Wetlands Ecol Manage
https://doi.org/10.1007/s11273-019-09688-x

ORIGINAL PAPER

Bacterial and fungal endophyte communities differ in trees of natural versus wastewater-treatment wetlands

Jennifer Janowsky · Elizabeth Kimbrough · Demetra Kandalepas · Gary Shaffer · Stephen K. Formel · Sunshine A. Van Bael

Received: 8 February 2019 / Accepted: 19 September 2019

Abstract Little is known about the microbiota that form symbioses with wetland plants. We describe how endophytes from leaves and roots of baldcypress trees changed along a nutrient gradient and differed in wastewater treated versus untreated wetlands. Cultured bacterial and fungal endophytes were most abundant and diverse in baldcypress roots (compared to leaves) for both treated and untreated wetlands. Bacterial endophyte abundance increased with increasing distance from the wastewater outfall pipe, while fungal endophytes decreased with increasing distance from the wastewater outfall pipe—showing a greater abundance of fungi where nutrients were greatest. Bacterial endophyte abundance and species richness were greater in the wastewater-treatment compared to untreated wetlands, but diversity metrics suggested this was due to incomplete sampling at some sites. Community composition of endophytes in the treated wetland differed from some but not all of the communities observed in untreated wetlands. Since baldcypress is a key restoration species for declining swamps in the southeastern US, our descriptive work provides a foundation for future studies to understand the functional roles of plant-microbial interactions and community patterns.

Keywords Baldcypress · Nutrient gradients · Symbiosis · *Taxodium distichum* · Wastewater-treatment wetland

Introduction

Wetlands provide a variety of benefits to coastal systems worldwide, but many of these systems are under severe stress from over-population and sea-level rise (Day et al. 2000, 2007; Couvillion et al. 2011). As a result, wetland loss has become a major problem along Louisiana's Gulf coast (Boesch et al. 1994), primarily due to over-management of the subsiding Mississippi River Delta. Baldcypress (*Taxodium distichum* (L.) Richard) in the Cupressaceae family is often used in restoration planting, including in treated, or wastewater treatment wetlands (Lundberg et al. 2011). Waste-water treatment wetlands implement the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11273-019-09688-x) contains supplementary material, which is available to authorized users.

J. Janowsky · E. Kimbrough · S. K. Formel · S. A. Van Bael (✉)
Department of Ecology and Evolutionary Biology, Tulane University, 6823 St. Charles Avenue, New Orleans, LA 70118, USA
e-mail: svanbael@tulane.edu

D. Kandalepas · G. Shaffer
Department of Biological Sciences, Southeastern Louisiana University, 808 N. Pine St., Hammond, LA 70402, USA

Published online: 28 September 2019

addition of secondarily-treated wastewater as a means of increasing productivity and thereby creating additional plant growth and sediment deposition (Bodker et al. 2015; Shaffer et al. 2015; Hunter et al. 2018). When baldcypress was grown in wastewater treatment wetlands, elevated nutrient levels increased tree growth by up to four times greater than in untreated reference areas (Lundberg et al. 2011).

Little is known about how nutrients interact with the symbionts of baldcypress, namely the endophytes–microscopic fungi and bacteria that live within the tissues of all plants without causing disease symptoms (Wilson 1995). Endophytes are distinct from mycorrhizas because they exist entirely inside of plant tissues (Wilson 1995), rather than extending mycelium into the soil. In studies with mostly crops as hosts, endophytes are known to confer advantages to their hosts, including increased resilience to stress (Friesen et al. 2011; Redman et al. 2011; Porras-Alfaro and Bayman 2011) and increased nutrient uptake (Hodge et al. 2001). For example, colonization by dark septate endophytes in roots has been associated with increased nitrogen and phosphorus in the shoots of host plants, as well as increased shoot and root biomass (Newsham 2011; Mayerhofer et al. 2013). Roots have been observed to change their morphology with endophyte inoculation, resulting in longer and thinner roots with greater capacity for nutrient uptake (Peskan-Berghofer et al. 2004). Whether these types of relationships occur between endophytes and baldcypress remains unknown. Only a few studies have documented the presence of endophytes in wetland plants (e.g., Weishampel and Bedford 2006; Kandalepas et al. 2010; Kandalepas 2012; Kandalepas et al. 2015) but have not assessed their function.

