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REGULAR ARTICLE

Disease protection and allelopathic interactions of seed-transmitted endophytic pseudomonads of invasive reed grass (*Phragmites australis*)

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Abstract
Background and aims Non-native *Phragmites australis* (haplotype M) is an invasive grass that decreases biodiversity and produces dense stands. We hypothesized that seeds of *Phragmites* carry microbes that improve seedling growth, defend against pathogens and maximize capacity of seedlings to compete with other plants.
Methods We isolated bacteria from seeds of *Phragmites*, then evaluated representatives for their capacities to become intracellular in root cells, and their effects on: 1.) germination rates and seedling growth, 2.) susceptibility to damping-off disease, and 3.) mortality and growth of competitor plant seedlings (dandelion (*Taraxacum officionale* F. H. Wigg) and curly dock (*Rumex crispus* L.)).
Results Ten strains (of 23 total) were identified and characterized; seven were identified as *Pseudomonas* spp. Strains Sandy LB4 (*Pseudomonas fluorescens*) and West 9 (*Pseudomonas* sp.) entered root meristems and became intracellular. These bacteria improved seed germination in *Phragmites* and increased seedling root branching in *Poa annua*. They increased plant growth and protected plants from damping off disease. Sandy LB4 increased mortality and reduced growth rates in seedlings of dandelion and curly dock.
Conclusions *Phragmites* plants associate with endophytes to increase growth and disease resistance, and release bacteria into the soil to create an environment that is favorable to their seedlings and less favorable to competitor plants.

Keywords Bioherbicide · Ecosystem engineering · Microbiome · *Pseudomonas* · Reactive oxygen · Rhizophagy · *Phragmites* · Symbiosis

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Introduction

Plants are inhabited by communities of non-pathogenic bacteria, fungi and in some cases algae (Arnold and Lutzoni 2007; Hardoim et al. 2008; Rodriguez et al. 2009; Compant et al. 2010). Many of the inhabitants of plants are endophytes, colonizing internal tissues of plants (Stone et al. 2000; Schulz and Boyle 2006). Clay (1988) proposed that endophytes are functional, often defending plants from predation. This concept became known as the 'defensive mutualism concept.' Rodriguez et al. (2008) expanded that idea with the 'habitat adapted symbiosis concept' showing that many of the non-pathogenic microbes associated with plants serve to adapt plants to their environments and increase

tolerance to biotic and abiotic stresses (Hurek et al. 2002; Redman et al. 2002; Puente et al. 2009). Some invasive plants contain endophytes that may enhance their invasive properties. Rout and Chrzanowski (2009) demonstrated that invasive *Sorghum halepense* (L.) Pers. contains nitrogen-fixing endophytic bacteria that enable it to colonize low fertility soils. Soares et al. (2015) showed that the invasive vine *Hedera helix* L. contained an endophyte that protected it from disease and increased its growth. It seems evident that symbiotic associations of invasive plants play critical roles in enabling their hosts to thrive.

Invasive *Phragmites australis* (Cav.) Trin. (haplotype M), native to Eurasian wetlands, is an introduced grass in North America that displaces entire communities of native flora and fauna. This plant is highly competitive and generally outcompetes other plants to produce large nearly monospecific stands. It is increasingly becoming clear that microbes that form symbiotic associations with *Phragmites* play roles in increasing its growth and invasive character (Fischer and Rodriguez 2013; Clay et al. 2016). Soares et al. (2016a) recently demonstrated that endophytic bacteria isolated from tiller meristems were capable of increasing nitrogen assimilation into plants in greenhouse experiments. In another study, Soares et al. (2016b) showed that fungal endophytes that enter into roots of *Phragmites* growing in saline soils enhance salt tolerance in the host, enabling it to thrive in high saline soils. Ernst et al. (2003) demonstrated that a seed-borne fungal endophyte in genus *Stagonospora* enhanced biomass accumulation in experiments in microcosms. It is thus evident that symbiotic microbes may impact the growth of *Phragmites*. In order to better understand how seed-transmitted bacteria may impact competitiveness of *Phragmites,* we initiated studies to compare effects on several well-studied model grasses that could be grown in axenic conditions.

In this research, we hypothesized that seeds of *Phragmites* carry components of their microbiome that are defensive against pathogens and maximize capacity of seedlings to grow and compete with other plants. Further, we hypothesized that *Phragmites* employs its seed microbiome as a tool to engineer its soil ecosystem to make it more favorable to *Phragmites* seedlings and less favorable to pathogens and plant competitors. To begin to evaluate these hypotheses, we sought to answer the following questions: 1.) What are the identities of the seed-vectored bacteria? 2.) Do the seed-vectored bacteria become endophytic within plant tissues? 3.) Do the bacteria promote host growth? 4.) Are the bacteria defensive against pathogens? 5.) Are the bacteria ecosystem engineers and functioning to inhibit competitor plant species?

Materials and methods

Seed collection

Populations of *Phragmites australis* (invasive haplotype M) were identified by morphological examination at four sites in New Jersey, including Seabright, Sandy Hook peninsula (40°23′52″N, 73°58′34″W) and towns of South River (40°42′46″N, 74°0′21″W), West Windsor (40°15′57″N, 74°36′52″W) and Robbinsville (40°13′31″N, 74°37′34″W). Seeds were collected in November of 2015 from plants at each site.

Seed preparation

Seeds were cleaned of associated awns, lemmas and paleas by rubbing seeds between a fine mesh screen and wooden block. Seeds were washed with agitation in sterile water for 5 min and then the water was discarded. Seeds were washed a second time for 5 min after which the seeds were placed onto the surface of agar plates containing trypticase soy agar (TSA), Luria Bertani Agar (LBA) and Yeast Extract Sucrose Agar (YESA). The plates were incubated at room temperature for a 24–48 h period. Colonies were removed and streaked to obtain pure cultures. Ten morphologically unique isolates (from 23 total) were selected for further study based on color, texture and size of colonies in culture.

