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PHRAGMITES INVASION

Evaluation of the functional roles of fungal endophytes of *Phragmites australis* from high saline and low saline habitats

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Abstract Non-native *Phragmites australis* decreases biodiversity and produces dense stands in North America. We surveyed the endophyte communities in the stems, leaves and roots of collections of *P. australis* obtained from two sites with a low and high salt concentration to determine differences in endophyte composition and assess differences in functional roles of microbes in plants from both sites. We found differences in the abundance, richness and diversity of endophytes between the low saline collections (18 species distributed in phyla Ascomycota, Basidiomycota and Stramenopiles (Oomycota); from orders Dothideales, Pleosporales, Hypocreales, Eurotiales, Cantharellales and Pythiales; Shannon H = 2.639; Fisher alpha = 7.335) and high saline collections (15 species from phylum Ascomycota; belonging to orders Pleosporales, Hypocreales, Diaporthales, Xylariales and Dothideales; Shannon H = 2.289; Fisher alpha = 4.181). *Peyronellaea glomerata*, *Phoma macrostoma* and *Alternaria tenuissima* were species obtained from both sites. The high salt endophyte community showed higher resistance to zinc, mercury and salt stress compared to fungal species from the low salt site. These endophytes also showed a greater propensity for growth promotion of rice seedlings (a model species) under salt stress. The results of this study are consistent with the 'habitat-adapted symbiosis hypothesis' that holds that endophytic microbes may help plants adapt to extreme habitats. The capacity of *P. australis* to establish symbiotic relationships with diverse endophytic microbes that enhance its tolerance to abiotic stresses could be a factor that contributes to its invasiveness in saline environments. Targeting the symbiotic associates of *P. australis* could lead to more sustainable control of non-native *P. australis*.

Keywords *Phragmites australis* · Invasive · Endophytic fungi · Heavy metal · Salt stress

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Introduction

Endophytic fungi are ubiquitous in plants where they associate with multiple plant tissues (Bacon and White 2000; Currie et al. 2014). These microorganisms are frequently examined as potential sources of bioactive metabolites (Zhao et al. 2011), enzymes of industrial interest (Grawe et al. 2015); they also play roles in biological control of plant pathogens (Compant et al. 2010), plant growth promotion (You et al. 2012) and bioremediation (Li et al. 2012a, b). Endophytes play important ecological roles for environmental adaptation of their hosts including increasing resistance against pathogens and herbivores (Clay and Schardl 2002), assisting in mineral nutrition of the plant (Kipfer et al. 2011), mitigating the effects of water stress (Atala et al. 2012), and affecting plant responses to other biotic and abiotic stresses (Clay and Schardl 2002; Rodriguez et al. 2008; Cheplick and Faeth 2009; Soares et al. 2015). Symbiotic relationships can have a direct or indirect impact on the structure, function and composition of plant communities, the expansion of host niches (Rudgers et al. 2015), and food webs and ecosystem processes (Clay and Holah 1999; Aschehoug et al. 2014) including invasion processes. Mutualisms often are an important mechanism used in invasion processes (Callaway et al. 2011), so the competitive ability of invasive species can be increased with mutualistic associations established with the autochthonous microbiota (Reinhart and Callaway 2006; Jordan et al. 2008; Andonian and Hierro 2011) or endophytic microorganisms (Aschehoug et al. 2012, 2014). Rodriguez et al. (2008) and Redman et al. (2002) proposed that symbiosis with endophytic microbes may help plants adapt to particular environmental stresses; they termed this hypothesis 'habitat-adapted symbiosis'.

Common reed (*Phragmites australis* subsp. *australis* (Cav.) Trin. Ex Steud.) is a perennial grass native to Eurasian wetlands (Holm et al. 1977; Meyerson and Cronin 2013). In North America, this non-native macrophyte is highly productive and often outcompetes native plants to create large expanses with very low plant and wildlife biodiversity (Silliman and Bertness 2004; Meyerson et al. 2009). Unlike the native genotype (*Phragmites australis* subsp. *americanus*), the non-native *Phragmite australis* subsp. *australis* (*P. australis*) is considered an invasive species and very aggressive (Gessner et al. 1996; Saltonstall 2002; Windham and Meyerson 2003; Lambertini et al. 2012; Meyerson and Cronin 2013). Success in invasion has been related to the ecological capability of *P. australis* to tolerate and grow in a range of soil salinity and fertility levels (Haslam 1972; Lissner et al. 1999a, b; Meyerson et al. 2000), its wide dispersion efficiency (Kettenring and Mock 2012; Meyerson et al. 2012), high genetic diversity (Saltonstall 2002; Lambertini et al. 2006; Fer and Hroudova 2009), and microbial symbiosis (Kowalski et al. 2015). It also has been shown to tolerate heavy metals and other stresses (Quan et al. 2007; Bonanno and Giudice 2010). The ability of *P. australis* to cope with salt stress plays a crucial role in colonization of coastal habitats (Achenbach and Brix 2014), but it is unclear how much the plant's microbiome influences the resistance to salt stress.

