbs (12 h on, 12 h off).

Seed plate monitoring and culture isolation

Plated seeds were monitored for germination (WA plates only) and fungal growth (WA and CMA plates) daily for two weeks. Both germination and fungal growth were documented on the first day of germination or when fungal growth became apparent. Germination was defined as the emergence of the shoot from the seed coat. Every seed was checked for germination at the end of the 14-day period via microscopy to identify more cryptic cases of germination due to the small size of *Phragmites* seeds (0.12 mg per seed). Mycelium resulting from fungal growth from seeds was transferred onto fresh CMA plates to obtain sufficient mycelium for DNA extractions. Isolates were incubated for 14 days under the same conditions used for seed germination.

Seedling planting, sterilization, and plating

Seedlings from WA plates were planted within seven days of germination. The seedlings were carefully removed from the agar and rinsed thoroughly using sterile distilled water to remove external fungal hyphae that may have grown from the seed and any adhering water agar. Individual seedlings were then planted in autoclaved greenhouse soil in independent cells within seedling trays with clear plastic tops to maintain high soil moisture and humidity levels. Seedlings were then grown for 30 days in incubators at 24 C with a 12 h light:dark cycle. After 30 days, surviving seedlings were washed with sterile distilled water to remove any soil from roots. The length of both the shoot and longest root was measured and the number of leaves was counted on each seedling. Biomass was not determined because fresh tissue was required for endophyte isolation. To isolate endophytes from seedlings, seedlings were surface sterilized (rinsing in 70% ethanol for 30 s, 0.5% hypochlorite for 30 s, 95% ethanol for 30 s, and sterile water for 30 s). The roots and shoots of each seedling were then separated and placed on separate CMA plates, which were sealed and allowed to incubate for 14 days using the same methodology and conditions for seed-derived endophytes.

Molecular techniques

DNA was extracted directly from mycelium obtained from fungal isolates from seeds and seedlings using PowerPlant® Pro DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. For potential future use, we also collected vouchers from each of the isolates and stored the samples in 750 mL sterile distilled water at room temperature in 2.0 mL microcentrifuge tubes. The fungal ITS region (ITS1, 5.8 s rRNA, and ITS2) of the nuclear ribosomal DNA was amplified by the Polymerase Chain Reaction (PCR) using the GoTaq® HotStart DNA Polymerase (Promega Corporation, Madison, WI, USA) as per the manufacturer's recommendations in a 25 μL reaction with 1 μL of template (1–25 ng of DNA). The primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used since this region is commonly used for fungal species identification (White et al. 1990). We used a Tetrad PTC-225 Peltier Thermal Cycler (MJ Research, MA, USA) to perform the PCR reaction using the thermal cycler program recommended by Promega, except for changes to the denaturation time (30 s), annealing temperature and time (60.2 °C for 30 s), and extension time (40 s) (Clay et al. 2016). Amplicons were prepared for sequencing using the MicroElute® Cycle-Pure Kit (Omega Bio-tek, Inc., GA, USA) following manufacturer's instructions. Lastly, all amplicons were sequenced in the forward and reverse direction via Sanger Sequencing at the Indiana Molecular Biology Institute at Indiana University, Bloomington, IN.

Bioinformatic analysis

To determine the identity of the isolates, sequences were manually inspected and edited using CodonCode Aligner v. 6.0.2 (CodonCode Aligner Company). Forward and reverse reads were paired manually, and then these sequences were grouped into operational taxonomic units (OTUs) based on the following parameters: ≥ 95% sequence similarity over ≥40% of the paired sequences being compared (Del Olmo-Ruiz and Arnold 2014). Identification of consensus sequences was performed

using the Ribosomal Database Project (RDP) Bayesian Classifier with the Warcup ITS training set (Deshpande et al. 2016). BLAST results from the sequence data are also reported in Supplemental Table 1 for direct comparison with a previous paper on *Phragmites* endophytes from adult plant tissues where endophyte taxa were identified using BLAST (Clay et al. 2016).

