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Overwintering seeds as reservoirs for seedling pathogens of wetland plant species

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Abstract. Seed germination and seedling establishment are central to the distribution and abundance of plant species in wetlands. While fungal and oomycete pathogens are known to affect seed viability and emergence, relatively little is known about which fungi and oomycetes are associated with seeds in the soil or how these species affect seeds and seedlings. We characterized the fungi and oomycetes associated with overwintering seeds in wetlands and determined their potential to influence seed germination and subsequent seedling mortality. Fungi and oomycetes did not affect seed germination, despite the isolation of high frequencies of known seed and seedling pathogens in the fungal genera *Alternaria*, *Peyronellaea*, *Epicoccum*, and *Fusarium*. However, many of the most frequently isolated fungal species from overwintering seeds were highly virulent to seedlings. While both native and nonnative plant species were tested, we did not observe consistent differences in either seed germination or seedling susceptibility based on the invasive status of plants tested, contrary to what we expected given several established hypotheses for invasive success. The high seedling virulence of fungi from overwintering seeds coupled with the differential abundance of some of the more pathogenic fungi among seeds of different plant species, led us to the conclusion that the fungal pathogens that colonize seeds in the seed bank over winter are likely to strongly impact subsequent seedling establishment in wetlands the following spring despite not reducing overwintering seed germination in the seed bank or differently effecting invasive plant species.

Key words: fungi; invasive plants; marsh wetlands; oomycetes; seed banks; seedling survival; soil pathogens.

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Introduction

Seed germination and seedling establishment play important roles in the subsequent distribution and abundance of plant species in many ecosystems (Fenner and Thompson 2005). Most seeds eventually come in contact with soil, forming a transient to persistent seed bank population, which represents the sum of all viable seeds, dormant, and nondormant, in and on the soil. The success of seedlings emerging and establishing from the seed bank may greatly determine plant community composition following disturbances (Templeton and Levin 1979, Csontos 2007). Wetlands, in particular, have diverse and long-lived seed banks where seeds remain dormant until appropriate conditions trigger their germination and growth (Heerdt and Drost 1994, Zedler and Kercher 2004).

Despite the importance of seed banks in wetland plant community dynamics (Wang et al. 2009, Baldwin et al. 2010), relatively little is known about how soil microbes in the seed bank affect germination and subsequent

seedling establishment. While in the soil, seeds encounter and acquire a rich microbial community that includes saprobic, mutualistic and pathogenic bacteria, fungi and oomycetes (Wagner and Mitschunas 2008). Previous research into seed bank dynamics suggests that pathogenic soil fungi are especially important in reducing the persistence of seeds (Blaney and Kotanen 2001*a*,*b*, Schafer and Kotanen 2003, 2004, O'Hanlon-Manners and Kotanen 2004, Kotanen 2007, Orrock and Hoisington-Lopez 2009, Gallery et al. 2010, Orrock et al. 2011), degrading the seed in its dormant state and/or directly infecting germinating seeds and developing seedlings (Crist and Friese 1993). Seeds and seedlings in wetland soils are likely to encounter a wide variety of potential pathogens. While these interactions may play a role in seedling establishment of particular plant species, little is known about the specific seed–pathogen interactions that impact seed germination and subsequent seedling establishment, with several exceptions (Crist and Friese 1993, Leishman et al. 2000, Kluger et al. 2008, Gallery et al. 2010, Mordecai 2012).

Such interactions with pathogens may be of particular importance to plant invasions as seed bank dynamics have been implicated in the invasiveness of introduced plant species (Klironomos 2002, Meyer et al. 2008, 2010, Reinhart and Clay 2009, Orrock et al. 2011, Mordecai 2013). Invasive plant species are known to change not only the richness and evenness of seed bank plant communities at the sites they colonize (Gioria et al. 2014) but they also can alter soil and rhizosphere microbial communities (Van Der Putten 1997, Nelson and Karp 2013). In some cases, introduced invasive plants encounter fewer virulent pathogens (Reinhart et al. 2010, Callaway et al. 2011) and are less likely than native plant species to experience negative feedbacks at the seedling stage (Diez et al. 2010). Although soil microbes have the potential to influence interactions between native and nonnative species, our understanding remains incomplete with regard to the plant–microbe interactions and plant developmental stages (infection in the seed bank to post-germination seedling infection) for which such interactions are most influential.

A more thorough understanding of the relative virulence of specific pathogens to plant species in seed bank communities is necessary to better understand how such pathogens potentially influence plant demography and community dynamics in freshwater wetlands. To that end, experiments were designed to test the following hypotheses: (1) Specific fungal and oomycete pathogens significantly reduce the germination of overwintering seeds. (2) Invasive plant species experience higher rates of seed germination than native species following overwintering. (3) Unique fungal and oomycetes species associate with seeds of different plant species. (4) Seeds of invasive plant species are less susceptible to fungi and oomycetes than seeds of native species.