The microbial endophyte communities of *P. australis* have been examined in several previous studies (Wirsel et al. 2001; Li et al. 2010; Angelini et al. 2012; Fischer and Rodriguez 2013; Kim et al. 2014; Hipol and Cuevas 2014; Sim et al. 2015; Sauvêtre and Schröder 2015). The differentiation of the composition and functions of the microbiota associated with *P. australis* in the invasion process are still unknown. We are not aware of any previous studies that compare the structure and diversity of the endophytic fungal community from *P. australis* between high salt and low salt sites and examines the differentiation of these communities by functional traits.

Our hypothesis is that the capacity of *P. australis* to tolerate high salt soils is partially the result of endophytic fungi that enhance host tolerance to salt stress. We use culture dependent methods to assess the community structure and diversity of endophytic fungi associated with *P. australis* (haplotype M from one site in New Jersey) in high salt and low salt soils. In developing this work, we attempted to answer the following questions: Do endophytic fungal communities in grasses at the two sites differ in structure and composition? Do these communities themselves differ in the degree of resistance to environmental stress? Do these communities differ in the capacity to mitigate stress in the plant?

Materials and methods

Collection sites

P. australis plants were collected from two sites along the shore in Sandy Hook, New Jersey. Five plants were

collected in each site. In the inland population in *site 1*(40.45°N; −73.99°W) approximately 500 m from the shoreline, the sparse population margins in dry sand at the edge of a dense stand in fresh standing water was sampled; and at *site 2* (40.47°N; −74.00°W) a dense population was located on the shore approximately 4 meters from the water at high tide. Whole plants (1.5–2.0 m height) with undamaged leaves, roots and rhizomes were collected, brought to the laboratory in polythene bags, and processed the same day. Plants were confirmed as the invasive haplotype M morphologically by Dr. Bernd Blossey in the Department of Natural Resources at Cornell University. For analysis of soil sodium content at both sites, soil was sampled at a depth of 10 cm with approximately six samples arbitrarily taken from around the base of plants. Sand was sampled at various places and levels and combined at each site. Samples were sent to the Rutgers University Soil Test Laboratory and analyzed for exchangeable cation extraction which would specifically provide Na^{+}, Ca^{++}, Mg^{++}, and K^{+} values (typically as milli-equivalents/100 g). The lime requirement index and soil pH also were tested (McLean 1982).

Isolation and identification of microorganisms

In the laboratory, apical meristems, fully expanded apical leaves and roots (approximately 0.5–1-mm-thick) were sampled from each plant for endophyte analysis. The plant material was superficially disinfected with 70 % ethanol for 1 min, 2.5 % sodium hypochlorite for 5 min (meristematic tissues and leaves) or 7 min (roots), and then rinsed 5 times with autoclaved distilled water. The edges of the leaf fragments were removed and fragments of tissues (2 × 2 mm) were placed on Petri plates containing 10 % trypticase soy agar (10 % TSA), yeast extract-sucrose agar (1 % yeast extract + 1 % sucrose; 1 % YES) and potato dextrose agar plus three antibiotics (ampicillin + tetracycline + streptomycin—50 μg ml^{-1} for each antibiotic; PDA + 3). We used three media to maximize the likelihood that endophytes in samples would be cultured and detected. Meristem and root fragments (approximately 2-mm-long) were analyzed on the same three media types. The plates were incubated at room temperature and growth was assessed as it occurred. We analyzed 504 fragments (252 from *site 1* and 252 from *site 2*) of each tissue on the media (7 plates/12 fragments each plate). Representative isolates were stored at −80 °C in the Department of Plant Biology and Pathology, Rutgers University, New Brunswick, New Jersey, USA.

The endophytic fungi growing from the samples were grouped into morphotypes following the method used by Lacap et al. (2003). To confirm the grouping, microscopic traits were observed via slides obtained from microculturing. DNA was extracted from a representative of each morphological group with QIAGEN miniprep kits according to the manufacturer's recommendations. The validation of morphotypes was confirmed by fingerprinting analysis (data not shown) using molecular markers inter-retrotransposon amplified polymorphism (IRAP) and inter-simple sequence repeat ISSR with primers CLIRAP1 (5′-CGT CGA GAA ACA GCT CAC GA-3′) CLIRAP 4 (5′-CTT CCA TTG GCA AGG TGC-3′) (Santos et al. 2012) and BH1 (5′-GTG GTG GTG GTG GTG-3′), respectively. The primers ITS5 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) were used for amplification of the ITS region (White et al. 1990). The PCR products were purified with QIAquick PCR Purification Kit and sequenced by the Sanger method (Genewiz, South Plainfield, New Jersey). The sequences were aligned and fixed in MEGA 6 (Tamura et al. 2013). The ITS sequences were compared with sequences deposited in GenBank database using BLASTn (http://www.ncbi.nlm.nih.gov). Identifications were based on Blastn and morphological analysis. ITS sequences with ≥97 % identity (Morris et al. 2008) and morphological similar characteristics were grouped in same species. Colonization frequency (CF%) was determined as the percentage of the endophyte-infected fragments of the total observed fragments in each site. Diversity was measured as Shannon–Wiener and Fisher's alpha index to characterize the species diversity (Fisher et al. 1943; Spellerberg and Fedor 2003) and species evenness was estimated with Pielou's evenness index (Pielou 1966) in Past 3.x (Hammer et al. 2001).