Disassociation of hosts and their symbionts has been observed when particular stressors are removed. For example, in agricultural systems where nitrogen is added as fertilizer, legumes have been shown to disassociate with their rhizobial symbionts due to reduced nutrient stress (Gibson and Harper 1985; Imsamde 1986; Streeter and Wong 1988; Murray et al. 2016). Since wastewater treatment wetlands create nutrient-rich habitat, it is possible that there is a reduced need for baldcypress to associate with endophytes. Therefore, it may be expected that treated baldcypress show decreased association with endophytes compared to those in a natural, or non-treated environment. Alternatively, more endophytes may be observed in nutrient rich areas because biogeochemical cycling happens at a greater rate and provides more niches for diverse microbial taxa.

Our objectives in this study were (1) to culture endophytes from the leaves and roots of baldcypress in a wastewater-treatment wetland and (2) to compare the endophyte community of baldcypress in a wastewater-treatment wetland to that of baldcypress growing in untreated sites.

For the first aim of this study, the abundance, diversity and community composition of both leaf and root endophytes were assessed to observe the extent to which these variables changed along a man-made treatment gradient. For the second aim of the study, bacterial endophytes were isolated from roots of baldcypress at four natural wetlands, and compared to baldcypress endophytes in the treated wetland. Our study is one of the first field studies of the baldcypress endophyte community, with previous reports focusing on greenhouse experiments, individual endophyte taxa, or arbuscular mycorrhizal fungi (Li et al. 1996; Kandalepas et al. 2010; Kandalepas 2012; Kimbrough et al. 2019). This research should inform future wetland design and help predict how endophyte communities respond to discharges of excess nutrients.

Methods

Site descriptions

Our study included one treated wetland where baldcypress was planted and four wetlands where baldcypress grew naturally. The treated wetland in Hammond, Louisiana, known as Four Mile Marsh, is located approximately 5 km south of Ponchatoula, Louisiana (Fig. S1). The city of Hammond began discharging into the pre-existing marsh with municipal disinfected, secondarily-treated wastewater in fall of 2006 to stimulate plant growth and prevent saltwater intrusion into the wetland. A 10 km long buried pipeline carries the treated wastewater from the South Sewage Treatment Plant in Hammond to the marsh site, and an outfall pipe distributes this wastewater into the wetland along the length of the pipe (Fig. S1). Previous research at the Hammond site demonstrated the presence of a nutrient gradient, where elevated nutrient levels at the outfall pipe return

to background levels where the wetland ends (Hunter et al. 2009; Shaffer et al. 2015). Another study of the Hammond wetland (Lundberg et al. 2011) noted greater growth in trees closest to the outfall pipe where wastewater entered the marsh. The *T. distichum* sampled for endophytes were planted in 2006 as described in Lundberg et al. (2011). At the same time, a control plot of trees was established by Lundberg et al. (2011) in a nearby marsh. Lundberg et al. (2011) referred to this nearby marsh as the reference site, as it was not in direct contact with the wastewater. Trees at the treated wetland and its reference site, 8 years old at most, are likely younger than those sampled at other wetlands in our study (Shaffer et al. 2015).

The four sampled wetlands with unmanipulated populations of *T. distichum* were all in southeastern Louisiana. Three of these wetlands are entirely freshwater: John Lafitte National Historical Park and Preserve (Marrero, Louisiana), Tickfaw State Park (Springfield, Louisiana), and Honey Island swamp (Slidell, Louisiana). The remaining wetland is slightly saline (average salinity 0.6 ppt): the Bonnet Carré Spillway off of Lake Pontchartrain (LaPlace, Louisiana) (Fig. S2).

Field sampling

Collection for the Hammond wetland was done in June of 2014, and collections at the other four wetlands were done during September and October of 2014. At the treated wetland (Hammond), we sampled transects perpendicularly to the outfall pipe in three locations contained in the subunits described by Lundberg et al. (2011). On each of these three transects, we sampled at 0 m, 200 m, 400 m, and 600 m away from the outfall pipe (Fig. S1). We also sampled four trees from an untreated reference within the Hammond area (Fig. S1). We took two leaf and two root samples consisting of a 10 cm branch or at least 15 cm of roots from each tree. Samples of the same tissue type (leaf, root) from each tree were mixed and processed together as one sample.