Methods

Seed container set-up

Ten plant species were selected for seed burial: *Asclepias incarnata*, *Calamagrostis canadensis*, *Carex comosa*, *Carex frankii*, *Epilobium glandulosum*, *Muhlenbergia glomerata*, *Lythrum salicaria*, European (nonnative, invasive) haplotype of *Phragmites australis* (hereafter referred to as *P. australis*), North American (native, noninvasive) haplotype of *Phragmites australis* (hereafter referred to as *P. a. americanus*), and *Phalaris arundinacea.* Seeds of some plant species (*P. australis*, *P. a. americanus*, *Ph. arundinacea*) were collected locally at sites within 10 km of each other in a marsh wetland at the Montezuma Wetlands Complex in upstate New York. All other seeds were ordered from Prairie Moon Nursery, Winona MN. These species were selected because they reflect a phylogenetic range of native and nonnative plant species as well as a range of species frequencies (common to rare). All seeds were surface sterilized by dipping in a 70% EtOH solution followed by a sterile water rinse prior to burial. Following this treatment, subsets of surface-sterilized seeds were placed on plates containing 2% water agar (WA) to check for any potential fungal or oomycete seedborne pathogens. Very few contaminants were observed and we assume that their impact on our results is minimal. Initial germination percentages of seeds were not recorded because stratification during burial is known to dramatically alter (typically increase) seed germination percentages upon recovery, making prestratification germination rates insignificant.

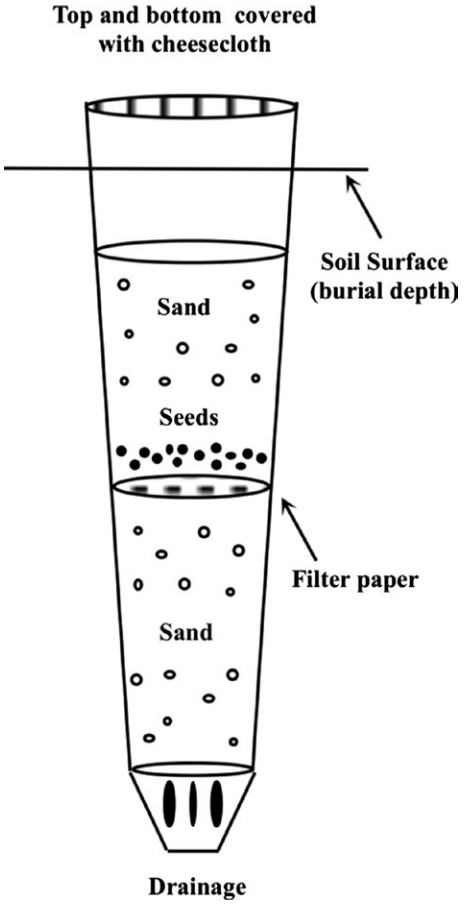

Fig. 1. Seed burial container set-up.

Seeds were prepared for burial by filling conical containers (Stewe and Sons, Ray Leach "Cone-tainer"™ SC7U) with ~50 ml sterile, sieved 0.25–0.5 mm quartz sand (Fig. 1). Prior to filling containers with sand, sterile cheesecloth squares were placed over the drainage holes to prevent the loss of the container contents but allow the flow of water. A sterile 5.5 cm diameter filter paper was placed on the sand surface upon which seeds (number varied from ~25 to 100 depending on plant and seed size) were placed. An additional ~50 ml sand was then added to nearly fill containers. Sterile cheesecloth squares were affixed over the top of containers to exclude granivores and detritus. Nine containers were established for each of the 10 plant species at each of 10 different wetland sites (for a total of 900 containers). These burial sites reflected a range of local wetland conditions and vegetation types (Table 1). For each plant species, three of the nine containers at each site were nontreated (dipped in water), three were treated by drenching with the fungicide (anti-fungal) difenoconazole (1.18 mL/gallon), and the remaining three were treated by drenching with the stramenicide (anti-oomycete) mefenoxam (0.07 mL/gallon).

Seed container burial, overwintering, retrieval and germination assessment

Three quadrats were established at each field site, each within 3 m of the other two. Each container was buried in its own 20 × 20 cm square of the quadrant in a randomized design. Seeds were buried in January and collected the following April. Container recovery was staggered by site to reflect the order in which they were buried. Recovered containers were transported to the lab and stored at ~ 4°C prior to processing. At the time of container retrieval soils at most sites, and the seed containers themselves, were saturated with water due to heavy spring rains. However, one site was flooded making it impossible to retrieve containers until after the experiment concluded and these were excluded from subsequent analyses.

Seeds were extracted from each individual container by emptying the top layer of sand, containing the filter paper with seeds, into a beaker of water. The seeds were then elutriated under a stream of running water until seeds either floated or were released into the supernatant. Seeds were then collected by decanting the water and vacuum-filtering through a Whatman #1 filter paper. Seed retrieval efficiency using this method was unexpectedly high and essentially all seeds buried were eluted from containers following overwintering. Some of the retrieved seeds from plots at a few sites were already germinated upon collection and these were excluded from further analysis. After seeds were isolated, they were stored at 4°C (generally 1–2 d) on sterile filter paper in sterile glass Petri dishes until placed into germination assays or plated on isolation media.

Seed germination was assessed by placing a subsample of 12 ungerminated seeds from each container onto WA in wells of a 96-well microtiter plate (1.5 ml WA per well). For the purposes of our study, seed germination is defined as seeds with a clearly emerged radicle and seedlings that died postgermination were not discounted. Plates were covered, sealed with Parafilm®,

Table 1. Seed burial sites used in this study.