Metal and salt resistance of isolates

Resistance and susceptibility to metal toxicity and salt stress were examined for each isolate using the PDA medium amended with $HgCl_2$ (1 mM), $ZnSO_4{\cdot}7H_2O$ (10 mM) (Aleem et al. 2003) and NaCl (200 mM). In parallel, cultures without metal and salt were

performed as the control treatments. For each factor, the endophytic strains were incubated at 28 °C for 10 days. The radial growth was evaluated from two perpendicular measurements (in mm) that passed through the center of the inoculated portion every other day. The tolerance index (TI), an indication of the organism response to stress was calculated from the growth rate of the strain exposed to metals or salt divided by the growth rate in the control plate. TI > 1 and TI $= 1$ indicated a resistance strain and TI < 1 indicated a sensible strain. A TI $= 0$ result indicated that the strain was killed by stress. Three replicates were used for each fungal species and control. The TI data were subsequently analyzed using the multivariate ordination technique principal component analysis (PCA) to assess whether different endophytic communities differ by tolerance stress. PCA scores were further analyzed by ANOVA using R version 3.0.2 (R Core Team 2013) to determine the significance of separations observed in PCA plots to examine similarities between TI and fungi species site.

Effect of endophytic community on rice under salt stress

The ability of the strains to assist the host under salt stress was evaluated in rice seedlings (*Oryza sativa* L.) because it was not possible to remove native microbes from *P. australis* seedlings (see Fischer and Rodriguez 2013); furthermore, *P. australis* and rice have similar responses against pollutants (Chu et al. 2006) and anoxic conditions (Armstrong and Armstrong 2001). To reduce microbial populations on rice seedlings, seeds were immersed in distilled water and maintained at 60 °C for 30 min. The seeds were superficially disinfected with sodium hypochlorite 2.5 % for 15 min and then rinsed 5 times with autoclaved distilled water. Then, the cultivable endophyte-free (E−) seeds were germinated in 10 % TSA for 5 days. To create the experimentally inoculated plants, seedlings (E−) were placed in Petri dishes containing fungal endophytes cultured on potato dextrose agar and allowed to remain in contact with fungal hyphae for 12 h to ensure adequate inoculation. Then, seedlings were transplanted into magenta boxes containing autoclaved vermiculite:soil mix (3:1) moistened with Murashige and Skoogs salt solution (Sigma-Aldrich) plus 0 or 200 mM NaCl. Fafard® Growing Mix 2 was autoclaved 2× for 1 h each autoclaving (1 atm at 121 °C). Magenta boxes were incubated at ambient laboratory temperature in a 12-h alternating light/dark cycle. Eight seedlings were used for each treatment. Plants in vitro were collected after 20 days and rinsed in tap water. The lengths of shoots and roots were measured. Growth promotion efficiency (GPE) was estimated to elucidate the relative effect of tested strains on plants according to Almoneafy et al. (2014). The experiment was set up as a completely randomized design. Data of plant growth and others parameters were analyzed using R version 3.0.2 (R Core Team 2013). Logarithm and natural logarithm transformed data were used for the ANOVA when non-homogeneity of variances was observed.

Results

Isolation and identification of microorganisms

Soil from *site 1* exhibited lower values of sodium concentration 25.0 mg/kg soil (low sodium concentration—LS) compared with the soil collected from *site 2*, which was 175.2 mg/kg soil (high sodium concentration—HS) (Table 1). The two sites were representative of distinct environments in salinity. Populations of *P. australis* were very uniform in these environments, as well as other soil chemical properties. Therefore, the choice of the two sites is based on the homogeneity of the two collection sites to assess our hypothesis about the effect of salinity on the community of endophytic fungi.

There was no fungal growth from fragments of leaves and meristems over a period of 45 days. We observed abundant growth of fungi from fragments of roots. These strains were isolated and separated into 33 morphotypes from 225 strains of endophytes isolated from 252 fragments of the roots from site 1 (LS) (18 morphotypes) and 252 fragments of the roots from site 2 (HS) (15 morphotypes).

The colonization frequency (CF) of roots differed significantly (*t* test: t 4.2716, *p* 0.00011) according to collection site. Regardless of the medium used, roots collected in LS resulted in CF 15.10 ± 10.67 % colonization, while roots in HS reached 32.45 ± 6.56 % colonization. Considering each culture medium and collection site, the CF in PDA did not differ between the sites, unlike of CF in 10 % TSA and 1 % YES. The CF obtained in different media for HS did not differ between

Table 1 Soil characterization by cation exchange analysis

	pH	Lime requirement index	Mg[a]	K[a]	Ca[a]	Na[a]
Site 1/LS	6.43	7.88	0.49 (59.5)	0.13 (52.8)	0.56 (112.6)	0.11 (25.0)
Site 2/HS	6.66	7.94	0.30 (36.3)	0.06 (22.8)	0.56 (113.1)	0.76 (175.2)

[a] Meq/100 g (mg/kg soil)

culture media. But the CF in the roots of LS varied with the culture medium used (ANOVA: F 9.269, p 0.002). The CF in PDA resulted in 27.38 ± 10.64 % of the fragments with endophytes (Tukey's test, $p < 0.05$) compared with 10 % TSA (9.52 ± 9.37 %) and 1 % YES (8.33 ± 4.45 %).