Bacterial identification and characterization

Genomic DNA from bacteria was isolated using GenElute Bacterial Genomic DNA Kits (Sigma Aldrich, St. Louis, MO). Bacterial identifications were made by obtaining 16S rDNA sequences after methods employed by Lane (1991) (Table 1). For Sandy LB4, we used the recA gene to identify the bacterium to species (Petti 2007). Sequences were compared to GenBank accessions using BLASTn (http://www.ncbi.nlm.nih.gov). Cultures on LB agar were also observed using an ultraviolet mineralogical lamp (UVGL-21, long wave

UV-366 nm, UVP, San Gabriel, California, USA). Phosphate solubilization was assessed by culturing bacteria on Pikovskaya agar, while protease testing was done by culturing in skim milk agar.

Endophytism determination

Two of the *Phragmites* seed bacteria (*P. fluorescens* Sandy LB4 and *Pseudomonas* sp. West 9) were arbitrarily selected along with control bacteria *Microbacterium oxydans* B2 (GenBank KP860310; from *Phragmites* shoots) and *Bosea thiooxidans* TBN (GenBank: KX692270; from a non-grass host *Fallopia japonica* (Houtt.) Ronse). These bacteria were screened to determine their capacity to become endophytic in grass roots. Bermuda grass (*Cynodon dactylon* (L.) Pers.) was used to evaluate endophytism because seeds could be surface sterilized, and seedlings would readily grow in sterile agarose medium. Seeds without husks were surface disinfected by agitation in 4% NaOCl solution for 45 mins. Seeds were washed thoroughly by agitation in several washes of sterile water until chlorine odor could not be detected on seeds. Seeds were then placed onto 0.7% agarose in Petri dishes. To inoculate seeds, one drop of a suspension of bacteria (at approximately 0.5 OD, 600 nm) was placed onto each seed. Each treatment contained 5 replicates, and each replicate consisted of a Petri dish with 10–15 seeds. An axenic control was also done to observe the behavior of seedlings without bacteria. Plates were incubated at room temperature for 7–10 days with a 12-h alternating light/dark cycle.

Visualization of bacteria in/on seedling roots

To visualize reactive oxygen associated with bacteria in/on seedling roots, agarose (0.7%; Low melting point; Sigma-Aldrich, St. Louis, MO) plates bearing seedlings were flooded and stained with a solution of 3, 3-diaminobenzidine (DAB) using SIGMAFAST® 3, 3-diaminobenzidine tablets from Sigma (White et al. 2014) and incubated at ambient temperature overnight for staining. Plates were washed of excess DAB and seedling roots were examined through the reverse of the Petri plate using a compound light microscope. Because plant cells secrete reactive oxygen onto bacteria that penetrate into plant cells, intracellular penetration of root cells may be indicated by dark red or brown staining (H_2O_2 positive) within root hairs and/or root parenchyma cells. Reactive oxygen staining permits better visualization of intracellular bacteria than use of other bacterial stains. To better visualize bacteria within root cells, squash preparations of seedling roots were made by removing seedlings from agarose after DAB staining, excising roots, counterstaining staining roots using aniline blue (0.01%, aqueous) and then squashing roots beneath a glass coverslip.

Plant growth promotion experiments

Experiment 1: Assessment of capacity for plant growth promotion

To assess plant growth promotion, *P. fluorescens* Sandy LB4, *Pseudomonas* sp. West 9, *M. oxydans* B2 and *B. thiooxidans* TBN (from *Fallopia japonica* Siebold & Zucc) were inoculated onto previously surface disinfected (40 mins agitation in 4% NaOCl) seeds of *P. annua* by dipping seeds in a water suspension of bacteria (at approximately 0.5 OD, 600 nm). Controls were dipped in sterile water. Seeds were planted in sterile soil in magenta boxes (Sigma-Aldrich, St. Louis, MO) with approximately 15 plants/treatment and placed under fluorescent lights in the laboratory for 37 days. Plants were harvested by removal from soil and roots wiped of excess soil. Shoot and root lengths were then measured and recorded (Table 2).

Experiment 2: Seed germination and seedling root architecture

Experiments were conducted to evaluate *P. fluorescens* Sandy LB4 effects of seed germination and early seedling root architecture of *Poa annua* and *Phragmites australis*. In these experiments, seeds of *P. annua* were surface disinfected as above before being plated onto agarose media; 128 seeds were inoculated with a suspension of Sandy LB4 and 130 seeds were not inoculated. Seeds of *P. australis* were cleaned of adherent fibers as above, surface disinfected by agitation in 4% NaOCl for 30 min, rinsed in sterile water and scarified by making an incision using a sterile razor, then plated on agarose. Approximately 40 seeds of *Phragmites* were inoculated with a suspension of Sandy LB4, and a control set of 40 seeds was not inoculated. Seeds were monitored daily for 7 days for the

parameters percent germination and the percentage of seedlings showing two lateral roots.

Disease protection experiments

Experiment 3: Disease protection (co-culture experiments)

To assess potential for disease protection, bacteria *P. fluorescens* Sandy LB4, *Pseudomonas* sp. West 9 and *M. oxydans* B2 were co-cultured on LBA with soil fungi *Sclerotinia homeocarpa* or *Fusarium oxysporum* for 7 days. Formation of zones of inhibition was noted.

Experiment 4: Damping off disease control experiment

To evaluate whether the *Phragmites* bacteria could protect seedlings from disease, an experiment was set up using the damping off pathogen *F. oxysporum*. In this experiment, surface disinfected seeds of *P. annua* were inoculated by dipping in a water suspension (at approximately 0.5 OD, 600 nm) of strains Sandy LB4, West 9 or *M. oxydans* B2, then planted in magenta boxes containing sterile peat soil in that had been mixed with 10-mL of a conidial suspension of *F. oxysporum*. Controls were dipped in sterile water prior to planting. Each treatment included 25 replicates (*P. annua* seeds). After 15 days, seedlings evident on the surface of the soil were counted. Also presence of fungal mycelium on the surface of soil was determined. To test for movement of the bacteria into soil, a sterile probe was placed into soil between plants in each replicate, then the probe was streaked on a fresh Petri plant containing LB agar and incubated for 24 h prior to examination.