Site	Abbreviation (#)	Longitude	Latitude
Resource Ecology and Management Centre, Cornell University, Ithaca NY	1	76°28′5.40″W	42°26′30.31″N
Wildflower Garden, Cornell Plantations, Cornell University, Ithaca NY	2	76°28′9.13″W	42°27′1.92″N
Malone Unit, Department of Environmental Conservation, Savannah NY	3	76°44′43.55″W	43° 4′27.34″N
Martin's Marsh 1, Department of Environmental Conservation, Savannah NY	4	76°42′33.33″W	43° 5′7.11″N
Martin's Marsh 2, Department of Environmental Conservation, Savannah NY	5	76°42′27.60″W	43° 5′2.03″N
Martin's Marsh 3, Department of Environmental Conservation, Savannah NY	6	76°42′36.16″W	43° 5′2.05″N
Teal Pond 1, Department of Environmental Conservation, Savannah NY	7	76°42′20.62″W	43°5′7.98″N
Teal Pond 2, Department of Environmental Conservation, Savannah NY	8	76°42′17.83″W	43°5′9.51″N
Teal Pond 3, Department of Environmental Conservation, Savannah NY	9	76°42′18.03″W	43°5′5.41″N
Private Residence, Cayuga View Rd., Trumansburg NY	10	76°37′39.43″W	42°32′42.44″N

and placed in a growth chamber set to alternating light (12 hours at 30°C) and dark (12 h at 10°C), conditions (Ekstam and Forseby 1999). At 3 weeks, plates were examined and germination assessed by counting the number of seeds with emerged radicles out of the total seeds plated.

Isolate collection and pathogenicity bioassays

Fungi and oomycetes were collected from six seeds per container for each plant species/treatment combination at eight different sites, one plot at each (~432 seeds in total). Each seed was placed in one of six wells of a 12-well tissue culture plate filled with an anti-bacterial selective medium (Nelson and Hsu 1994). Fungal and oomycete mycelia were allowed to emerge from seeds at which time mycelia were hyphal-tipped and transferred to WA plates. The hyphal tip transfers were repeated several times to reduce contamination and ensure that each isolate reflected a single fungus or oomycete strain. Isolates were then stored on WA at 18°C until use in pathogenicity assays.

To test the pathogenicity and virulence of fungi and oomycetes, seedling survival (defined as plants that developed healthy cotyledons over the 3-week period of our assay) was assessed following inoculation with each of the collected isolates. Pathogenic isolates were defined as those that significantly decreased seedling survival relative to noninoculated (control) seeds. Virulence, on the other hand, was defined as the degree of this decrease in survival relative to other isolates tested on the same plant species. Four plant species were selected for these bioassays: *P. australis*, *L. salicaria*, *M. glomerata*, and *E. glandulosum*. These plants were chosen because of their ease of growth, high seed germination, and because they reflect a range of plant species (native and nonnative, grasses, and forbs). Due to a failure in germination of some *L. salicaria* seeds, several pathogen interactions with this plant species are missing. For each isolate, a small (~1 mm diameter) colonized agar disk was placed in the center of each of four plates of WA and allowed to grow for approximately 1 week at 18°C until hyphae had covered nearly all of the agar surface. Ten surface-sterilized seeds of a given plant species were then placed on the surface of one plate for each isolate, as well as on the surface of noninoculated WA plates to act as a control for seedling survival. This also served as a check for any contaminating seedborne pathogens, of which few were observed. Plates were sealed in Parafilm® and placed in a growth chamber set to the previously described conditions. Three weeks later, plates were examined and percent seedling survival was assessed. This bioassay gave fungi and oomycetes great advantage by putting them at high density and directly in contact with seeds. While this is unlikely to mimic the field situation in soils, it enables a quick assessment of whether or not the tested fungi are potential pathogens.

Isolate sequencing and identification

Fungal and oomycete isolates were identified by sequencing the internal transcribed spacer (ITS) region. To prepare fungi and oomycetes for DNA extraction, isolates were grown on 10 cm diameter Petri dishes containing potato dextrose agar (PDA). The agar surface was covered with a sterile cellophane membrane to facilitate hyphal removal once colonies had reached the edge of plates. Hyphae were then placed in microcentrifuge tubes,

and lyophilized. DNA was extracted from lyophilized hyphae using a QIAGEN® DNeasy Plant Mini Kit following the manufacturer's protocols. DNA was stored at −20°C until subsequent processing. For PCR amplifications, we used ITS 1 and ITS 4 primers to amplify the ITS region of each isolate (White et al. 1990). All PCR reactions contained 10 mmol/L Trizma HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.2 pmol of each primer, 200 pmol of each dNTP, 1 unit of Sigma REDTaq Genomic Polymerase, and 0.5 μl of template DNA per 25 μL reaction. DNA was amplified using a Bio-Rad T100™ (Bio-Rad Laboratories, Hercules, California, USA) thermal cycler. PCR amplicons were purified using the Promega® SV 96 PCR Purification Kit.

Sequencing was performed at the Cornell University Life Sciences Core Laboratories Center on an Applied Biosystems Automated 3730 DNA Analyzer (Life Technologies Corporation, Carlsbad, California, USA) using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequences were compiled and edited in Sequencher 5.2.4 (Gene Codes Corp., Ann Arbor, Michigan, USA). Sequence affinities to known taxa were determined by BLAST searches of the NCBI GenBank database on Nov. 3, 2014.

All sequences were aligned in MAFFT (Katoh and Standley 2013), trimmed in gblocks and converted to PHYLIP format in readseq. A maximum likelihood phylogenetic tree was built using raxML blackbox to determine how well this region resolved sequences. Along with our isolate sequences, GeneBank voucher sequences for the ITS region were added to alignments for each species we detected in our preliminary BLAST search and we included these in the tree. The sequenced ITS region resolved most of these genera to species very well, with the exception of *Alternaria alternata, A. tenuissima, A. s*p., and *A. brassica*. To minimize confusion, these were grouped as *A. alternata,* whereas *A. rosea* and *A. infectoria* were grouped as *A. infectoria.*

Statistical analysis

Generalized linear mixed models (GLMM) with a binomial distribution were employed to test whether biocide treatment influenced seed germination. For each plant species, the main effect of biocide application on seed germination was tested with site as a random variable. Differences between biocides were evaluated *a posteriori* by aggregating biocide treatments unless significant differences were present (Crawley 2013). Starting with the full model [which included soil treatment (nontreated, fungicide-treated, or stramenicide-treated)] models were reduced in a backwards-stepwise process to determine the best model and significance via log-likelihood tests at $P < 0.05$. R version 3.0.1 (R Development Core Team 2013) and the add-on package "lme4" (Bates et al. 2013) was used for all mixed models.