We obtained 225 isolates comprising 30 species in 23 genera from both HS and LS plant populations (Table 2). Analyses of ITS rDNA sequences revealed the correspondence of the morphotypes with distinct species of filamentous fungi. Roots from plants at the HS and LS sites were colonized by 14 and 16 genera, respectively. The sequences have been deposited in GenBank under accession numbers: KT827254–KT827285.

The endophyte community from plants at the LS site showed higher species richness (Table 2): 18 species of fungi, distributed into five classes (Dothideomycetes, Sordariomycetes, Agaricomycetes, Eurotiomycetes and Oomycetes) and six orders (Dothideales, Pleosporales, Hypocreales, Eurotiales, Cantharellales and Pythiales). Sordariomycetes, Pleosporales and Hypocreales, were represented by the greatest numbers of species. The endophyte community in roots from the HS sites showed lower richness, consisting exclusively of species of Ascomycota (Table 2), almost equally distributed in the Dothideomycetes and Sordariomycetes classes, and included orders Pleosporales, Hypocreales, Diaporthales, Xylariales and Dothideales; orders Pleosporales and Hypocreales were the most frequently isolated.

The species *Peyronellaea glomerata*, *Phoma macrostoma* and *Alternaria tenuissima* were encountered in both low and high saline sites (Table 2). The species richness difference between the plants at the two sites is reflected in the indices of diversity. The indices were higher for plants at the low saline site (Shannon H = 2.64; Fisher alpha = 7.33) compared to those at the high saline site (Shannon H = 2.29; Fisher alpha = 4.18). The dominant species in the low saline site were *Peyronellaea glomerata*-FN3 (relative abundance 19.23 %) and *Fusarium sporotrichioides*-FN23 (relative abundance 19.23 %); and *Fusarium oxysporum*-FI28 (relative abundance 28.57 %) and *Peyronellaea glomerata*-FI47 (relative abundance 20.40 %) were more frequently isolated in plants from the high saline site (Table 2).

Stress tolerance

The addition of mercury ($HgCl_2$ 1 mM) improved growth (TI ≥ 1) of *Phomopsis* sp.-FI26, *Phomopsis mali*-FI15, *Fusarium oxysporum*-FI28 and *Paraphoma radicina*-FN8 (Table 2). The other species were sensitive to mercury resulting in TI values <1. The resistance profiles on $ZnSO_4.7H_2O$ (10 mM) were differentiated by isolation source. Approximately 61 and 26.5 % of the endophytic microbes from the low and high saline sites, respectively, did not grow in the medium containing Zn^{2+} (IT = 0) (Table 2). The addition of zinc to the culture medium improved the *Phomopsis* sp.-FI26 growth (TI > 1). The other strains showed TI < 1 values indicating sensitivity to $ZnSO_4{\cdot}7H_2O$ (10 mM). The TI values varied according to the host source, ranging from 0.05 to 0.33 (species from low saline site) and 0.07–0.94 (species from high saline site), respectively.

The profiles of salt tolerance in endophytes differed based on whether plants grew in a low or high salt soil. The addition of 300 mM NaCl favored growth (TI > 1) of approximately 87 % of fungi obtained from the high saline site. Only *Fusarium avenaceum*-FI25 and *Pseudoseptoria obscura*-FI48 were sensitive (TI < 1) to salt stress. In contrast, only *Alternaria* sp.-FN20, *Fusarium sporotrichioides*-FN23 and *Ilyonectria radicícola*-FN28, isolated from the low saline site, were resistant (TI > 1) to NaCl 300 mM. The other species (approximately 83 %) were sensitive (TI < 1) to NaCl. The PCA analysis indicated that the set of effects (TI values: Hg^{2+}, Zn^{2+} and NaCl) could differentiate fungal community from the *site 1* = LS and *site 2* = HS (Fig. 1). The PC1 and PC2 axes

Table 2 Endophytes from *Phragmites* roots collected in high (HS) and low (LS) saline sites and index tolerance (IT) to heavy metals and salt stress