Competitor inhibition experiments

Effects of Phragmites bacteria on mortality and growth of competitor plants

Dandelion (*Taraxacum officionalis*) was used to screen *Phragmites* microbes for any antagonism leading to increased mortality in the dandelion seedlings. Here, dandelion seeds were rubbed against a nylon screen to remove awns and associated fibers. Seeds were surface disinfected for 30 mins in 0.4% NaOCl, then rinsed in five changes of sterile water to remove residual chlorine. Seeds were used in a series of experiments to determine bacterial effects on seedling mortality (% dead) after germination on agarose media and bacterial effects on growth of seedlings in soil.

Experiment 5: Competitor inhibition experiment (*Phragmites* mixture 1)

Eleven dandelion seeds were placed onto the surface of agarose medium (0.7%; Low melting point; Sigma-Aldrich, St. Louis, MO) in Petri dishes. One drop of bacterial mixture 1 (including equal parts of *M. oxydans* B2, *P. fluorescens* Sandy LB4 and *Pseudomonas* sp. West 9 at approximately OD 0.5, 600 nm) was applied to the surface of each seed (with 9 replica plates). Controls were moistened with sterile water only. Plates were then incubated at approximately 21C for two weeks in alternating light/dark conditions (10-h light/14-h dark). Seedlings were then examined to determine whether they were alive (light green) or dead (brown).

Experiment 6: Competitor inhibition experiment (*Phragmites* mixture 1 and 2 in soil)

In a second experiment, dandelion seeds were cleaned of external fibers, surface disinfected as above and placed onto sterile soil in magenta boxes (10 seeds/treatment). Treatments included bacterial mixture 1 (as indicated above) and bacterial mixture 2 (including strains: *Pseudomonas* sp. WY6m, *Pseudomonas* sp. Sandy LB6, *Pseudomonas* sp. Sandy Y8, *Pseudomonas* sp. RoLB13w). A control was not inoculated with *Phragmites* bacteria. To inoculate seeds with bacteria, 2 mL of a bacterial suspension at approximately 0.5 OD 600 nm was mixed into soil in magenta boxes. Seeds of *P. annua* were also pre-moistened with bacterial suspension or water (control) then placed into soil and boxes incubated for four weeks under alternating light/dark conditions (10-h light/14-h dark) and 21C. After four weeks, seedlings were removed from soil, weighed and root and shoot lengths determined.

Experiment 7: Competitor inhibition experiment (examination of individual bacterial strains)

A preliminary screen was conducted using individual strains of the bacteria included in mixture 1 (as indicated above). In this preliminary individual screen, 14 disinfected dandelion seeds collected from plants

growing locally were placed on agarose media in Petri dishes. Seeds were inoculated as above with each bacterium; control seeds were not inoculated. Three replica plates were made for each treatment and control. Plates were then incubated at approximately 21C for two weeks in alternating light/dark conditions (10-h light/14-h dark), after which mortality was assessed in each plate.

Experiment 8: Competitor inhibition experiment (*P. fluorescens* Sandy LB4)

A larger experiment was conducted using strain Sandy LB4. In this experiment, dandelion seeds were cleaned and surface disinfected as above, then placed on 0.7% agarose in 38 Petri dishes (15 seeds/dish). Seeds on nineteen of the plates were inoculated using a suspension of Sandy LB4 as above, and the other nineteen were not inoculated. Plates were incubated for two weeks under the conditions of the previous experiment, after which seedling mortality was assessed.

Experiment 9: Competitor inhibition experiment (effects on seedlings of curly dock (*Rumex crispus*)

Seeds of curly dock collected in South, River, New Jersey were cleaned of bracts and associated wings before being surface disinfected for 40 min in 4% NaOCl. Ten seeds were then placed onto each of five Petri plates containing 0.7% agarose. Each seed was inoculated with a drop of bacterial suspension (strain Sandy LB4). Five control replica plates were prepared where seeds were not inoculated. Plates were incubated at approximately 21C for one week in alternating light/dark conditions (10-h light/14-h dark) after which seedlings were stained using DAB and examined microscopically for evidence of bacteria within root cells.

Experiment 10: Competitor inhibition experiment (curly dock in soil)

A test was conducted in which 2 mL of individual bacteria (Sandy LB4, West 9 and *M. oxydans* B2) was mixed into previously sterilized soil in magenta boxes, and then ten surface disinfected seeds of curly dock were placed into the soil 2–3 mm beneath surface. A control treatment was not inoculated with bacteria. After two weeks, percentage of seedlings emerging to the soils surface was calculated.

Statistical analyses

In experiments 1 and 6, the differences between treatments were assessed by Duncan's multiple range test (SPSS version 20, IBM, USA). Treatment differences in experiments 5 and 8 were assessed using t-test options in Macanova 5.06 (University of Minnesota, Minnesota, USA). All differences were tested at the $p < 0.05$ level of significance. Results from other experiments were either qualitative or expressed as simple percentages and were not subjected to statistical analyses.

Results

Isolates

All of the plated seeds of *Phragmites* yielded bacteria. A total of 10 bacterial isolates were selected for testing. Of these, seven were shown to belong in genus *Pseudomonas* based on sequence data (Table 1). Two strains examined closely, *Pseudomonas* sp. West 9 and *P. fluorescens* Sandy LB4, were shown to become endophytic in seedlings of Bermuda grass and *P. annua*. These bacteria colonized root tip meristems and became intracellular in root cells, inducing formation of root hairs on seedling roots.

Modes of colonizing Bermuda grass seedling roots

Strains Sandy LB4 and West 9 were both shown to enter root cells at the root meristem and induce reactive oxygen (Figs. 1, 2, 3, 4, 5 and 6). Squash preparations of root tips showed presence of bacteria within meristematic cells. In older root cells, the spherical bacteria were seen to stain brown (positive indication of reactive oxygen) using the reactive oxygen stain DAB. Spherical bacterial L-forms were evident in root hairs and parenchyma cells that comprised the main axis of the root (Figs. 3–6). In older parts of the root, the L-forms were seen to swell and the contents lacked protein as evident by failure to stain blue using the aniline blue counterstain. In still older root parts, including root hairs and parenchyma, the internal bacterial inclusions were no longer evident (Fig. 6).