Statistical analyses

OTU accumulation curves were calculated for seed samples from all three sites (R Core Team 2013, vegan package, program "specaccum"). Community data were then rarified to account for the small differences in sample sizes among sites (resulting from a few contaminated plates) and to obtain Chao estimators of species richness ("specpool" function), which provide a metric for highly skewed community abundance distributions. To test for differences in endophyte community composition among sites (where seed collections were bulked by site), we conducted permutational multivariate analysis of variance (PERMANOVA, 999 permutations) and two-dimensional non-metric multidimensional scaling (NMDS) for visualization purposes (function "Adonis"). Only non-singleton OTUs were used for analyses of community similarity. Fishers Exact Test (function "fisher.test",) was used to determine differences in germination success among sites and by endophyte infection (for seeds producing no OTUs or OTU1, 2 or 3). Finally, to determine if endophytes affected seedling growth, ANOVA was used to test whether there was a significant effect of isolating an endophyte from the seed on total seedling length at 30 days or a significant effect of any specific endophyte on seedling length. Given that there were only three seedlings from the SAN site, only seedlings from the TC and CC sites were included in this analysis.

The relationship among all experimental procedures and analyses is presented in a flow chart (Fig. 1).

Results

A total of 290 endophyte isolates were obtained from *Phragmites* seeds. Sixty-six isolates were from 150 seeds plated on CMA and 224 isolates were from 750 seeds plated on WA (Table 1). In some cases two or more morphologically distinct endophytes grew from a single seed on WA. A total of 31.9% of seeds on WA germinated, but endophyte isolates were obtained from both germinated and non-germinated seeds (Table 2). Germination rate for seeds producing an endophyte isolate was 36.2% and ranged from 22.5% (CC) to 49.2% (SAN) among sites. By contrast, the germination rate of seeds that did not produce an endophyte isolate was 20% and ranged from 6.5% for site SAN up to 32.4% from site CC. Thus, over all sites, mean germination was significantly higher for seeds producing endophyte isolates compared to those that did not (Fisher's Exact Test, $P < 0.0001$, Table 2), although for site CC germination of seeds producing isolates was slightly lower than that of seeds not producing isolates ($P = 0.126$). Fisher's Exact Test indicated a highly significant difference ($P = 0.003$) in germination rates among seeds infected by OTU 1 (33%), OTU 2 (30%), and OTU 3 (60%) when all sites were pooled together (Fig. 2). When seeds from individual sites were analyzed independently, only SAN still showed a significant effect of OTU identity on germination rate ($P = 0.005$).