Site[a]	Strain[b]	Species	# Identity blast (%)	Media[c]	RA[d] (%)	TI-rice	TI-NaCl	TI-Hg	TI-Zn
HS	FI31	*Alternaria tenuissima*	KP942908.1 (100)	B	4.08	2.23	1.03	0.56	0.00
HS	FI6	*Arthrinium arundinis*	KF144889.1 (100)	A, B	4.08	2.12	1.38	0.47	0.74
HS	FI10	*Bipolaris buchloes*	KJ909765.1 (99)	A, B	6.12	1.92	1.14	0.33	0.40
HS	FI1	*Curvularia trifolii*	KP067238.1 (100)	A	2.04	0.50	1.03	0.64	0.25
HS	FI7	*Diaporthe cf. nobilis*	KC343146.1 (99)	A, B, C	4.08	0.92	1.19	0.47	0.00
HS	FI25	*Fusarium avenaceum*	KC464345.1 (99)	A, C	4.08	0.41	0.95	0.52	0.65
HS	FI28	*Fusarium oxysporum*	KF577910.1 (99)	A, B, C	28.57	0.41	1.29	1.00	0.94
HS	FI21	*Paraphaeosphaeria michotii*	KJ939279.1 (97)	B	2.04	2.05	1.27	0.24	0.00
HS	FI47	*Peyronellaea glomerata*	KM114267.1 (97)	A, B, C	20.41	1.29	1.00	0.71	0.38
HS	FI36	*Phoma macrostoma*	DQ474092.1 (99)	A, B	4.08	0.50	1.24	0.29	0.42
HS	FI15	*Phomopsis mali*	AB665315.1 (99)	B, C	4.08	1.91	1.09	1.31	0.00
HS	FI26	*Phomopsis* sp.	AY745019.1 (100)	B, C	8.16	2.10	1.12	1.89	2.67
HS	FI48	*Pseudoseptoria obscura*	KF251219.1 (96)	B	2.04	0.61	0.83	0.60	0.55
HS	FI41	*Purpureocillium lilacinum*	HQ607867.1 (100)	A	2.04	2.32	1.38	0.38	0.92
HS	FI4	*Septoriella hubertusii*	KF251230.1 (98)	B	4.08	1.90	1.30	0.62	0.08
LS	FN20	*Alternaria alternata*	KM486070.1 (99)	A	3.85	1.70	1.07	0.42	0.00
LS	FN26	*Alternaria tenuissima*	HM051071.1 (99)	A	3.85	0.93	0.68	0.30	0.00
LS	FN11	*Aureobasidium* sp.	KF367567.1 (99)	C	3.85	0.94	0.00	0.56	0.00
LS	FN7	*Ceratobasidium* sp.	HQ269825.1 (99)	C	3.85	0.86	0.71	0.43	0.16
LS	FN27	*Curvularia spicifera*	HF934915.1 (99)	A	3.85	0.70	0.79	0.58	0.33
LS	FN9	*Dothideomycetes*	JQ759888.1 (99)	B	3.85	0.99	0.43	0.25	1.30
LS	FN23	*Fusarium sporotrichioides*	DQ093674.1 (99)	A, C	19.23	0.45	1.12	0.40	0.00
LS	FN18	*Gliocladium viride*	GU903310.1 (99)	B	3.85	1.06	0.79	0.77	0.00
LS	FN28	*Ilyonectria radicicola*	KF856956.1 (100)	A	3.85	0.51	1.05	0.50	0.00
LS	FN16	*Paecilomyces* sp.	GU108582.1 (98)	A	3.85	0.87	0.55	0.74	0.00
LS	FN17	*Paraphaeosphaeria sporulosa*	JX496084.1 (99)	A	3.85	0.68	0.82	0.33	0.28
LS	FN8	*Paraphoma radicina*	KP174683.1 (99)	B	3.85	0.85	0.71	1.25	0.00
LS	FN3	*Peyronellaea glomerata*	KM114267.1 (100)	B, C	19.23	0.84	0.53	0.46	0.00
LS	FN19	*Phoma macrostoma*	DQ474117.1 (99)	A	3.85	0.77	0.55	0.41	0.05
LS	FN24	*Pythium dissotocum*	AY598634.2 (99)	A	3.85	2.30	0.88	0.53	0.00
LS	FN5	*Pythium inflatum*	AY598626.2 (99)	A	3.85	0.95	0.52	0.81	0.00
LS	FN15	*Setosphaeria rostrata*	KT265240.1 (100)	C	3.85	0.31	0.69	0.20	0.16
LS	FN14	*Trichoderma hamatum*	KF856960.1 (100)	A	3.85	0.50	0.70	0.31	0.00

[a] Collection site: *HS* high saline and *LS* low saline

[b] FI = fungus from roots collected in HS; FN = fungus from roots collected in LS

[c] Medium for isolation: A = PDA; B:1 %YES; C:10 %TSA

[d] Relative abundance

explain 82.94 % of the variations found. The two endophytic communities were different in the response to Hg^{2+}, Zn^{2+} and NaCl in accordance to the mean test for the values of PC1 ($t = -3.5756$, $df = 31$, p value $= 0.001169$) and PC2 ($t = 3.4341$, $df = 31$, p value $= 0.001709$).

The ability to protect rice seedlings from salt stress was clearly different between the two fungal communities (Tables 2, 3). *A. tenuissima*-FI31, *Arthrinium arundinis*-FI6, *Paraphaeosphaeria michotii*-FI21, *Phomopsis* sp.-FI26, *Purpureocillium lilacinum*-FI41, *Septoriella hubertusii*-FI4 *Phomopsis* mali-

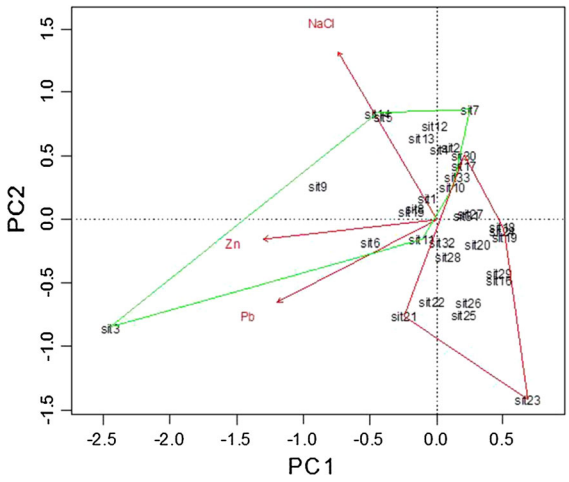

Fig. 1 PCA-ordination plot of tolerance index (Hg^{2+}, Zn^{2+} and NaCl) of endophytic fungal species from low and high salt sites. Clusters indicate values from species groups: *green* (endophytic fungal high salt site) and *red* (endophytic fungal low salt site). The *red arrows* are the tolerance index variables