To better understand the effects of the seed-colonizing fungi and oomycetes on seedling survival, the pathogenicity and virulence among selected fungal and oomycete isolates were examined at 3 weeks by comparing seedling survival in the presence of the pathogens with the noninoculated control seedlings. The ten most frequently isolated fungal species were selected and effects calculated as mean percentage seedling survival of species "y" when inoculated with fungal species "x" divided by mean percentage seedling survival of species "y" in noninoculated control plates. Seedling survival when inoculated with isolates of these species was also compared to noninoculated seedlings using a one-way ANOVA followed by Student's *t* test ($P < 0.05$) in JMP to determine significant differences between means. Percent seedling germination was normally distributed and thus we treated this as a linear rather than binomial term indicating survival or death of each seedling. The same procedure was used to analyze the cumulative effects of all isolates from each source plant species on seedling survival. Isolates were grouped by the plant species from which they were isolated and effects values were calculated as mean percentage seedling survival of species "y" when inoculated with isolates from plant species "x" divided by mean percentage seedling survival of species "y" in noninoculated controls.

Results

Effect of soil fungi and oomycetes on seed germination

Overall germination following overwintering was variable across plant species. *C. comosa* exhibited the lowest seed germination (2%), whereas *L. salicaria* exhibited the highest (89%). No clear trends in germination were observed

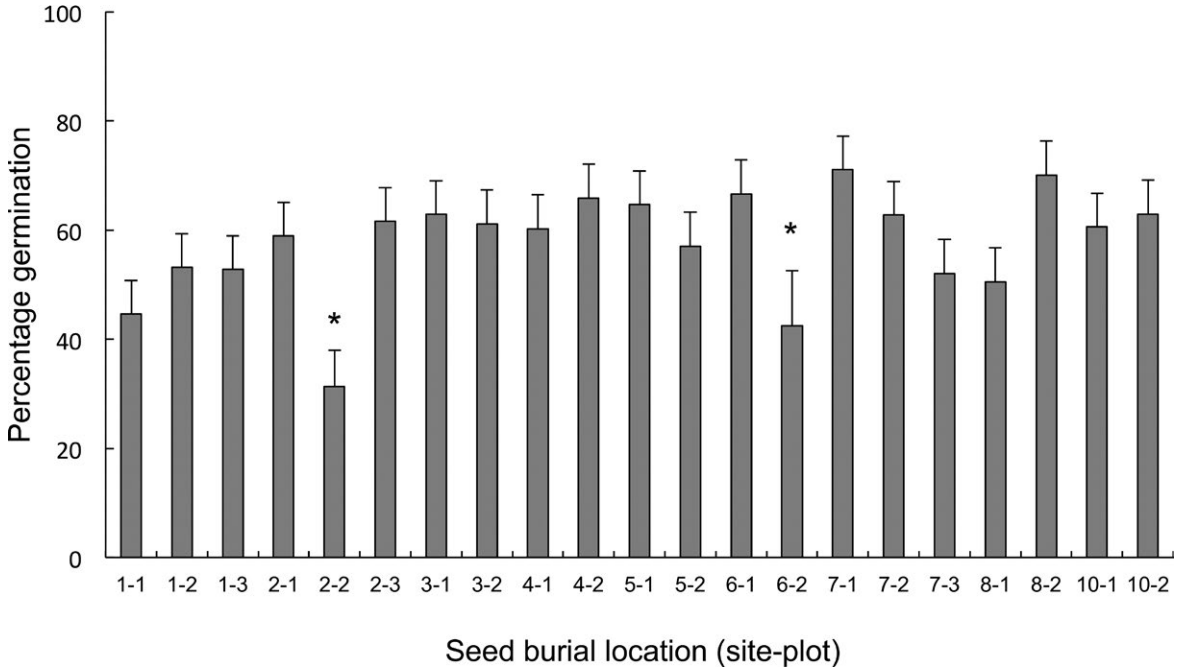

Fig. 2. Percentage germination of all seeds after overwintering at 10 different sites. Field sites are designated as a "*x-y*" value where x = site number and y = the plot number at that site. Data are means (+1SE) with significant differences ($P < 0.05$) represented by an asterisk (*).

between forbs and grasses or between native and nonnative species. Furthermore, little site-to-site variation in germination rates was observed, despite large differences in soil flooding status and plant community composition at each of the different sites (Fig. 2).

Germination of most plant species was unaffected by fungicide or stramenicide treatment (Fig. 3). However, for the three plant species where seed germination was significantly altered by treatment, it was lower for *E. glandulosum* when treated with fungicide, higher for *L. salicaria* when treated with fungicide, and lower for *Ph. arundinacea* when treated with stramenicide. Although statistically significant, effect sizes were very small.

Diversity of seed-isolated fungi and oomycetes

Over 200 fungi and only three oomycetes were isolated and identified from seeds across all plant species and sites (Table 2). Nearly half of the fungal isolates (104) were collected from stramenicide-treated seeds (no oomycetes were isolated from stramenicide-treated seeds as predicted), whereas 98 and 11 fungal isolates came from nontreated and fungicide-treated seeds, respectively (Table 3). The most frequently isolated fungal genera were species of *Alternaria* (80 isolates), *Peyronellaea* (57 isolates), *Epicoccum* (22 isolates), and *Fusarium* (13 isolates). The remaining 26% of isolates were distributed among 32 different species, each of which accounted for less than 2% of the total number of isolates (Table 2). All oomycetes isolated were species of *Pythium* (*P. citrinum, P. logandrum*, and *P. heterothallicum*).