At each non-manipulated wetland site, we collected samples from 12 adult trees at least 10 m apart. A minimum of three apparently healthy root samples of 15–20 cm in length were dug and cut from each tree base. Roots were gently washed on site in surrounding water to remove agglomerated soil. Samples were transported in cold storage and then refrigerated upon arrival to Tulane University. We processed the roots and leaves and cultured for endophytes within 24 and 36 h of collection, respectively.

Root endophyte culturing

Following collection, we removed 10–15 apparently healthy roots that were close to the main stems of each root sample. We selected fine, non-woody root samples (< 1 mm in diameter) from these segments, cut them into segments 3–5 cm long and rinsed them of residual soil. Previous pilot work in our laboratory suggested that this size and fine diameter were appropriate for isolating only one fungal or bacterial strain. In a metal tea strainer, roots were surface sterilized in 70% ethanol for 10 s, 50% commercial bleach solution (2.625% sodium hypochlorite) for 2 min, followed by three rinses in sterile deionized water. Under sterile conditions, the ends of root segments were trimmed and discarded ($\sim$ 5 mm from the end of each root segment) and the remaining root segments were cut into 5 mm pieces for culturing (Kandalepas et al. 2015).

For each plant, we plated 18 5 mm sections onto two media types. Nine segments were plated onto 2% malt extract agar (2% BD Bacto malt extract, Franklin Lakes, New Jersey) which selects for fungi, and nine segments onto nutrient agar (Oxoid, Cheshire, England; recipe as per manufacturer guidelines) which selects for bacteria (Mugerwa et al. 2013; Kandalepas et al. 2015). In total, about 90 mm of root length (18 pieces $\times$ 5 mm length) was plated per tree. Plates were stored flat at room temperature (24 °C) in the dark. We regularly inspected the roots with a stereoscope to see whether bacteria and fungi grew from the root surface rather than from within the root segment. Segments showing bacterial or fungal growth from outside the root piece were removed and discarded to exclude contaminants. We isolated new growths, grew them in individual plates into pure culture, and extracted DNA after the culture grew sufficiently to take a tissue sample.

Leaf endophyte culturing

We removed four apparently healthy leaves from each sample and rinsed them in tap water to remove particulate matter and other surface residue. The leaves were submerged using a metal tea strainer in

95% ethanol for 10 s, followed by 2 min in 10% commercial bleach solution (0.252% sodium hypochlorite), then 2 min in 70% ethanol (Arnold et al. 2000). Following sterilization, leaves were cut into 2 mm squares under sterile conditions. For each plant, 32 total sections were plated onto two media types, 16 pieces onto 2% malt extract agar and 16 pieces onto nutrient agar. Plates were stored at room temperature (24 °C) on the lab bench, out of direct sunlight. New growths were isolated, grown in individual plates of pure culture, and DNA was extracted (see below) after the culture grew sufficiently to take a tissue sample.

Sanger sequencing of morphospecies

Total genomic DNA was extracted from each of the pure cultures. Cultures from the Hammond wetland were extracted using a Qiagen DNAeasy Plant Mini Kit (Germantown, MD) following the TissueLyser protocol. A Mobio Ultraclean Microbial DNA Isolation Kit (Germantown, MD) was used for the isolations from the natural wetlands following the protocol in the kit.

Following isolation, genomic DNA was used in polymerase chain reaction (PCR) mixtures following Hoffman and Arnold (2010) and protocol from Kandalepas et al. (2015).