FI15 and *Bipolaris buchloes*-FI10 all increased stress tolerance with TI-rice >1.9 (Table 2). *Pythium dissotocum*-FN24 was the only species isolated from LS site that showed TI-rice >1.9 (Table 2). Nine species from the high saline site induced increased length (root + shoot) of the rice seedlings GPE values ranging from 72.0 to 131.8 % (Table 3). Only two isolates from the LS site resulted in GPE 62.0 and 119.1 % (Table 3). Other species of endophytes resulted in negative effects on the rice seedlings, drastically reducing total seedling lengths and consequently resulting in negative values of GPE (Table 3). In total, seven and four endophytic species from low and high saline sites, respectively, had pathogenic (i.e., reducing growth of seedlings over the uninoculated controls) effects in rice under salt stress. The other species (nine and two from low and high saline sites, respectively) did not result in significantly different effects compared to the control (no inoculation seedlings) (Table 3).

Discussion

Our results show that variation in soil salt content between the two sites studied may influence the endophytic community in *P. australis* root systems.

Table 3 Results of experiments with endophytes in growth promotion of rice plants under salt stress

Species	Media	SD[a]	GFP[b]
Alternaria tenuissima-FI31	18.2*	2.88	123.3
Arthrinium arundinis-FI6	17.2*	1.36	111.9
Bipolaris buchloes-FI10	15.6*	1.12	91.6
Curvularia trifolii-FI1	4.1*	0.59	−49.6
Diaporthe cf. nobilis-FI7	7.5	1.22	−8.2
Fusarium avenaceum-FI25	3.3*	0.26	−58.8
Fusarium oxysporum-FI28	3.4*	0.33	−58.7
Paraphaeosphaeria michotii-FI21	16.7*	1.63	104.7
Peyronellaea glomerata-FI47	10.0	1.46	22.4
Phoma macrostoma-FI36	4.1*	0.67	−49.9
Phomopsis mali-FI15	15.6*	1.20	91.3
Phomopsis sp.-*FI26*	17.2*	2.20	110.4
Pseudoseptoria obscura-FI48	14.0*	2.03	72.0
Purpureocillium lilacinum-FI41	18.9*	1.64	131.8
Sclerostagonospora phragmiticola-FI4	15.5*	2.43	90.4
Alternaria sp.-*FN20*	13.2*	1.07	62.0
Alternaria tenuissima-FN26	7.2	1.41	−11.5
Aureobasidium sp.-*FN11*	7.3	1.30	−10.2
Ceratobasidium sp.-*FN7*	6.6	1.43	−18.4
Curvularia spicifera-FN27	5.5*	1.60	−32.9
Dothideomycetes sp.-*FN9*	7.7	1.32	−5.9
Fusarium sporotrichioides-FN23	3.5*	0.45	−57.3
Gliocladium viride-FN18	8.2	2.66	0.9
Ilyonectria radicicola-FN28	4.0*	0.93	−51.0
Paecilomyces sp.-*FN16*	6.9	2.34	−14.9
Paraphaeosphaeria sporulosa-FN17	5.3*	1.10	−35.0
Paraphoma radicina-FN8	6.6	0.60	−19.1
Peyronellaea glomerata-FN3	6.5	1.22	−20.3
Phoma macrostoma-FN19	6.0*	1.15	−26.6
Pythium dissotocum-FN24	17.8*	1.67	119.1
Pythium inflatum-FN15	7.3*	1.88	−9.8
Setosphaeria rostrata-FN5	2.4*	0.45	−70.8
Trichoderma hamatum-FN14	3.9*	0.91	−52.2
C + NaCl	8.1	2.46	–
C − NaCl	25.3	2.72	–

Seedlings were inoculated with endophytic microbes and treated with NaCl (120 mM). Data are presented as the mean of 8 replicates

* Differ significantly from control C + NaCl (non-inoculated plant in NaCl—120 mM)

[a] Standard deviation

[b] Growth promotion efficacy (%)

Understanding whether and how endophytic microbes may be affecting competitive ability of plants is important basic information and may lead to new management strategies of invasive populations (Kowalski et al. 2015). Knowledge of the symbiotic microbial communities that enhance invasiveness contributes to our understanding of the roles that microbes play in enabling invasive plants to outcompete native species.