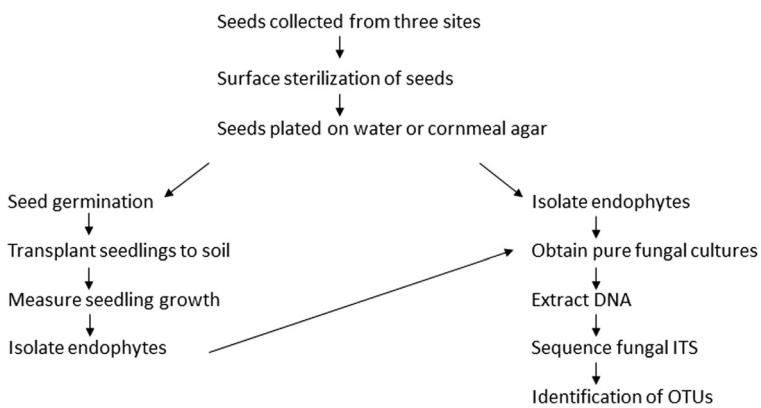

Fig. 1 Flow chart of experimental procedures and analyses used in this study

The most frequent isolate corresponded to an *Alternaria* sp. (OTU1) and comprised 54% of all isolates (Tables 1 and 3). The second most frequent OTU corresponded to a *Phoma* sp. (OTU2) and comprised over 20% of isolates. Both OTU1 and OTU2 were isolated from seeds from all three sites. The third most frequent isolate corresponded to *Penicillium corylophilum* (OTU3) and comprised 12% of all isolates. OTU3 was isolated primarily in one site (SAN), where it was the most frequent isolate, and was never isolated from seeds from the CC site. OTU2 and OTU7 both corresponded to *Phoma* sp. but were sufficiently divergent to be considered distinct taxa. Similarly, OTU3 and OTU10 both corresponded to *Penicillium corylophilum* but were also sufficiently divergent to be considered distinct taxa. OTUs 4–12 were less frequent, isolated from two to seven times across all sites, while OTUs 13–15 were singletons and isolated only once. Less common OTUs of particular interest include OTU4, which was most closely related to a *Phaeosphaeria* sp. (previously identified as *Stagonospora neglecta* with BLAST, Supplemental Table 1) and was isolated from all three sites.

Table 1 Distribution of fungal endophyte OTUs isolated from seeds of *Phragmites australis* collected from three different sites (Crane Creek (CC), Sandusky (SAN) and Turtle Creek (TC)). OTUs are listed in descending order of prevalence (#1 most common, #15 least common) with the number of isolates from each site. Identities of each OTU are provided in Table 3. Isolates were obtained from both germinated and ungerminated seeds, and from both water agar (WA, $N = 250$ seeds per site) and cornmeal agar plates (CMA, $N = 50$ seeds per site)

OTU	CC	SAN	TC	Total
1	55	23	79	157
2	31	16	13	60
3	0	27	8	35
4	1	5	1	7
5	1	3	2	6
6	1	1	4	6
7	4	1	0	5
8	1	0	2	3
9	1	0	1	2
10	1	0	1	2
11	0	1	1	2
12	2	0	0	2
13	0	0	1	1
14	0	0	1	1
15	1	0	0	1
From WA	72	59	93	224
From CMA	27	18	21	66
Total	99	77	114	290

Species accumulation curves generated for each site indicated that additional seed sampling would result in additional OTUs beyond those isolated from 300 seeds per site (Supplemental Fig. 2). Given the slightly different number of seed samples per site yielding endophyte isolates (resulting from <1% of plates becoming contaminated), data were rarified, and Chao estimators of diversity suggested that between 12 (SAN) and 36 (CC) additional OTUs could be isolated if larger numbers of seeds were sampled. This result is in general agreement with the actual number of OTUs isolated from seeds from each site (Table 1) where the fewest number of OTUs were isolated from seeds from the SAN site.

The endophyte communities isolated from *Phragmites* seeds were significantly different among sites (PERMANOVA, $r^2 = 0.088$, $P < 0.001$) although two-dimensional non-metric multidimensional scaling indicated substantial overlap of endophyte communities among sites, especially between CC and TC (Supplemental Fig. 3). Thus, while the endophyte communities among sites were statistically different, those differences were not dramatic.

Mortality of small seedling transplants was high, primarily within the first few days following transplantation, but 30 seedlings survived at least 30 days, when endophytes from shoots and roots were isolated. From these 60 tissue samples (30 seedlings x two tissues), 63 endophyte isolates were obtained and 47 isolates were successfully sequenced, corresponding to 11 OTUs (Table 4, Supplemental Table 2). Five of the 11 OTUs were singletons. The three most frequent isolates (OTUs 2, 3, and 1, respectively) were the same three most frequent isolates from seeds (Table 1), but their relative order differed. OTUs 4, 8, 9, 10, and 15 were also isolated from both seeds and seedlings, while four other OTUs (16, 17, 18, and 19) were isolated from seedlings but not from seeds. Most surviving seedling isolates came from either CC or TC (only two surviving seedlings were from SAN). PERMANOVA indicated no significant difference among endophyte communities in seedlings from the proximate CC and TC sites ($r^2 = 0.052$, $P = 0.276$). In one case (seed 632), the endophyte isolated from the seed corresponded to the endophyte isolated from both the shoot and roots of the seedling (Supplemental Table 2).