Some fungal species were more commonly isolated from particular plant species. For example, nearly all isolates of *Peyronellaea glomerata* were from *P. a. americanus* and *P. australis* seeds (Fig. 4). More than 50% of the *Alternaria alternata* isolates were from the *Carex* species (*C. comosa* and *C. frankii*). All of the other fungal species were isolated at relatively low frequencies from all the plants tested.

Virulence of fungal isolates

Many of the dominant fungal species isolated from seeds were pathogenic not only to the plant species from which they were isolated

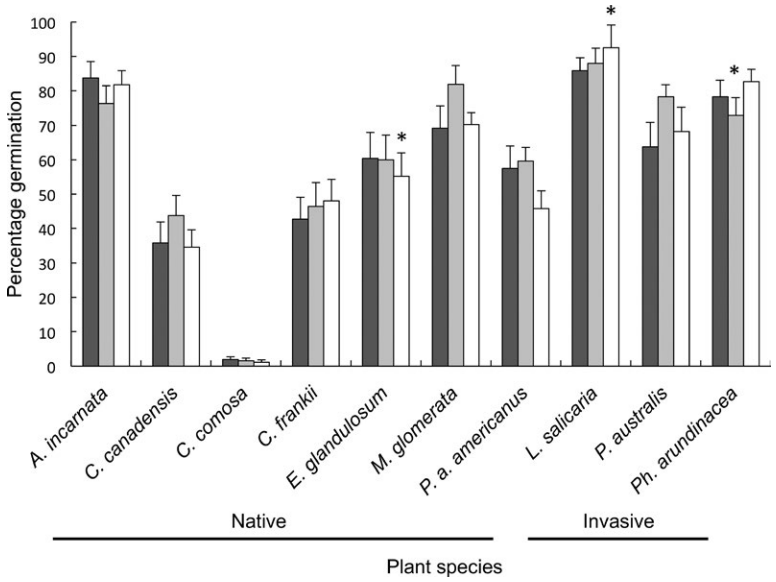

Fig. 3. Overwintering seed viability as influenced by biocide treatment. Nontreated seeds = dark gray bars, fungicide-treated seeds = light gray bars, stramenicide-treated seeds = white bars. Data are means (+1SE) with significant differences (GLMM) represented by an asterisk (*).

but also to the other plant species we tested (Table 4); however, there were wide variations in virulence. Among the most virulent pathogens were *Fusarium sporotrichiodes, Alternaria infectoria*, and *A. alternata* (Table 4). *Cadophora luteo-olivacea*, *Phaeosphaeria* species, *Pilidium concavum,* and *Stagonospora* were not pathogenic to any of the plant species tested.

The general susceptibility of each of the plant species to multiple pathogens varied. The mean seedling survival for each species when inoculated was 51% for *E. glandulosum*, 61% for *L. salicaria*, 56% for *P. australis*, and 77% for *M. glomerata.* These variations reflect different susceptibilities of the different plant species to individual isolates. For example, inoculation with *Fusarium sporotrichiodes* decreased seedling survival of *E. glandulosum* to 6% and *P. australis* to 0% but only decreased seedling survival of *M. glomerata* to 44%. Some pathogens such as *Alternaria alternata* and *Fusarium sporotrichiodes* significantly decreased seedling survival of all plant species tested, whereas others exhibited a narrower host range. For example, *Epicoccum nigrum* decreased seedling survival only of *P. australis*, whereas *Peyronellaea glomerata* decreased seedling survival of *M. glomerata, P. australis*, and *L. salicaria* but not *E. glandulosum.*

In general, the source plant species from which fungal pathogens were originally isolated was not correlated with their virulence (Table 5). The susceptibility of *P. australis, M. glomerata,* and *L. salicaria* to fungal isolates did not differ, regardless of the source plant. However, *E. glandulosum* was significantly more susceptible to fungi that came from seeds of *C. frankii*, *A. incarnata, C. comosa*, and *L. salicaria* than those from *P. australis*, *P. a. americanus,* and *Ph. arundinacea*. Additionally, plant species that served as source hosts for the isolates were no more susceptible to those isolates than nonsource host species. Fungal isolates from nonnative hosts were no more virulent to different plant species than those isolated from all but one of the native hosts. Instead, *E. glandulosum* was more susceptible to fungal isolates from native plant species than isolates from nonnative plant species.

Discussion

In this study, we determined the identity and pathogenicity of the fungi and oomycetes that colonize seeds of wetland plant species during overwintering in soil. Although previous studies have examined the potential roles of fungal and oomycete pathogens on the persistence of

Table 2. Fungal species isolated from different plant species.