The PCR reaction mixture included 12 µl Sigma REDTaq (Sigma Aldrich; 20 mM Tris–HCl, pH 8.3, 100 mM KCl, 3 mM MgC_{12}, 0.002% gelatin, 0.4 mM mixed deoxyribonucleotide triphosphates, stabilizers, 0.06 units Taq polymerase/ml), 1.0 µl of each primer, 1.0 µl DNA template, 1.0 µl of dimethyl sulfoxide and 9.0 µl of PCR-quality water (Hoffman and Arnold 2010). For fungi, the nuclear ribosomal internal transcribed spacer (nrITS) and the partial large subunit (LSU) were amplified using primers ITS1F (5′-CTTGGTCAT TTAGAGGAAGTAA) or ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) for the forward reaction, and LR3 (5′-GGTCCGTGTTTCAAGAC) or ITS4 (5′-TCCTCCGCTTATT GATATGC) for the reverse reaction. The PCR cycling protocol followed Arnold and Lutzoni (2007). For bacteria, the 16S ribosomal DNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG) and 1492RI (5′-GGTTACCTTGT TACGACTT) with the same cycling protocol as for fungi. PCR products were visualized with SYBR Safe DNA Gel Stain (Thermofisher Scientific, Waltham, MA) on 1% agarose gels, and submitted to Beckman Coulter Genomics (Boston, MA) for Sanger sequencing. Sequences were assembled and edited in Sequencher v5.0 (Gene Codes Corp., Ann Arbor, MI). We considered sequences with a minimum 97% similarity to be representative of the same operational taxonomic unit (U'ren et al. 2009). Voucher cultures of all operational taxonomic units were archived in the Van Bael lab at Tulane University, under isolate numbers 949-1320. Accession numbers for sequences were deposited in the NCBI Genbank archive from MN371994 - MN372028 and MN319606 - MN319618.).

Taxonomic classification methods

Putative taxonomic identities were assigned using representative sequences for each operational taxonomic unit, using two different methods. First, sequences were classified in QIIME2,version 2018.2, (Caporaso et al. 2010) using the scikit-learn naive Bayes machine-learning classifier (Bokulich et al. 2018; Pedregosa et al. 2011). Sequences were classified against pre-trained databases kindly provided by Greg Caporaso and Sydney Morgan via the QIIME2 online forum. For classification of 16S sequences the classifier was trained against the full-length SILVA 132 database (Glöckner et al. 2017). The classifier for ITS sequences was trained against the "dynamic" UNITE database, version 7.2, released Dec. 1, 2017 (Kõljalg et al. 2013), a version of UNITE that has been curated to cluster fungal sequences by a 97–99% sequence similarity according to the best judgment of the expert curators. To ensure that the highest quality classification was obtained, all sequences were assigned taxonomy in both the forward and reverse-complement direction, and the classification with the greatest confidence chosen. If the confidence values were similar (< 0.01 difference) then the one classified to the highest rank was chosen. To provide a balance between recall and precision, as recommended by the classifier's developers (Bokulich et al. 2018), 16S sequences were classified with a confidence threshold of 0.7, while ITS sequences were classified with a confidence threshold of 0.9 (Tables S1, S2). Sequences went through another round of classification with the confidence thresholds relaxed to 0.5 (16S) and 0.7 (ITS) to give additional

insight into the classification, although these are not included in the results.

In addition, sequences were classified with BLAST+, version 2.5.0 (Camacho et al. 2009) with default parameters. For classification of 16S sequences the reference database was the full-length SILVA 132 SSUParc database (Glöckner et al. 2017). ITS sequences were BLASTed against the "dynamic" UNITE database, version 7.2, released Dec. 1, 2017 (Kõljalg et al. 2013), a version of UNITE that has been curated to cluster fungal sequences by a 97–99% sequence similarity according to the best judgment of the expert curators. The reported result is the top BLAST hit, as all but six isolates had top hits with percent identities of $> 90\%$ and e-scores of approximately zero (Tables S1, S2). The six isolates mentioned above all had percent identities of $> 83\%$ and e-scores of approximately zero.

Statistical analyses

Isolation rates

Symbiont isolation frequency (or abundance) was calculated for each tree sampled, as the proportion of leaf or root segments from which a symbiont was isolated. A two-way analysis of variance was used to test for whether distance from outfall pipe or symbiont type (root bacteria, root fungi, leaf bacteria, and leaf fungi) explained the most variation in symbiont isolation frequency. The analysis was done with and without the reference site and found the same overall effects, so the analysis that includes the reference is presented. Significant effects were followed up with Tukey's Honest Significant Difference tests. A partial least squares regression (PLS) analysis (Eriksson et al. 1995) was used to look for relationships between endophyte isolation frequencies and water quality concentrations taken from the same points where trees were located. Water quality concentrations were used from Shaffer et al. (2015), which were collected at an earlier date than this study, but were representative of long term water quality data at the Hammond wetland (Shaffer et al. 2015). Values for water quality (mg/L) and isolation frequency were log transformed before the PLS analysis. The PLS analysis allowed constructing a model of collinear water quality parameters with an explanatory variable: endophyte isolation frequency (abundance), and was performed using the package *pls* in R (R Team 2019). Four separate PLS models were made for fungal/bacterial and leaf/root endophytes, but leaf endophyte data were too scarce so results are not reported. Kruskall-Wallis tests were used to compare endophyte abundance among wetlands. At Hammond, these tests were done with and without the reference samples to confirm that treatment was driving differences among wetlands, rather than differences in isolation rates.