We also tested whether there were differences between endophyte communities in seeds and seedlings. PERMANOVA indicated that there were significant

Table 2 Total number of seeds plated on water agar that a given OTU was isolated by site (Crane Creek (CC), Sandusky (SAN) and Turtle Creek (TC)), and the percentage of those seeds that germinated (in parentheses). Dashes indicate that the given OTU was not isolated from that site. The specific identities of OTUs are given in Table 3. The No OTU category indicates that no endophytes were isolated from those seeds

OTU	Site			All sites
	CC #seeds (%germ)	SAN #seeds (%germ)	TC #seeds (%germ)	#seeds (%germ)
1	41 (26.8%)	14 (35.7%)	63 (36.5%)	118 (33.1%)
2	21 (19.0%)	12 (25.0%)	10 (60.0%)	43 (30.2%)
3	-	27 (74.1%)	8 (12.5%)	35 (60.0%)
4	-	2 (50.0%)	1 (100.0%)	3 (66.7%)
5	1 (0.0%)	1 (0.0%)	2 (0.0%)	4 (0.0%)
6	1 (0.0%)	1 (0.0%)	3 (33.3%)	5 (20.0%)
7	3 (0.0%)	1 (0.0%)	-	4 (0.0%)
8	1 (0.0%)	-	1 (100.0%)	2 (50.0%)
9	1 (0.0%)	-	1 (100.0%)	2 (50.0%)
10	1 (100.0%)	-	1 (100.0%)	2 (100.0%)
11	-	1 (0.0%)	1 (100.0%)	2 (50.0%)
12	1 (0.0%)	-	-	1 (0.0%)
13	-	-	1 (0.0%)	1 (0.0%)
14	-	-	1 (0.0%)	1 (0.0%)
15	1 (0.0%)	-	-	1 (0.0%)
Total	72 (22.5%)	59 (49.2%)	93 (38.7%)	224 (36.2%)
No OTU	176 (32.4%)	185 (6.5%)	155 (21.9%)	516 (20%)
Grand Total	248 (29.4%)	244 (16.8%)	248 (28.2%)	740 (24.9%)

differences in communities from seeds and seedlings ($r^2 = 0.02$, $P = 0.003$), as well as significant differences between sites (the two seedlings from SAN were excluded, $r^2 = 0.08$, $P = 0.001$), but no interaction between

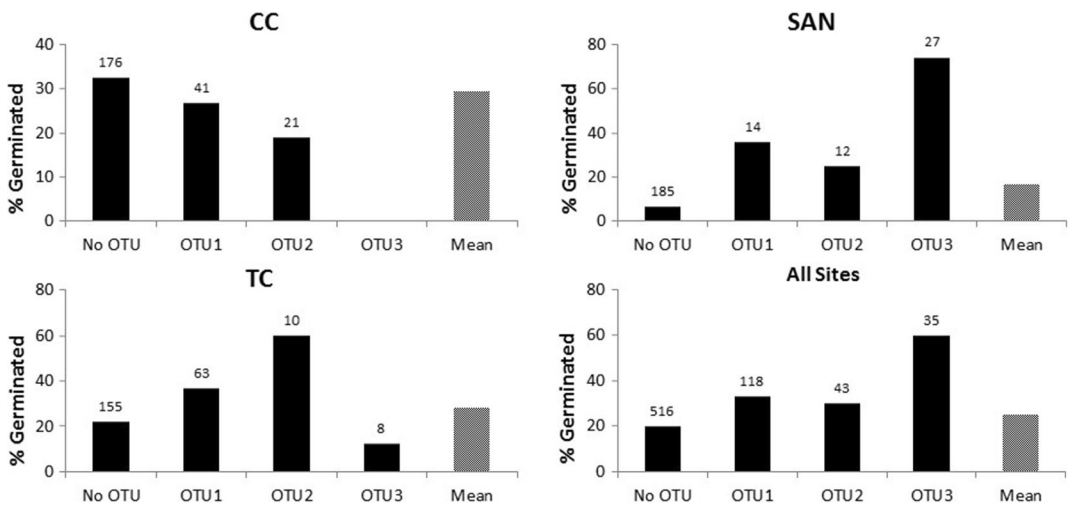

Fig. 2 Germination rate of seeds from different sites (Crane Creek (CC), Sandusky (SAN) and Turtle Creek (TC)) and infected by OTU1, OTU2 or OTU3, or no OTU (black bars). Sample sizes are given above each bar. The mean germination rate of all seeds from each site is also presented (grey bars)