Table 1 Identity of bacteria isolated from *Phragmites australis* seeds and their characteristics

Collection Site, Strain ID	UV	P[a]	Prot.[b]	Genus and Species	GenBank No.
Sandy Hook, Sandy LB4	BF*	Y	Y	*P. fluorescens*	KX665565
Sandy Hook, Sandy LB6	WF	Y	Y	*Pseudomonas* sp.	KX650502
Sandy Hook, Sandy Y8	WF	Y	N	*Pseudomonas* sp.	KX650875
Sandy Hook, Sandy Y7w		Y	N	*Pseudomonas* sp	KX650497
Robbinsville, RoY12	NF	Y	N	*Pantoea* sp.	KX650498
Robbinsville, RoLB13w	WF	Y	Y	*Pseudomonas* sp.	KX650501
West Windsor, WY9y	NF	–	–	*Enterobacter* sp.	KX650499
West Windsor, West 9	WF	Y	N	*Pseudomonas* sp.	KX650874
South River, RiY3	NF	Y	–	*Pseudomonas* sp.	KX650500
South River, RiLB4	NF	Y	N	*Pantoea* sp.	KX752781

**BF* bright fluorescence, *WF* weak fluorescence, *NF* no fluorescence observed

[a] Phosphorus solubilization, Y = yes; − = missing data

[b] Protease production based on milk agar clearing; Y = yes; N = no; − = missing data

Seedlings derived from seeds that had not been inoculated with either Sandy LB4 or West 9 did not show root hair development or presence of bacterial L-forms within root cells (Figs. 1 and 2).

- Experiment 1: Plant growth promotion capacity

Pseudomonas fluorescens Sandy LB4, *Pseudomonas* sp. West 9 and *M. oxydans* B2 were found to increase growth of *Poa annua* in the magenta box experiment. Roots and shoots of seedlings bearing these three bacteria were shown to be significantly longer ($p < 0.05$) than those without bacteria or inoculated with *B. thiooxidans* TBN (from Japanese knotweed (*Fallopia japonica* Siebold & Zucc); family Polygonaceae; Table 2).

- Experiment 2: Seed germination rate and root architecture

In the germination and root growth study where we used axenic seeds of *Poa annua* and *Phragmites australis* to determine bacterial effects on seed germination and seedling root branching, we found that the germination rate was only slightly increased in *P. annua* seeds bearing Sandy LB4. However, there was a slightly greater tendency for roots bearing the bacterium to branch compared to roots without the bacterium. *Phragmites* seeds bearing strain Sandy LB4 showed a much higher germination rate compared to seeds without bacteria (Table 3).

- Experiment 3: Production of fungal inhibitors

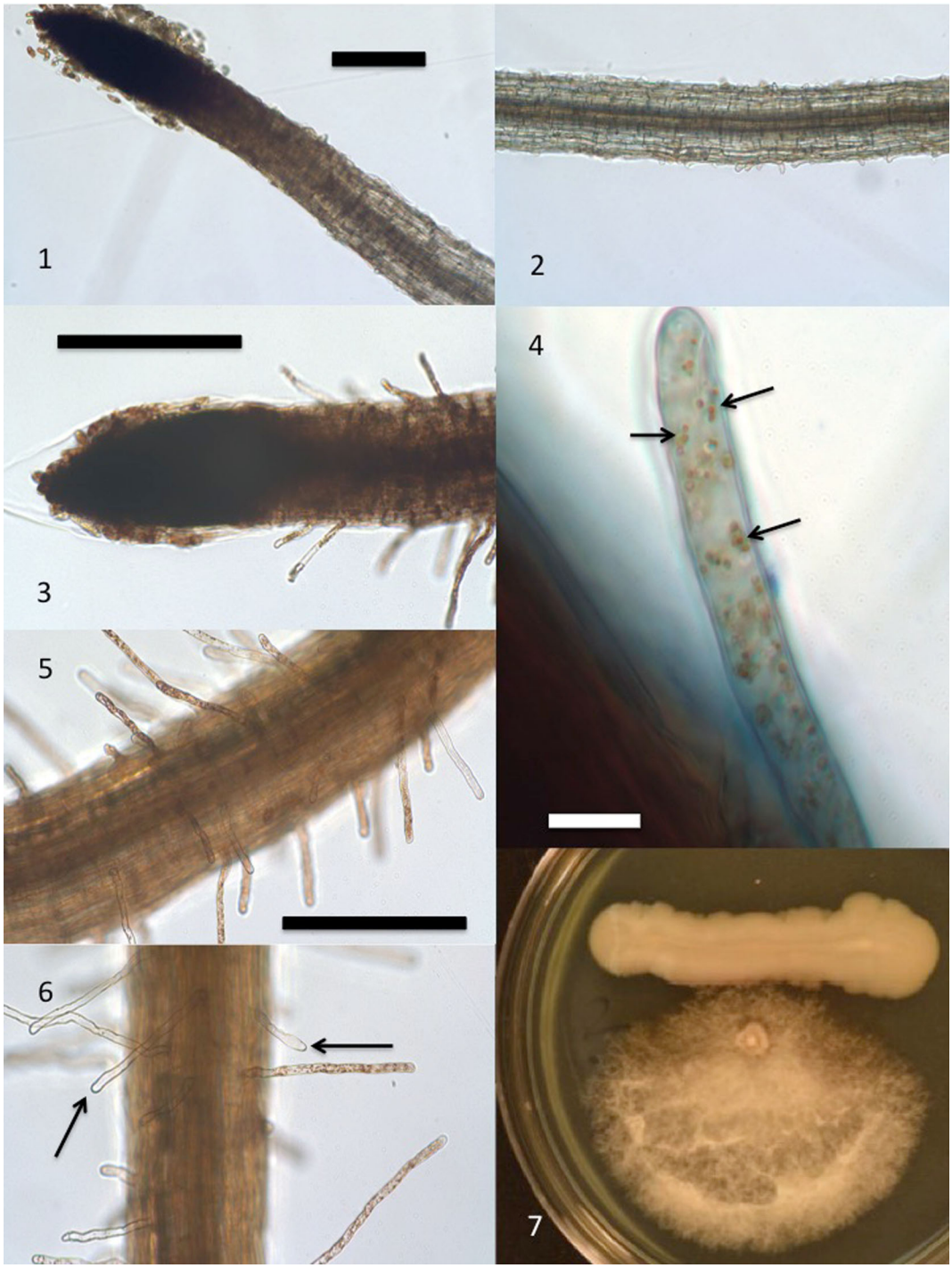
Inhibition zones were evident in co-culture experiments, where West 9 and Sandy LB4 were cultured with pathogens *S. homeocarpa* and *F. oxysporum* and appeared to inhibit them in culture (Fig. 7). *Microbacterium oxydans* B2 did not produce inhibitory substances in culture.