Distribution and diversity of endophytes in *P. australis* plants

We could not isolate endophytes from young leaves and shoot meristematic tissues even after 45 days of incubation. This may be explained in that we used very young leaf and meristem tissues. Many fungal endophytes, especially the class 2 and 3 endophytes, colonize plant tissues as they age and may not be present in younger tissues (Rodriguez et al. 2009). Although other authors have reported fungal isolation from leaves of *P. australis*, the ages of leaves were not always clear (Wirsel et al. 2001; Neubert et al. 2006; Angelini et al. 2012; Fischer and Rodriguez 2013; Sim et al. 2015). Venkatachalam et al. (2015) also did not detect endophytes in leaves of *Cymodocea serrulata* and *Thalassia* sp. when analyzing 100 leaf fragments from 10 different grasses species.

We found that roots of *P. australis* plants from both sites were colonized abundantly by endophytic microbes. Despite the lower CF in the roots from the LS site, endophytic microbial diversity (Shannon diversity) was higher than that supported by the HS site. Biotic and abiotic factors influence the CF, richness and diversity of endophytes (Arnold 2007; Arnold and Lutzoni 2007). The Shannon's diversity values 2.64 (roots from LS site) and 2.28 (roots from HS site) were between 1.5 and 3.5; therefore, the Shannon's index for each *P. australis* population in the present study was similar to those reported in another study on endophytes from *Hevea brasiliensis* (Gazis and Chaverri 2010).

We recovered a high diversity of endophytes from the *P. australis* root samples, with isolates from roots belonging to species of the phylum Ascomycota, Basidiomycota and Oomycota (Stramenopiles), including 23 genera and 30 species (Table 2). These results clearly indicate that a high diversity of endophytic microbes is associated with roots of *P. australis*. The endophytic community differentiated between high and low saline sites in high-level taxa as observed in other macrophytes (Sandberg et al. 2014). In general, endophytic fungi of plant root systems tend to be highly diverse (Vandenkoornhuyse et al. 2002). The dominant species we encountered in plants of the HS site were *Fusarium oxysporum* and *Peyronellaea glomerata*, and in plants of the LS site were *Fusarium sporotrichioides* and *P. glomerata. F. oxysporum* has been reported as an endophytic fungus in nearly 100 plant species (Kuldau and Yates 2000). *F. sporotrichioides* (Szécsi et al. 2013) and *P. glomerata* (Zhang et al. 2012) are endophytes found in a lower diversity of hosts.

The endophytic species isolated in our study were not previously detected in *Phragmites* roots (Wirsel et al. 2001; Angelini et al. 2012; Kim et al. 2014; Hipol and Cuevas 2014; Sim et al. 2015). Fischer and Rodriguez (2013) found *Arthrinium arundinis*, *F. oxysporum* and *F. sporotrichioides* in shoots of non-native *P. australis* collected in Michigan. *Paraphaeosphaeria michotii* was found in leaf sheaths of *P. autralis* along a salinity gradient in Belgium (van Ryckegem and Verbeken 2005).

Species of genera *Arthrinium*, *Diaporthe*, *Fusarium*, *Phoma* and *Trichoderma* have been previously isolated from *P. australis* roots (Wirsel et al. 2001; Angelini et al. 2012; Sim et al. 2015). Species of the genera *Phomopsis*, *Alternaria*, *Paecilomyces* and *Xylaria* have been isolated as endophytes from many other grass plants (Kleczewski et al. 2012; Márquez et al. 2012). We suggest that composition of the endophytic communities in *P. australis* may differ due to various factors, including plant genotype, tissue type, soil composition, geographic and seasonal factors (Collado et al. 1999; Higgins et al. 2007; Suryanarayanan et al. 2011; U'Ren et al. 2012; Gehring et al. 2014).

Invasive species may thrive due to escape from soil-borne phytopathogens when they are introduced into new habitat (Keane and Crawley 2002; Parker and Gilbert 2007; Reinhart et al. 2010). Some species in our study are known causative agents of plant diseases, including *F. oxysporum* (Guo et al. 2014), *A. tenuissima* (Blodgett and Swart 2002) and *F. sporotrichioides* (Mielniczuk et al. 2004). Many grasses are rich sources of endophytic *Fusarium* (Szécsi et al. 2013). The detection of known plant pathogens is relatively common in endophytic fungal communities

(Moricca et al. 2012; Sun et al. 2012; Wearn et al. 2012; Pawlowska et al. 2014) reinforcing the idea that the symbiosis between plant and endophyte oscillates between parasitism and mutualism (Schulz and Boyle 2006). The root colonization of pathogenic strains varies depending on the plant species and the *forma specialis* as in *F. oxysporum* colonization (Alabouvette et al. 2009). Since none of the *P. australis* plants in our study showed symptoms of disease, it seems possible that pathogenicity by these and other root endophytes may be suppressed in the symbiotic relationship.

Root endophytes and plant stress tolerance

Salt-affected soils are characterized by excess levels of soluble salts (salinity) and/or Na^+ in the solution phase as well as in a cation exchange complex (sodicity) (see Qadir et al. 2007). Plants are exposed to water stress, ion toxicity and disturbances in mineral nutrition when they are growing under hypersaline conditions (Sabzalian and Mirlohi 2010). Although our study was limited, the differences between the endophyte communities obtained from the two sites are proposed to be caused, at least in part, by the site conditions that played a role in influencing which microbes survived and associated with host roots. In this respect, it is notable that soils (predominantly sand) associated with the plants at the inland site contained significantly less salt than we measured in sand associated with the plants at the other site ($Na^+ = 0.11$ meq/100 g at LS site (*site 1*) vs. $Na^+ = 0.76$ meq/100 g at HS site (*site 2*).