Table 3 OTUs isolated from seeds and seedlings of *Phragmites australis* with their closest match and confidence level for each hierarchical level. Identification of consensus sequences was performed using the RDP Bayesian Classifier with the Warcup ITS training set. OTUs are designated in order of prevalence (#1 most common). Isolates were obtained from both germinated and ungerminated seeds, seedlings, and from both water agar and cornmeal agar plates. OTUs 16–19 were isolated only from seedlings while OTUs 5, 6, 7, 10, 11, 12, 13 and 14 were isolated only from seeds

OTU	Closest Match	Confidence Level (%)	Accession Number
1	Alternaria/ *Alternaria alternata*	100/47	KU366278.1
2	Didymellaceae/Didymella/ *Phoma glomerata*	100/84/78	KF367493.1
3	Eupenicillium/ *Penicillium corylophilum*	100/78	KP016813.1
4	Pleosporales/Phaeosphaeriaceae/ Phaeosphaeria/ *Phaeosphaeria* sp. JF_2013	100/68/53/20	AJ496630.1
5	Nectriaceae/Gibberella/ *Fusarium pseudograminearum*	100/92/34	HG970335.2
6	*Leptosphaerulina chartarum*	100	HQ607815.1
7	Didymellaceae/Didymella/ *Phoma glomerata*	100/88/86	KF367493.1
8	Davidiella/*Cladosporium pseudocladosporioides*	100/62	KU182497.1
9	Chaetomium/ *Chaetomium globosum*	100/98	GQ376099.1
10	Eupenicillium/ *Penicillium corylophilum*	100/82	KP016813.1
11	Dothideomycetes/ Pleosporomycetidae/ Pleosporales/ Pleosporales_Incertae sedis/ Pyrenochaeta/ *Pyrenochaeta gentianicola*	100/97/97/62/38/37	KF800484.0
12	*Phaeosphaeria herpotrichoides*	100	JX981464.1
13	Dothideomycetes_Incertae sedis/ Myxotrichaceae/ Gymnostellatospora/ *Gymnostellatospora japonica*	100/53/53/53	HG937009.1
14	Pleosporales/ Didymellaceae/ Leptosphaerulina/ *Leptosphaerulina chartarum*	100/98/98/98	HQ909081.1
15	*Peziza ostracoderma*	100	HQ829059.1
16	Aspergillus/ *Aspergillus awamori*	100/36	KP196574.1
17	*Aspergillus puniceus*	100	AY373863.1
18	*Hypoxylon investiens*	100	JQ761712.1
19	Eupenicillium/ *Penicillium corylophilum*	100/82	KP016813.1

the two factors. Despite the significant differences, r^2 values were relatively low and two-dimensional non-metric multidimensional scaling (Stress =0.00057, Supplemental Fig. 4) indicated high overlap of these communities.

The growth of seedlings was also measured and analyzed with respect to whether an endophyte grew in the water agar during germination, as well as the identity of the endophyte or endophytes isolated from the seed or seedling (Supplemental Table 2). ANOVA indicated that there was a significant effect of isolating an endophyte from the seed on total seedling length at 30 days (P = 0.015), but there was no effect of any specific endophyte on seedling length (P = 0.10).

Discussion

Our results demonstrate that fungal endophytes frequently occur within the seeds of invasive *Phragmites* and have a measurable effect on seed germination and seedling growth. Endophytes were isolated from one-third of 900 seeds tested, and from both germinated and non-germinated seeds. The presence of OTUs 1, 2, and 3, which comprised over 85% of all isolates, significantly increased germination relative to seeds where no endophyte was isolated. Less common OTUs might also have important effects for host biology, but they would be harder to detect. While relatively few seedlings survived 30 days, we obtained endophyte isolates from 100% of seedlings and typically the same OTU was obtained from both the shoot and roots. Whether an endophyte isolate was obtained from a seed had a significant effect on seedling length after 30 days of growth. Co-dispersal of *Phragmites* and fungal endophytes in seeds could contribute to invasiveness by ensuring that at least some of the plant's microbiota will be immediately present upon colonization of new sites.