- Experiment 4: Damping off disease protection

In the experiment to evaluate the capacity of *Phragmites* bacteria to protect *P. annua* seedlings from damping off caused by *F. oxysporum*, we showed that the incidence of damping off was least in the pathogen and bacterial free treatment with 92% (23) of the seedlings surviving after 15 days. After 15 days, Sandy LB4 protected *P. annua* seedlings the most against damping-off with 80% (20) of the seedlings surviving; followed by West 9 with 64% (16) of the seedlings surviving, *M. oxydans* B2 with 60% (15) of the seedlings surviving and the pathogen + no bacterium treatment with 48% (12) of the seedlings surviving.

- Experiments 5 and 6: Effects of *Phragmites* bacteria on growth of seedlings of competitor species

In experiment 5, we showed that a mixture of *Phragmites* bacteria (including *M. oxydans* B2, *Pseudomonas* sp. West 9 and *P. fluorescens* Sandy LB4) increased



Figs. 1–7 Bermuda grass roots stained for H_2O_2 using DAB stain. 1. Axenic (not inoculated) Bermuda grass root tip showing absence of root hairs (bar =50 μ). 2. Axenic (not inoculated) Bermuda grass root behind tip showing absence of hairs and brown staining (for scale reference bar in Fig. 1). Fig. 3. Root tip of seedling inoculated with *Pseudomonas sp.* West 9 showing abundant hair formation behind meristem and brown staining due to presence of H_2O_2 (bar =50 μ). 4. Root hair showing internal L-forms of *Pseudomonas* sp. West 9 (bar =5 μ). 5. Root behind tip of seedling inoculated with *Pseudomonas* sp. West 9 showing abundant hair formation and brown staining due to presence of H_2O_2 (bar =50 μ). 6. Older part of root of seedling inoculated with *Pseudomonas* sp. West 9 showing clearing of root hairs due to degradation of bacteria internally (for scale reference bar in Fig. 5). 7. Coculture of bacterium *P. fluorescens* Sandy LB4 (above) and *Sclerotinia homeocarpa* (below) showing inhibitory zone

Table 2 Growth promotion of *Poa annua* in soil for 37 days (15 plants in each treatment)

Treatment	Shoot length (mm)[1]	Root length (mm)
No bacteria	88.87 ± 23.53[a]	17.27 ± 8.59 [a]
Bosea thioxidans TBN	86.2 ± 32.35[a]	13.73 ± 5.01[a]
P. fluorescens Sandy LB4	122.33 ± 27.38 [b]	23.8 ± 8.54 [b]
Pseudomonas sp. West 9	119.33 ± 20.43[b]	27.33 ± 14.56[b]
Microbacterium oxydans B2	113.73 ± 25.41[b]	20.87 ± 6.79 [a, b]

[1] Data is given as mean ± standard deviation. Means followed by the same letter within a column are not significantly different according to the Duncan multiple range test ($p < 0.05$)

mortality in dandelion seedlings compared to the non-inoculated control ($p < 0.05$ Student's t-test). The bacterial mixture had 83.4 ± 15.7% mortality compared to the axenic control that had 16.4 ± 22.8% mortality (Fig. 8).

In experiment 6, conducted in soil in magenta boxes, we found that inoculation of soil with bacterial mixture 1 (including Sandy LB4, West 9 and *M. oxydans* B2) resulted in seedlings that were significantly smaller ($p < 0.05$ in Duncan's multiple range test) in terms of shoot and root lengths and lighter than those of non-inoculated seedlings or those grown in soil containing *Phragmites* bacterial mixture 2 containing four other bacterial isolates (Table 4; Fig. 9).

- Experiments 7 and 8: Identification of strains showing dandelion seedling inhibition

In experiment 7, *P. fluorescens* Sandy LB4, *Pseudomonas* sp. West 9 and *M. oxydans* B2 were tested for their effect on mortality of dandelion seedlings on agarose. In this test, the most potent bacterium for increasing mortality of dandelion seedlings was Sandy LB4 (71%) > *M. oxydans* B2 (35%) > West 9 (31%) > no bacterium control (23%). On the basis of this test, we concluded that Sandy LB4 was the most potent of the bacteria increasing mortality in dandelion seedlings and a larger study (experiment 8) was set up to gather statistical data for effect of Sandy LB4 on dandelion seedling mortality. In the larger study, a statistically significant ($p < 0.05$ in student's t-test) increased mortality of 71.1 ± 15.3% was shown for Sandy LB4-inoculated seedlings compared to only 6.8 ± 7.4% for the non-inoculated control treatment.

- Experiments 9 and 10: Effects of *P. fluorescens* Sandy LB4 on curly dock (*Rumex crispus*) seedlings

In experiment 9, we evaluated the colonization of seedlings of *R. crispus* by Sandy LB4. Here, we found that Sandy LB4 actively colonized seedlings, inducing root hair development in the inoculated seedlings. Staining the seedlings using the reactive oxygen stain DAB and microscopic examination of roots showed that root hairs appeared to be occluded by large dark clusters of swollen degrading L-form bacteria (Figs. 10 and 11). In experiment 10, conducted in soil, we showed that curly dock seedling emergence was: no bacterium treatment (7 seedlings; 70% emergence) > West 9 (3 seedlings; 30% emergence) > *M. oxydans* B2 (2 seedlings; 20% emergence) > Sandy LB4 (0 seedlings; 0% emergence) Table 4.