Endophytes often increase the competitive ability of plants, especially under stress (e.g., Saikkonen et al. 2013). Osmotic and specific ionic stresses from salinity can cause stunted growth and reduced plant yield (Todaka et al. 2015). Excess salinity in the soil can compromise the availability of water and nutrients for the plants by affecting osmotic potential of the soil solution. High sodium levels also cause degradation of the soil structure, dispersion clay, and toxicity in plants, and may even prevent the germination of seeds and inhibit root development (Smith et al. 2009).

Many of the endophytes from the LS site were not found to be resistant to salt, while many endophytes found in plants from the HS site were found to be resistant to salt. This, in tandem with results of experiments showing that the endophytes from plants in the HS environment promote the growth of plants (rice) under salt stress, lend support to the 'habitat-adapted symbiosis hypothesis' proposed by Rodriguez et al. (2008). According to this hypothesis, plants living under stressful conditions may enhance their resistance to stress by associating with endophytes that increase their tolerance to stress. Our use of rice to screen *P. australis* endophytes for their capacity to enhance salt tolerance is supported by studies that have demonstrated that endophytic fungi that provide increased stress tolerance in one host also may provide stress tolerance in other more distantly related hosts (Waller et al. 2005; Redman et al. 2011). In addition, rice and *P. australis* are both grasses, and several studies have shown that they possess similar physiological characteristics (see Armstrong and Armstrong 2001, 2005; Soukup et al. 2002; Serghat et al. 2005; Chu et al. 2006).

Most saline resistant plants have biochemical and physiological mechanisms to reduce this abiotic stress (Munns and Tester 2008). The community of endophytic fungi obtained from plants at the HS site (>50 % of species resulted in TI-rice >1.9) may contribute more effectively to enhanced salt tolerance than the community from roots of *P. australis* collected at the LS site (~5 % of species). It seems reasonable that some plants may naturally associate with endophytic microbiota to enhance their competiveness or invasiveness (Nuñez et al. 2009; Molina-Montenegro et al. 2015; Van der Putten et al. 2007; Kowalski et al. 2015). Nevertheless, due to the limitations of our experiments expanded studies examining a greater number of sampling sites and assessment of endophyte effects on changes in stress tolerance of seedlings of *P. australis* rather than rice would be desirable to confirm our experimental results.

Potential applications of *P. australis* endophytes

Redman et al. (2011) argued that endophytes may be useful in mitigating impacts of climate change and expanding agricultural production into stressful environments. Redman et al. (2011) also demonstrated that endophytic fungi isolated from *Leymus mollis* and *Dichanthelium lanuginosum* promoted plant growth in rice seedlings under salt stress similar to what we observed for the *P. australis* root endophytes. Fungal endophytes isolated from hosts on saline soils may

have potential application to increase tolerance of agriculturally and horticulturally important plants to salt or arid environments (Waller et al. 2005; Khan et al. 2011; Yin et al. 2014; Azad and Kaminskyj 2015). *P. australis* has also been used widely to treat industrial wastewater containing heavy metals in wetland systems (Jean and De 1997; Vymazal and Kropfelova 2005). The success of *P. australis* in invasion of diverse environments has been related to phenotypic, genetic and reproductive plasticity (Haslam 1972; Lissner et al. 1999a, b; Saltonstall 2002; Lambertini et al. 2006; Fer and Hroudova 2009; Kettenring and Mock 2012; Meyerson et al. 2012; Cronin et al. 2015). However, it is possible that the capacity of *P. australis* to grow in heavy metal contaminated sites could stem at least in part from endophytic microbes. In this respect it has been shown that *P. australis* is tolerant to toxic concentrations of Zn (Weis and Weis 2004) and Hg (Stoltz and Greger 2002). Our analysis revealed the presence of several heavy metal tolerant taxa of fungi. These heavy metal-tolerant fungi could contribute to the capacity of non-native *P. australis* to grow in heavy metal contaminated sites. Future experiments with our isolated strains are needed to evaluate the effect of *P. australis* endophytes on plant resistance to heavy metals ($HgCl_2$ and $ZnSO_4{\cdot}7H2O$). If such endophytes are confirmed, they could be used to produce cultivars of plants for bioremediation of contaminated sites (Stoltz and Greger 2002; Idris et al. 2004; Weis and Weis 2004; Li et al. 2012a, b).

Conclusions

This study shows that endophytic microbes of *P. australis* are diverse, may be distinctive at low and high taxonomic levels, and are influenced by ecosystem conditions. Endophytic fungal communities from *P. australis* differed depending on soil saline conditions. Experiments using rice revealed that some of the endophytes found at the high salinity site increased tolerance of rice seedlings to elevated levels of salinity. This result is consistent with the hypothesis of 'habitat-adapted symbiosis' (Rodriguez et al. 2008), where endophytes are hypothesized to help plant hosts adapt to specific, often harsh, habitats. Our results suggest that endophytes play a role in increasing the capacity of *P. australis* to grow in high salinity soils, probably contributing to invasion in saline environments.

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