We found significant differences in the makeup of endophyte communities among sites. Given that the two

most common OTUs were largely consistent among sites, sites differed primarily in rarer community members. For example, OTU3 was never isolated from the CC site yet was the most common isolate from the SAN site (Table 1). The effects of endophyte infection on seed germination also varied by site. OTU3 was associated with higher seed germination in seeds from the SAN site compared to the overall mean, but seeds from the TC site infected by the same endophyte had reduced germination (Fig. 2). Similarly, seeds from the SAN site infected by OTU2 had reduced germination compared to the overall mean, but seeds from the TC site had elevated germination when infected by the same OTU. In grasses infected with *Epichloë*-type endophytes, seed germination was higher in *Epichloë*-infected vs. uninfected lines of tall fescue and perennial ryegrass (Clay 1987), but Gundel et al. (2006) reported that endophyte infection had little effect on germination behavior in annual ryegrass, *Lolium multiflorum*. Afkhami and Rudgers (2008) examined a range of cool-season grasses and found variable effects but several clear cases of *Epichloë*-infection enhancing germination. Our results demonstrate that, over all sites, seed germination was higher for seeds that produced an endophyte isolate vs. seeds that did not, but the effect of an OTU on germination varied among sites. We also found that there were significant differences in seedling growth after 30 days depending on whether an isolate was obtained from the seed or not, but seedling sample sizes were much lower than those for seeds. Cool-season grasses infected by *Epichloë* endophytes often exhibit greater growth of seedlings than non-symbiotic conspecifics (Clay and Schardl 2002).

In a recent study, we isolated fungal endophytes from leaves, stems, and rhizomes from invasive *Phragmites* from two of three same sites sampled here although from two years earlier (Clay et al. 2016). The most common OTU isolated from adult tissues (*Sarocladium strictum*) was never isolated from the seeds or seedlings tested here. This suggests that this endophyte does not occur in *Phragmites* seeds, despite its high prevalence, although seasonal or annual variation between sampling periods could result in different endophyte communities. For example, Del Olmo-Ruiz and Arnold (2014) found significant differences in endophyte communities from tropical ferns between sampling years. By contrast, two other common OTUs from Clay et al. (2016) are closely related to OTUs 1 and 2 here (*Alternaria* and *Phoma*), which are frequently isolated from seeds and resulting seedlings. In agreement with the results of Ernst et al. (2003), we also found that OTU4 (*Phaeosphaeria* sp., *Stagonospora neglecta* using the Genbank database and BLAST) was common in adult tissues and was also isolated from seeds and seedlings. Overall, the results presented here, in combination with those from Clay et al. (2016), indicate that some endophytes colonizing adult *Phragmites* tissues are transmitted through seeds and colonize resultant seedlings while other common endophytes in adult tissues were never isolated from seeds.

The presence of endophytic microbes in seeds has been reported in a variety of systems including monocots and dicots, woody and herbaceous plants, and crop species (Bloomberg 1966; Ganley and Newcombe 2006; Hodgson et al. 2014; Shen et al. 2014; Truyens et al. 2015; Parsa et al. 2016). In addition to the seed-transmitted, alkaloid toxin-producing fungal endophytes of cool season grasses, morning glories, and locoweeds (Panaccione et al. 2014), other studies suggest that a wider range of microbes are transmitted in seeds. For example, Hodgson et al. (2014) documented endophytic fungi in seeds of six forb species in Britain including *Alternaria alternata*, the most commonly isolated endophyte here. In some of the species that Hodgson et al. (2014) examined, fungal endophytes were also isolated from pollen, suggesting endophytes infected seeds by co-growth with pollen tubes. Although not examined here, endophytic bacteria have also been isolated from seeds in a variety of plant species (Coombs and Franco 2003; Compant et al. 2011; Truyens et al. 2015).