Diversity indices

The diversity in symbiont samples was calculated using the Fisher's alpha index, to account for a different number of samples per tree. These indices allowed a comparison of symbiont diversity among symbiont types and with respect to distance from the outfall pipe. Endophyte diversity levels were too few to calculate the Fisher's alpha index for each individual tree, so this study aggregated the symbionts into categories of interest (such as distance from outfall pipe, or wetland) and calculated one index per category. These calculations allowed comparison of endophyte diversity among and within wetlands. Additional analyses of species richness, Shannon and Simpson's diversity were performed using the iNEXT program (Chao et al. 2016) for asymptotic diversity based on Hill numbers (Chao and Jost 2015). This method allows low-biased estimates of diversity for incomplete sampling data (Chao and Jost 2012, 2015).

Community composition

For analyses of community composition, singletons (operational taxonomic units with only one individual) were removed from the dataset. In the treated wetland dataset (Hammond), a Bray–Curtis distance matrix was used for nonmetric multidimensional scaling (NMDS; Clarke 1993). This allowed visualizations of the similarities among trees with respect to distance from the outfall pipe. This was followed up with analysis of similarity (ANOSIM; Clarke 1993) to test whether distance from the outfall pipe influenced community composition of endophytes. The same testing protocol with NMDS and ANOSIM using site rather than distance from the outfall pipe was used to compare endophyte community composition in Hammond to unmanipulated wetlands in southeastern Louisiana. Community analyses were done using the

software program PRIMER version 6.0 (Clarke and Gorley 2006).

Results

Hammond wetland

Community description

Baldcypress tissues harbored diverse communities of endophytes. Culturing resulted in 217 bacterial and fungal isolates from the roots and leaves of the Hammond baldcypress. Successful PCR products for 125 of these samples were sequenced. Overall, 32 unique operational taxonomic units were grouped by sequence similarity, 19 being bacteria and 13 being fungi (Tables 1, S1). *Bacillus* was the most common genus isolated in roots, with 19 sequences grouped in *Bacillus* species 1. There were also seven leaf bacteria sequences grouped in *Bacillus* species 1. A root fungus, *Neonectria* sp. was most common fungal taxon with four grouped sequences. For leaves, *Xylaria cubensis* was most common fungus with three grouped sequences (Tables 1, S1).

Isolation rates

We found that symbiont type, not distance from the outfall pipe, explained the most variation in endophyte isolation frequency (Two-way ANOVA, distance $F_{3,41} = 2.17$, $p = 0.10$; symbiont type $F_{3,41} = 51.5$, $p < 0.001$, interaction not included, Fig. 1). Tukey HSD tests showed that root bacteria were significantly greater in number than root fungi, leaf fungi and leaf bacteria ($p < 0.001$ for all comparisons). Root bacterial isolation frequency was greatest at 200 m, beyond which frequency decreased with distance (Fig. 1). The PLS model showed that root bacteria abundance was positively correlated with distance from the outfall pipe and negatively correlated with NH_4, Si and PO_4 (Fig. 2a). PLS generates a metric called "Variable importance in projection" (VIP) to indicate differences among variables for the model solution. For bacteria, only distance from the outfall pipe had a VIP value > 1, signifying that it was highly important for the model projection, while the water quality variables had VIP values between 0.7 and 1, signifying that they were moderately influential for the model solution (Fig. 2). In contrast to bacteria, the PLS model showed that root fungal endophyte abundance was positively

Table 1 Putative operational taxonomic units for symbionts that were sequenced, with their abundance in baldcypress trees in the Hammond assimilation wetland