Discussion

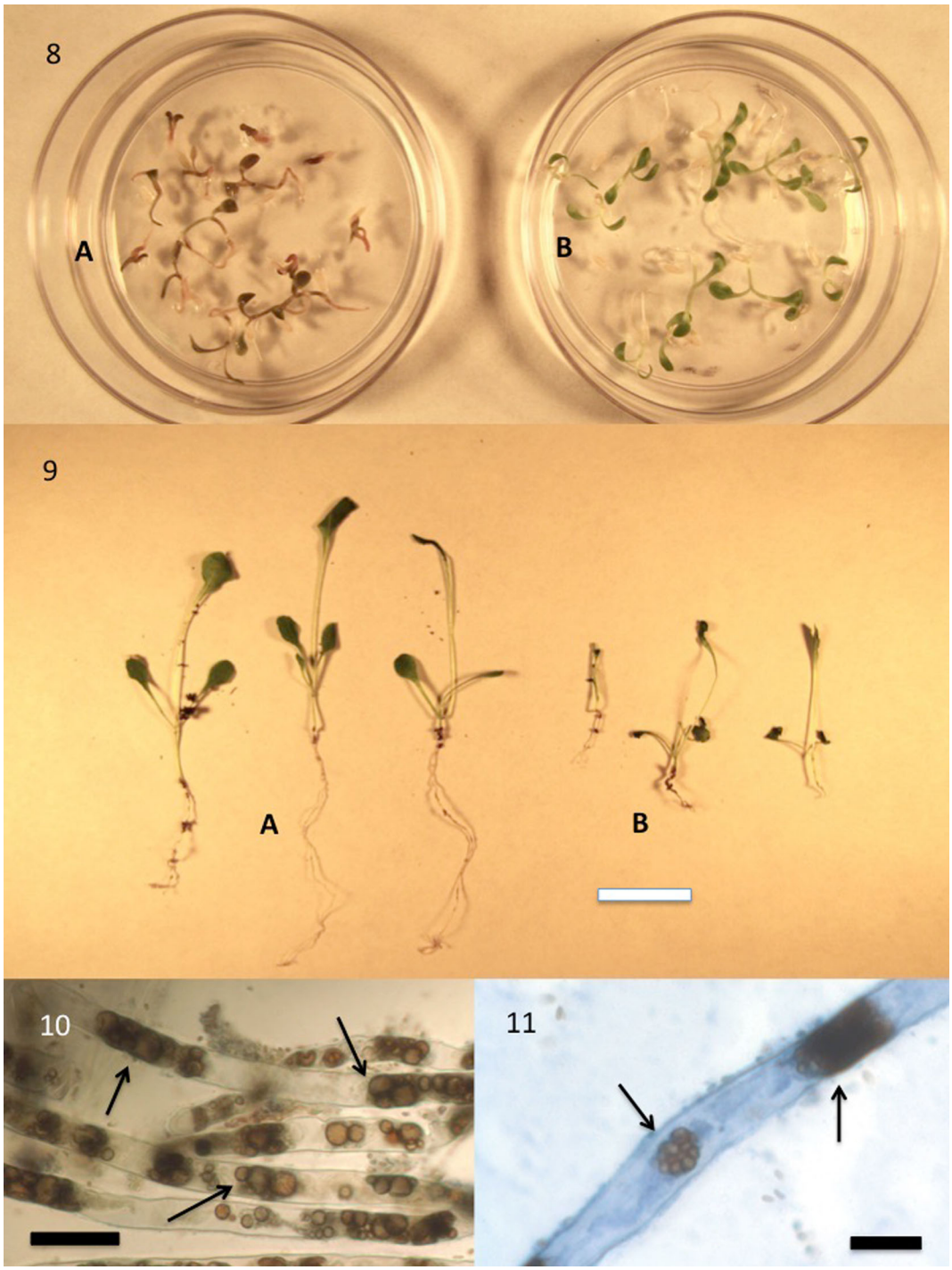
Endophytic nature of seed-associated bacteria

Our survey of *Phragmites* seeds from four sites suggests that *Pseudomonas* spp. dominate the cultivable bacteria on seeds in our collections. Whether, *Pseudomonas* spp. are widespread on seeds of *Phragmites* from diverse

Table 3 *Poa annua* and *Phragmites* seed germination and growth with *Pseudomonas fluorescens* Sandy LB4

Treatment	Germ. (%)	With-2 lateral roots (%)	Total
P. annua/Sandy LB4	95%	58	122
P. annua/no bacteria	90%	50	117
Phragmites/Sandy LB4	97%	___*	40
Phragmites/no bacteria	37.5%	___	40

**Phragmites* seedling roots did not continue to grow in agarose and root branching data was not taken. Data were not subjected to statistical analysis



Figs. 8–11 Dandelion and curly dock seedlings. 8. Results of mortality experiment showing high mortality of dandelion seedlings inoculated with *P. fluorescens* Sandy LB4 (A) while dandelion seedlings that were not inoculated remain living (B). 9. Results of experiment in soil showing larger non-inoculated dandelion seedlings (A) while seedlings inoculated with *P. fluorescens* Sandy LB4 remain much smaller (bar =10 μ). Figs. 10 and 11. Oxidized bacteria within root hairs of curly dock seedling stained for H_2O_2 using DAB stain. 10. Root hairs of curly dock seedling inoculated with *P. fluorescens* Sandy LB4 showing abundant internal oxidized L-form bacteria (arrows) occluding root hairs (bar =7 μ). 11. Close-up of curly dock root hair showing bacterial occlusions in hairs (bar =7 μ)

Table 4 Dandelion seedling growth inhibition in soil by *Phragmites* bacterial mixtures

Treatment	Seedling mass Wet weight (mg)	Shoot length (mm)	Root length (mm)
No bacteria	27.03 ± 9.54[*a]	42.44 ± 12.6 [a]	40 ± 17.11 [a]
Mix 1[1]	14.91 ± 8.71 [b]	23.88 ± 11.86 [b]	14.63 ± 5.21 [b]
Mix 2	26.36 ± 6.78 [a]	40.89 ± 10.71 [a]	43.33 ± 19.55 [a]