Symbiotic interactions with microbes, including both mutualists and pathogens, are increasingly recognized as important factors for the success or failure of invasive plant species (Mitchell and Power 2003; Reinhart et al. 2003; Nunez et al. 2009; Flory and Clay 2013; Traveset and Richardson 2014). The role of fungal endophytes specifically in plant invasions has been documented in several systems (Rudgers et al. 2005; Aschehoug et al. 2012) and hypothesized in others (Kowalski et al. 2015). For example, in invasive tall fescue grass and spotted knapweed (*Centaurea stoebe*), endophyte symbiosis increases their competitive ability against native species (Rudgers et al. 2005; Aschehoug et al. 2012). In *Phragmites australis* populations in Europe, Ernst et al. (2003) reported that seed-borne *Stagonospora* endophytes enhanced biomass production in controlled environmental conditions. In the present study,

Stagonospora neglecta was the fourth most common OTU isolated from seeds (Supplemental Table 1, Table 3), but it is not known whether this endophyte enhances growth of North American populations of invasive *Phragmites*. In theory, disrupting endophyte symbioses in invasive plant species represent a potential mechanism of invasive control (Kowalski et al. 2015).

There are several caveats for this study that suggest future research directions. In our survey of fungal endophytes in seeds of invasive *Phragmites*, a relatively small number of sites were evaluated relative to the widespread distribution of *Phragmites.* Future studies should examine a wider range of sites to determine if the fungal endophyte diversity and effects on germination and seedling growth are reflective of larger-scale patterns. Moreover, seed germination was relatively low and the small seedlings suffered high mortality, limiting our analyses of seedling communities. Future studies could use a larger number of seeds to obtain a larger number of seedlings, and seedling growth should be measured over a longer time period than 30 days. Demonstrating whether fungal endophytes are seed transmitted from generation to generation would require growing plants for two or more generations to determine if endophytes in seeds colonize seedlings and then adult plants, and the seeds produced by those plants. Independent of sample sizes and time frames, culture-based methodologies may be a conservative measure of the diversity of endophyte communities in seeds if growth of one endophyte inhibits emergence of additional endophytes from the same seed, although we did isolate multiple endophytes from some seeds. Future studies could utilize culture-independent, next-generation sequencing approaches to identify all endophytes in seeds independent of their isolation (Oono et al. 2015). Finally, seeds were collected here without knowledge of the underlying genetic structure of *Phragmites* populations. Future studies could use genetic markers to identify individual clones in conjunction with seed collection from those clones.

In conclusion, many fungal endophytes are common in seeds and seedlings of non-native *Phragmites*. Thus, dispersal of seeds and colonization of new habitats is often concurrent with dispersal and colonization of fungal microbiota in seeds, which can affect both seed germination and seedling growth. However, the most common endophyte previously found in adult *Phragmites* tissues was never found here in seeds from the same sites, suggesting that only some endophytes colonize seeds and resulting seedlings. Whether the fungal microbiota of seeds facilitates or inhibits the invasive success of *Phragmites* remains to be determined, but some endophyte taxa related to those identified here are known colonists of other weedy and invasive plant species (Aschehoug et al. 2012; Hodgson et al. 2014). Microbial symbiosis should therefore be taken into account in efforts to control this and other invasive plant species.

Table 4 Distribution of fungal endophyte OTUs isolated from seedlings of *Phragmites australis* collected from three different sites (Crane Creek (CC), Sandusky (SAN) and Turtle Creek (TC)). OTUs are listed in descending order of prevalence (#2 most common, #19 least common) with the number of isolates from each site. Identities of each OTU are provided in Table 3

OTU	CC	SAN	TC	Total
2	10	0	3	13
3	4	2	5	11
1	7	0	2	9
16	2	0	2	4
15	0	1	2	3
9	0	0	2	2
4	0	0	1	1
8	1	0	0	1
17	1	0	0	1
18	1	0	0	1
19	1	0	0	1
Total	27	3	17	47

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