Fungal species	Plant species†										Total
	A. in	C. ca	C. co	C. fr	E. gl	L. sa	M. gl	amer	aust	P. ar	
Alternaria alternata	12	4	12	24	3	6	7	5	2	1	76
Alternaria infectoria	1		1		1	1					4
Aureobasidium sp.			1								1
Cadophora luteo-olivacea		1	3			1	2				7
Curvularia inaequalis								1			1
Cylindrocarpon sp.	1										1
Diatype stigma						1					1
Diplodia seriata								1			1
Epicoccum nigrum	4	1	2	2	5	1	1	1		6	23
Epicoccum sorghinum								1			1
Fusarium proliferatum								1			1
Fusarium solani	1		1	1							3
Fusarium sporotrichioides			1		4						5
Fusarium tricinctum	1		1	1	1						4
Leptosphaeria sp.										1	1
Leptosphaerulina trifolii					1						1
Mucor circinelloides					2						2
Mucor hiemalis									1		1
Nectria nigrescens						1					1
Nigrograna mackinnonii				1							1
Paraconiothyrium sp.	1										1
Paraphaeosphaeria neglecta						1					1
Paraphaeosphaeria sp.	1										1
Paraphaeosphaeria sporulosa										1	1
Penicillium aculeatum	1										1
Penicillium glabrum						1					1
Penicillium sp.										1	1
Peyronellaea glomerata			3	2	1	1		38	9	1	55
Phaeosphaeria poae	1		1				1			1	4
Phoma sp.								1	1		2
Pilidium concavum			1				1				2
Pythium heterothallicum									1		1
Pythium logandrum	1								1		2
Saccharicola bicolor		1									1
Sarocladium strictum							1				1
Sphaeropsis sapinea										1	1
Stagnospora trichophoricola							1				1
Trametes gibbosa					1						1
Trichoderma koningiopsis	1										1
Uncultured fungal endophyte				1							1
Total	26	7	27	32	19	14	14	49	15	13	216

† A. in = Asclepias incarnata, C. ca = Calmagrostis canadensis, C. co = Carex comosa, C. fr = Carex frankii, E. gl = Epilobium glandulosum, L. sa = Lythrum salicaria, M. gl = Muhlenbergia glomerata, amer = Phragmites australis (native genotype; non-invasive), aust = Phragmites australis subsp. australis (nonnative genotype; invasive), P. ar = Phalaris arundinacea.

seeds in seed banks (Crist and Friese 1993, Leishman et al. 2000, Blaney and Kotanen 2001*a*,*b*, Schafer and Kotanen 2003, 2004, Gallery et al. 2010, Mordecai 2012), our work focused primarily on the impacts of those seed-colonizing pathogens on subsequent seed germination and seedling survival. This work is especially significant for freshwater wetland ecosystems where the prevalence and impacts of plant pathogens are rarely acknowledged and, with the exception of only a few studies (Blaney and Kotanen 2001*a*, Schafer and Kotanen 2004, Nechwatal et al. 2008, Nelson and Karp 2013), have not been examined.

While fungi and oomycetes in our study had little to no impact on the germinability of seeds

Table 3. Fungal species isolated from different soil treatments.

Fungal species	Seed treatment			Total
	Fungicide†	None	Stramenicide‡	
Alternaria alternata	2	38	36	76
Alternaria infectoria	1	1	2	4
Aureobasidium sp.			1	1
Cadophora luteo-olivacea		6	1	7
Curvularia inaequalis			1	1
Cylindrocarpon sp.	1			1
Diatrype stigma			1	1
Diplodia seriata	1			1
Epicoccum nigrum		10	13	23
Epicoccum sorghinum			1	1
Fusarium proliferatum			1	1
Fusarium solani			3	3
Fusarium sporotrichioides		4	1	5
Fusarium tricinctum		1	3	4
Leptosphaeria sp.			1	1
Leptosphaerulina trifolii		1		1
Mucor circinelloides			2	2
Mucor hiemalis		1		1
Nectria nigrescens	1			1
Nigrograna mackinnonii			1	1
Paraconiothyrium sp.			1	1
Paraphaeosphaeria neglecta			1	1
Paraphaeosphaeria sp.		1		1
Paraphaeosphaeria sporulosa			1	1
Penicillium aculeatum			1	1
Penicillium glabrum	1			1
Penicillium sp.			1	1
Peyronellaea glomerata	1	27	26	54
Phaeosphaeria poae		1	3	4
Phoma sp.		1	1	2
Pilidium concavum		2		2
Pythium heterothallicum	1			1
Pythium logandrum	1	1		2
Saccharicola bicolor		1		1
Sarocladium strictum	1	1		2
Sphaeropsis sapinea		1		1
Stagonospora trichophoricola				0
Trametes gibbosa			1	1
Trichoderma koningiopsis			1	1
Uncultured fungal endophyte			1	1
Total	11	98	106	216

† Fungicide (anti-fungal). Sand and seeds drenched with difenoconazole (1.18 mL/gallon) prior to burial.

‡ Stramenicide (anti-oomycete). Sand and seeds drenched with mefenoxam (0.07 mL/gallon) prior to burial.

overwintered in the seed bank, individual fungal species had significant impacts on subsequent seedling mortality. Many of the fungi and oomycetes colonizing seeds during the overwintering period were generalist pathogens similar to those described previously (Crist and Friese 1993, Kluger et al. 2008). These same pathogens have also been described from many other agricultural and nonagricultural ecosystems, as well as wetland soils (Ellis et al. 1951*a*,*b*, Stenton 1953, Schafer and Kotanen 2004, Nelson and Karp 2013). Some of the most dominant genera of fungi (*Alternaria, Peyronellaea,* and *Fusarium*) were also among the most virulent to seedlings of a range of wetland plant species, further highlighting their likely importance to wetland seedling establishment.

With the limited number of native and nonnative plants tested, our results fail to support the more common hypotheses to explain invasiveness (i.e., spillover, spillback, or enemy release), consistent with the findings of others (Blaney and Kotanen 2001*a*). Nonnative plant species did not experience higher rates of seed germination than native species during the overwintering process. Neither were the fungal pathogens associated with seeds of nonnative plant species more virulent than those from seeds of native plant species, which is inconsistent with observations in forest and grassland systems (Diez et al. 2010, Reinhart et al. 2010, Callaway et al. 2011) or where nonnative plants increase populations of seed bank pathogens (Beckstead et al. 2010).