Putative taxa[a,b]	Leaf bacteria	Leaf fungi	Root bacteria	Root fungi	Total
Bacillus species 1[c]	7	0	19	0	26
Bradyrhizobium species	0	0	5	0	5
Lysinibacillus species	0	0	5	0	5
Serratia marcescens	0	0	5	0	5
Achromobacter species	0	0	4	0	4
Hypocreales species	0	1	0	3	4
Neonectria species	0	0	0	4	4
Bacillus species 2	0	0	3	0	3
Xylaria cubensis	0	3	0	0	3
Phyllosticta capitalensis	0	2	0	0	2
Bacillus species 3	0	0	2	0	2
Burkholderia kururiensis	0	0	2	0	2

[a]Confidence threshold was set at 0.9 for fungi and 0.7 for bacteria

[b]Singletons and accession information for the nearest match in the UNITE and SILVA databases are listed in Table S1. Putative taxa listed here were from the analysis using the scikit-learn naive Bayes machine-learning classifier in QIIME2. A BLAST using the UNITE and SILVA databases often led to greater taxonomic resolution, and these results are available in Table S1.

[c]*Bacillus* sp. 1–3 are unique operational taxonomic units for which only the genus is given

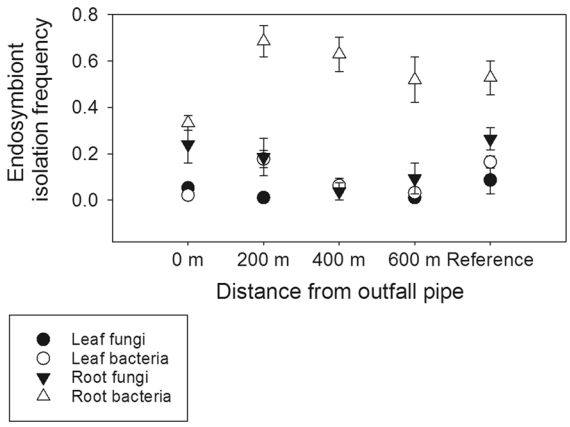

Fig. 1 Mean endophyte isolation frequency from leaves and roots of baldcypress at a wastewater-treatment marsh and a nearby reference wetland near Hammond, Louisiana. Endophytes are plotted with respect to their distance from the outfall pipe, as a mean isolation frequency ± one standard error (error bars). The reference wetland did not receive wastewater from the outfall pipe

correlated with NH_4, Si and PO_4 and negatively correlated with distance from the outfall pipe (Fig. 2b). The VIP values were > 1 for PO4, and distance from the outfall pipe, indicating that these variables were highly influential in the model explaining root fungal abundance. NH_4 and Si were only moderately important in the model (Fig. 2).

Diversity indices

Endophyte diversity (measured by the Fisher's alpha index) did not differ with distance from the outfall pipe. Fisher's alpha values for bacteria versus fungi largely followed patterns of of abundance (Fig. 1), so values are not presented. The observed species richness was 32 OTUs (Fig. S3a) and the estimated species richness was 98 ± 67 OTUs (Fig. S3b).

Community composition

Community composition was not significantly different with respect to distance from outfall pipe in the treated wetland (all endophytes ANOSIM R = − 0.02, p = 0.59, root bacteria only ANOSIM R = − 0.135, p = 0.90, NMDS not shown). Community composition also was compared by binning