*Data given as means ± standard deviation. Within columns means followed by the same letter are not statistically different according to the Duncan multiple range test ($p < 0.05$)

[1] Mix 1 included strains *Microbacterium oxydans* B2, *Pseudomonas fluorescens* Sandy LB4, and *Pseudomonas* sp. West 9; while mix 2 included strains: *Pseudomonas* sp. Sandy LB6, *Pseudomonas* sp. Sandy Y8, *Pseudomonas* sp. RoLB13w

collections of non-native and native populations has yet to be evaluated. Using representative strains *P. fluorescens* Sandy LB4 and *Pseudomonas* sp. West 9 to inoculate axenic Bermuda grass seedlings, we showed that on germination of seeds both bacteria colonize the root meristem and enter into meristematic cells. In the process of becoming intracellular, the bacteria lose their cell walls and their rod shapes, becoming wall-less spherical L-form bacteria (Aloysius and Paton 1984). Colonizing the root tip meristem enables the intracellular bacteria to become distributed in most or all cells that make up the root (Figs. 1–6). Intracellular bacteria were found to be generally surrounded by H_2O_2 (Fig. 4). The H_2O_2 is likely produced by the host plant and may be a mechanism to control or degrade the intracellular bacteria. As tissues of the root, including root hairs, became older, the intracellular bacteria swelled and their cytoplasmic contents disappeared. Presence of H_2O_2 around the intracellular bacteria suggests that the disappearance of bacteria was due to their degradation by the host cells at least in part through oxidation (White et al. 2012). In more mature root tissues, all evidence of the intracellular bacteria completely disappeared from plant cells (Fig. 6), suggesting that the intracellular bacteria were entirely degraded. Bacterial cells that do not enter the root tip cells do not appear to elicit a strong reactive oxygen response and may not be degraded; instead they likely remain as intercellular endophytes in the mature root tissues. While we did not examine shoot meristems for the presence of intracellular bacteria, they could be present there as well since they somehow become deposited on the seeds (Lamb et al. 1996).

Degradation of endophytic microbes as a nutritional source

Degradation of microbes in or on roots was proposed to be a mechanism of transfer of nutrients from microbes to plant (Paungfoo-Lonhienne et al. 2010; White et al. 2012; Beltran-Garcia et al. 2014). Paungfoo-Lonhienne et al. (2014) denominated the microbe consumption process 'rhizophagy' because roots consumed microbes. For a mechanism like rhizophagy to provide substantial nutrients to plants, the microbes must obtain their nutrients from outside roots, then enter roots and release their nutrients to the plant tissues. We have experimental evidence that in the case of the *Pseudomonas* endophytes of *Phragmites*, the bacteria move from plant out into the soil and perhaps may return to recolonize the plant root meristem. If colonization of roots by the soil bacteria is occurring with any regularity, a constant flow of nutrients from soil reserves to the plant may be occurring. In previous research, we hypothesized that microbes may aid plants by scavenging organic or inorganic nitrogen (White et al. 2015; Soares et al. 2016a). However, all of the pseudomonads we isolated from *Phragmites* seeds showed the capacity to solubilize phosphate when grown on Pikovskaya agar (Table 1; Sharma et al. 2013). Further, *P. fluorescens* and other fluorescent pseudomonads are known to secrete fluorescent siderophores called pyoverdines that actively bind iron (Visca et al. 2006). We confirmed that Sandy LB4 secreted a fluorescent pigment that fluoresced brightly in culture and that several other strains from *Phragmites* seeds (Table 1) fluoresced weakly in culture when viewed under an ultraviolet light, suggesting that several of our isolates possess the siderophores. Sandy LB4 was also shown to produce a secreted protease (Table 1) that might enable it to degrade microbial proteins present around roots and thus acquire organic nitrogen (White et al. 2015). The endophytic pseudomonads could function as nutrient scavengers for their *Phragmites* seedlings (Zolg and Ottow 1975) and could contribute to the large biomass accumulation typical of *Phragmites* stands. Whether

pseudomonads are in fact responsible for significant nutrients obtained by *Phragmites* will require additional experimentation to evaluate.

Intracellular endophytes and oxidative stress

Bacterial endophytes that internally colonize root meristems and stimulate plants to increase production of reactive oxygen may be oxidative stressors that trigger the host to express oxidative stress resistance features, making the host more resistant to oxidative stress. Perhaps consistent with this proposal is that multiple studies of fungal endophytes have shown that endophytes increase the oxidative stress resistance in the host (White and Torres 2009; Hamilton and Bauerle 2012; Hamilton et al. 2012). Oxidative stress protection could also account for some types of abiotic stress tolerance, including drought, salt and heavy metal tolerance that all result in increased oxidative stress in the plant host (Li et al. 2012). Future studies will be necessary to confirm this hypothesized linkage between endophytes that elicit reactive oxygen production by host tissues and oxidative stress protection of the host.

Growth promotion and endophyte-host compatibility

Using *P. annua* to test for growth promotion capacity (Experiments 1 and 2), we found that *Phragmites* isolates increased growth of grass while the bacterium (*B. thiooxidans* TBN) from a non-grass host (Japanese knotweed; family Polygonaceae) showed only partial compatibility with the grass. *B. thiooxidans* TBN stimulated root hair growth but did not increase overall plant growth of *P. annua*. Microscopic examination of Bermuda grass seedlings inoculated with this bacterium showed that it did not become endophytic in the grass host and was present only in patches on the surface of root hairs. We interpret that the *Phragmites* endophytes are compatible with the test grasses we used, but *B. thioxidans* TBN is only partially compatible with the grasses. Zeller et al. (2007) came to a similar conclusion regarding results of experiments where they examined cross compatibility of rhizobacteria between distantly related host plants. It seems likely that endophytes and their hosts may be co-adapted and that compatibility is more likely between closely related hosts.