Instead, our results support a growing body of research indicating fewer differences between native and nonnative plants with regard to plant traits (Park and Blossey 2008), their suitability as animal habitat (Martin and Blossey 2013) or in their associated microbial communities (Nelson and Karp 2013) than were previously assumed. For example, there were no major differences in the isolation frequency or virulence of fungal pathogens associated with seeds of the nonnative invasive plant *P. australis* compared to its native noninvasive conspecific *P. a. americanus*. This fits with research on the importance of plant genotype and factors such as plant exudate characteristics and root architecture (Philippot et al. 2013) on rhizosphere microbiota accumulation rather than invasive status.

The low frequency of oomycete isolations was surprising, given that wetland soils are known to harbor diverse communities of oomycete pathogens (Crocker et al. 2015), especially *Pythium* species that are commonly associated with seeds

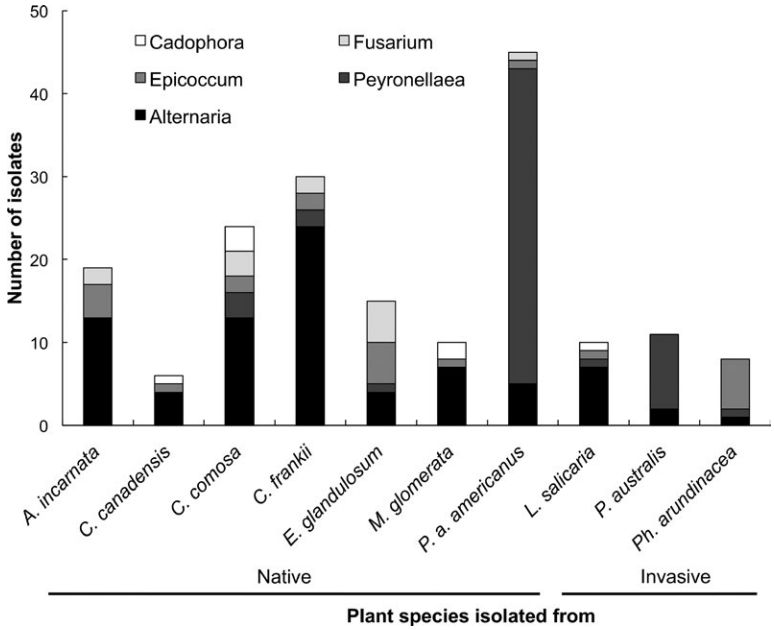

Fig. 4. Dominant fungal genera isolated from seeds of each plant species. Genera included: *Alternaria* = black, *Peyronellaea* = dark gray, *Epicoccum* = medium gray, *Fusarium* = light gray, *Cadophora* = white.

Table 4. Virulence of nine most frequently isolated fungal species to wetland plant species.

Fungal species	Plant species inoculated			
	Epilobium glandulosum	Muhlenbergia glomerata	Phragmites australis	Lythrum salicaria
Fusarium sporotrichiodies	**0.06***	**0.44***	**0.00***	nd
Alternaria alternata	**0.09***	**0.80***	**0.42***	**0.52***
Alternaria infectoria	**0.20***	**0.54**	**0.25***	0.59
Peyronellaea glomerata	0.80	**0.79***	**0.54***	**0.36***
Epicoccum nigrum	0.92	0.79	**0.49***	1.03
Phaeosphaeria sp.	0.99	0.79	0.50	nd
Stagnospora sp.	0.84	0.66	0.51	0.93
Cadophora luteo-olivacea	0.71	0.89	0.82	0.61
Pilidium concavum	0.64	0.87	0.68	0.98

Note: Virulence was calculated as (mean % seedling survival of plant species "y" when inoculated with isolates of fungal species "x")/(mean % seedling survival of plant species "y" in noninoculated control). Significant decreases in seedling survival when inoculated with a given species (Student's t test $P < 0.05$) are indicated with an asterisk (*) and bolded. Fungal species are ordered by overall virulence, "nd" indicated no data for fungus/plant combination.

(Martin and Loper 1999, Nelson and Karp 2013). Given the poor competitive ability of many *Pythium* species (Martin and Loper 1999), it is possible that fungal colonization of seeds may have preempted any subsequent oomycete colonization. Also, it is likely that the fungi growing out of colonized seeds in the laboratory may have competitively displaced any oomycetes emerging from colonized seeds in culture thus obscuring their presence. Extracting DNA directly from seeds and amplifying oomycete-specific sequences would have revealed their presence.

Often in studies of seed bank viability, the initial germination of seeds and the subsequent mortality or survival of seedlings are conflated (Blaney and Kotanen 2001*a*), making it challenging to determine the stages of early plant development where significant pathogen interactions occur. Here, we separated pathogen-induced overwintering seed degradation from seedling

Table 5. Virulence of fungi isolated from seeds of specific host plant species to different wetland plant species.

Plant species as source of isolates	Plant species inoculated			
	Epilobium glandulosum	Muhlenbergia glomerata	Phragmites australis	Lythrum salicaria
Asclepias incarnata	0.316^{d}	0.760^{b}	0.610^{b}	0.866^{a}
Calamagrostis canadensis	0.493^{b,c,d}	0.798^{a,b}	0.679^{a,b}	0.669^{a,b}
Carex comosa	0.473^{c,d}	0.741^{b}	0.535^{b}	0.640^{a,b}
Carex frankii	0.233^{d}	0.753^{b}	0.488^{b}	0.826^{a,b}
Epilobium glandulosum	0.535^{b,c,d}	0.736^{b}	0.515^{b}	0.634^{a,b}
Lythrum salicaria	0.315^{c,d}	0.839^{a,b}	0.536^{b}	0.572^{a,b}
Muhlenbergia glomerata	0.465^{b,c,d}	0.799^{a,b}	0.674^{b}	0.734^{a,b}
Phragmites australis americanus	0.697^{a,b,c}	0.726^{b}	0.534^{b}	0.429^{b}
Phragmites australis	0.902^{a,b}	0.868^{a,b}	0.674^{b}	0.360^{b}
Phalaris arundinacea	0.754^{a,b,c}	0.806^{a,b}	0.500^{b}	0.596^{a,b}

Note: Virulence was calculated as (mean % seedling survival of host species "y" when inoculated with isolates from plant species "x")/(mean % seedling survival of host species "y" in noninoculated control). Numbers in each column followed by the same letter are not significantly different (Tukey's test $P < 0.05$).

mortality. While this perspective presents two key different plant phases at which soil pathogens may play a role, it is still somewhat limited in that pathogens may be acting at other phases. For example, given that seed viability often decreases with increasing time in the seed bank (Schafer and Kotanen 2003), it is possible that any potential reductions in seed germination due to colonization by fungi and oomycetes would not be realized in the relatively short (4 months) residence time of our study. Furthermore, had seeds remained in soil well into spring, it is possible that higher levels of seed mortality would have been observed with potentially increasing levels of pathogen activity and thus infection in the field. Nonetheless, the observation that the fungi recovered from apparently healthy seeds could lead to high subsequent seedling mortality suggests that these fungi may persist on healthy seeds through the overwintering period yet have large detrimental impacts on subsequent seedling establishment in the field.

Given that the pathogens recovered from overwintering seeds also reside in the soils in which seedlings would develop the following spring, it is likely that these same pathogens could limit the establishment of seedlings, even if seeds were not colonized in the local seed bank but encountered after germination. However, because the pathogens that previously colonized seeds in the seed bank would have a temporal infection advantage to those colonizing seedlings that had not been previously exposed, it is likely that the more significant impacts on seedling establishment would come from pathogens persisting on precolonized seeds in the soil. Furthermore, nonpathogenic microbial colonization of seedlings that were not previously colonized by pathogens may protect newly emerging seedlings from further pathogen infection (Philippot et al. 2013).

Of the pathogenic fungi detected from overwintering seeds, species of *Alternaria, Peyronellaea,* and *Fusarium* comprised ~55% of all fungal isolates. These observations indicate that populations of lethal seedling pathogens can be maintained over the winter months in the seed bank with little or no impact on seed viability and germinability. This could support sufficient pathogen populations to allow for high seedling infection the following spring when seedlings are extremely susceptible and mortality rates can be high (Gilbert 2002). That different plant species varied in their susceptibility to a given fungal pathogen, and also altered the relative abundance of pathogens is consistent with other studies demonstrating the specific nature of microbial associations with specific plant species (Micallef and Colón-Carmona 2013, Peiffer et al. 2013, Berg et al. 2014, Aleklett et al. 2015, Lebeis 2015) and also suggests that these differential responses may have strong impacts on seedling establishment and possibly recruitment of different plant species.

We often isolated fungal species at different frequencies depending on the particular plant species. For example, *Peyronllaea glomerata,* one

of the more frequently isolated fungal species (~25% of all isolates), was almost exclusively isolated from both *P. australis* and *P. a. americanus*. There were many other fungal species isolated exclusively from specific plant species, however these were all very low in abundance and we cannot determine if there is specialization to any particular host plant. Furthermore, there are many other wetland plant species that we did not test and for which specialization could occur. *Peyronellaea glomerata* has previously been associated with *P. australis* (Wirsel et al. 2001, Wong and Hyde 2001) and *L. salicaria* (Nyvall 1995), both common wetland plant invaders. However, it is somewhat surprising that it was so infrequently isolated from other plant species, especially having been described as a ubiquitous fungus with a broad host range (Farr and Rossman 2013) that includes crayfish (Dörr et al. 2011).

In contrast, *Alternaria alternata* was isolated from many plant species, but most commonly isolated from either *Carex frankii* or *Carex comosa*. Given that the germination of *C. frankii* seeds was very low, it is possible that seeds were already infected with *A. alternata* and were nonviable when we received them (Nguyen et al. 2015). However, it is unlikely that seeds were the dominant source of *Alternaria* because of the high isolation frequencies from other plant species with higher germination rates.

Several of the fungal isolates we recovered from seeds were nonpathogenic but it is still possible that they, as well as other soil microbes, play important direct or indirect roles in seedling mortality or cause disease only under certain conditions. For example the fungi *Cadophora luteo-olivacea, Pilidium concavum*, and *Phaeosphaeria* sp. did not reduce seedling survival of the plant species we tested. However, each genus contains many pathogenic as well as nonpathogenic endophytic species (Farr and Rossman 2013) and may establish significant interactions with other wetland plant species that were not tested.

This research increases our understanding of the diversity of soil fungi associated with wetland seed banks and how particular seed bank fungal species affect seedling mortality. Our results suggest that the differential responses of seedlings to specific seed-associated pathogens have the potential to impact seedling establishment and possibly subsequent seedling recruitment in wetlands. These results further highlight the importance of studying specific pathogen interactions at specific stages of plant development to better understand the full impacts of pathogens on plant dynamics.

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