Suppression of soil borne disease

In co-culture experiments (Experiment 3), we demonstrated that the *Phragmites* pseudomonads produced unknown antifungal substances that inhibited two fungal pathogens (*S. homeocarpa* and *F. oxysporum*). In an experiment to assess protection from damping off disease caused by *F. oxysporum* (Experiment 4), we further showed that the pseudomonads reduced damping off disease in seedlings when compared to non-inoculated controls. In this same experiment, we showed that the two pseudomonads originally inoculated onto seeds moved into the soil for some distance away from seedlings, suppressing mycelial growth in and on the surface of the soil. Movement of seed-vectored microbes into the soil was previously shown for corn endophytes by Johnston-Monje et al. (2016). While in our study *M. oxydans* also moved out into the soil, it did not have any mycelial growth suppression effects and fungi grew readily over the surface of the soil in these treatments. From these experiments, we can conclude that the *Phragmites* pseudomonads may be effective in protecting *Phragmites* seedlings from disease and suppressing soil borne pathogenic fungi.

Some other *Pseudomonas* spp. that have been shown to be effective in biological control of diseases have been found to produce antifungal metabolites, including pyrrolnitrin, phenazines, pyoluteorin and hydrogen cyanide (Ligon et al. 2000; Compant et al. 2005). Whether any of our pseudomonads produce any of these antifungal metabolites has yet to be determined. Other endophytes protect plants from disease by increasing expression of plant resistance genes (Kloepper et al. 2004). Gond et al. (2015) showed that a *Bacillus amyloliquefaciens* endophyte in Indian corn induced increased expression of pathogen defense genes in the host. We presently cannot determine precisely how these endophytic bacteria protect plants from damping off disease.

Because *P. australis* seedlings are difficult to grow under laboratory conditions for many of our experiments, we used other species (*P. annua* and *C. dactylon*) with seeds that could easily be obtained, sterilized and grown in agarose media. While our test grasses were related to *P. australis*, it is entirely possible that the test grasses and *P. australis* may respond differently to presence of the microbes. For this reason, it will be important to confirm results of our experiments using seedlings of *P. australis* in future studies.

Endophyte inhibition of competitor species

Through a series of experiments (Experiments 5–10) using bacteria obtained from *Phragmites* to inoculate dandelion (*T. officionale*) and curly dock (*R. crispus*), we showed that the strain *Pseudomonas fluorescens* Sandy LB4, whether alone or in mixtures of bacteria, substantially increased mortality rates of dandelion seedlings (Table 4; Fig. 8) and in general reduced growth and vigor in both weed species (Figs. 9). A possible mechanism of antagonism against weeds may relate to secondary metabolites produced by the bacteria. Among the features of *P. fluorescens* is the production of hydrogen cyanide (HCN). HCN combines with cytochrome oxidase and prevents aerobic respiration. Differing sensitivities of hosts to *Pseudomonas* rhizobacteria-produced HCN was suggested by Zeller et al. (2007) to explain differing reactions of host and non-host species to the bacteria. Another possible mechanism to explain differential responses of host and non-host seedlings was evident when seedling roots were stained using DAB to visualize bacteria in roots. In grass roots, bacteria remained as small discrete spherical bodies (Fig. 4), likely flowing in the cytoplasm with the normal cytoplasmic streaming. However, the bacteria in the non-host species formed large clusters that appeared to occlude the root hairs (Figs. 10 and 11), likely blocking normal cytoplasmic streaming in the root hairs. Further examination is required to determine whether differential sensitivity to HCN or physical occlusion of absorptive root hairs or other structural issues is responsible for the different responses of host grasses and non-host dandelion and curly dock seedlings to *P. fluorescens* Sandy LB4.

Regardless of the mechanism of inhibition, the presence of some endophytes in *Phragmites* that show capability to inhibit competitor plants could provide a mechanism by which *Phragmites* excludes competitor species from its stands. The allelopathic exclusion of plants from *Phragmites* stands has been observed and previously attributed to secreted chemical inhibitors such as gallic acid (Rudrappa et al. 2007; Rudrappa et al. 2009; Weidenhamer et al. 2013) and phytotoxity of *Phragmites* residues (Uddin et al. 2014). It is possible that some of the allelopathic exclusion of competitors is a function of the growth promotional pseudomonads that are endophytic in plants and move out into soils, likely colonizing and inhibiting competitor plant species that are not adapted to them.

Effect of seed-vectored microbes on competitive capacity of the grass host

Kowalski et al. (2015) hypothesized that invasive *P. australis* utilized symbioses with microbes to enable it to outcompete native vegetation. Li et al. (2010) and Clay et al. (2016) showed that *P. australis* is inhabited by diverse community of endophytic microbes. Soares et al. (2016a) demonstrated that several bacterial endophytes of *Phragmites* appeared to function to increase nutrient uptake into plant roots. Further, Soares et al. (2016b) demonstrated that fungal endophytes of *Phragmites* play a role in adapting the host to high saline soils. In the present study, we demonstrate that *Phragmites* seeds may carry pseudomonads that are clearly multifunctional in that they may stimulate growth and increase nutrients available to the grass, but also may inhibit soil borne fungal pathogens, suppress diseases and inhibit competitor weed species. This research confirms our initial hypothesis that seeds of *Phragmites* carry components of their microbiome that are defensive against pathogens and maximize capacity of seedlings to grow and compete with other plants, and further, that *Phragmites* employs its seed microbiome as a tool to engineer its soil ecosystem to make it more favorable to *Phragmites* seedlings and less favorable to pathogens and plant competitors.

The seed-associated pseudomonads that we encountered in only four collections of the invasive haplotype M would seem likely to increase the competitive capacity of the host. However, *P. australis* is highly diverse in its genetics, ecology and biogeography, and our results in terms of microbiome composition and effects on hosts may not be generalizable across all locations and genotypes. In addition, many questions remain to be answered. For example: Are soils around *Phragmites* stands dominated and patrolled by the motile pseudomonad endophytes of the plant? Do these beneficial microbes suppress growth or pathogenicity of many potential pathogens in these soils? Do the pseudomonads of *Phragmites* function in consortia with division of functionality between members of a consortium? Future research is needed to evaluate the full impact of the seed associated microbes on the soil ecosystem.

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