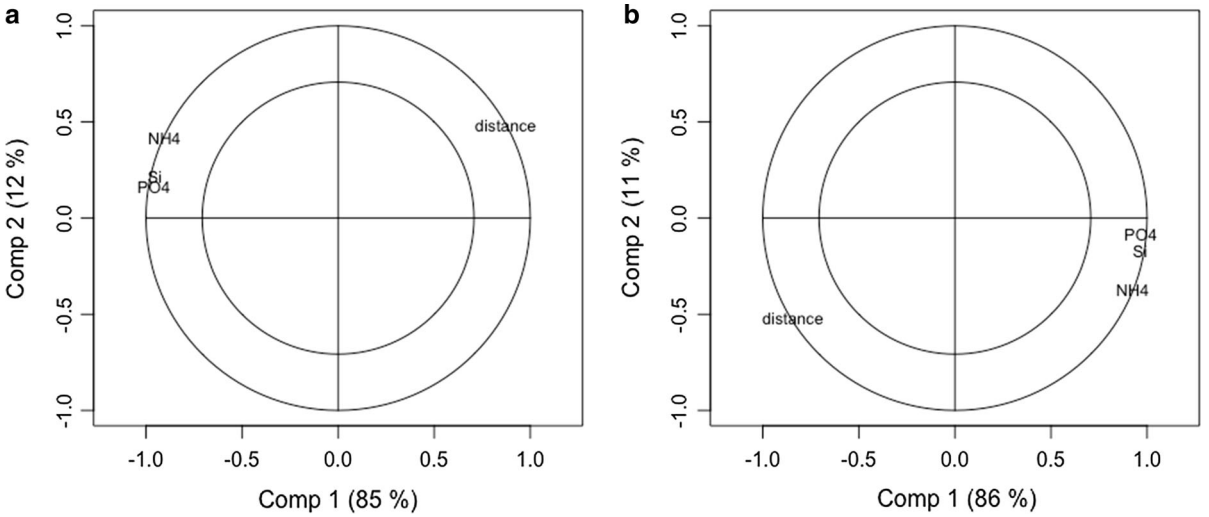

Fig. 2 Partial least squares correlations loading plot showing relationships among water quality indicators and **a** bacterial endophyte abundance and **b** fungal endophyte abundance. Variables in the upper right quadrant of each plot were positively correlated with the response variable, while lower left quadrant were negatively correlated with the response variable. Two components explained most of the variation in the model (e.g. Component 1 + Component 2 explained 97% for bacterial endophytes in **a**). The importance of a given predictor variable for endophyte abundance is proportional to its distance from the origin in the loading space. The circles represent the percent contribution of each variable. The inner circle represents 50% and the outer circle represents 100%, so those variables that are between the inner and outer circles are the variables that are significantly contributing to the overall model. For bacteria, the variable importance in projection (VIP) values for (component 1, component 2) were (**a**) distance (1.32, 1.32), NH_4 (0.67, 0.92), PO_4 (0.99, 0.84), and Si (0.91, 0.82). For fungi, the VIP values for (component 1, component 2) were (**a**) distance (1.07, 1.08), NH_4 (0.80, 0.95), PO_4 (1.10, 1.02), and Si (1.00, 0.93)

"untreated" (reference) and "treated" (0 m to 600 m) trees at Hammond and did not find a significant difference in endophyte community composition between the untreated and treated trees (ANOSIM $R = -0.03$, $p = 0.49$).

Hammond compared to more natural wetlands

Community description

Baldcypress in non-treated wetlands harbored less diverse communities of endophytes. Culturing resulted in 182 bacterial isolates from the roots of baldcypress outside of the treated wetland. Combined with the root bacteria of the treated wetland, 102 sequences were analyzed, and 27 total operational taxonomic units were represented. OTUs in the genus *Bacillus* were common at all sites, with *Bacillus* sp. 1 as the most common root bacterium among wetlands (Table 2). A unique OTU, *Bacillus* sp. 2, was the most common root bacterium among non-treated wetlands (Table 2).

Isolation rates

Root symbiont communities differed in treated wetland baldcypress versus baldcypress at other untreated sites. There was a significantly greater root bacterial abundance (proportion of root segments with culturable bacteria) in the treated site compared to other sites in southeastern Louisiana (Kruskal–Wallis, $H = 39.26$, $p < 0.001$). This was also true when the reference samples at the treated site were removed from the dataset (Kruskall-Wallis, $H = 34.34$, $p < 0.001$).

Diversity index

The treated site had a greater species richness and Fisher's alpha diversity measurement of root bacteria than all other untreated sites (Fig. 3). However, the greater OTU richness was likely due to a greater abundance of endophytes at the treated site, since additional metrics of Shannon and Simpson diversity showed that there was not a significant difference among sites (Fig. S3c).

Table 2 Comparison of root bacteria operational taxonomic units of baldcypress observed at the treated wetland (Hammond) compared to natural wetlands in southeastern Louisiana

Putative taxa[a]	Hammond	Honey Island	Jean Lafitte	Lake Pontchartrain	Tickfaw	Total
Bacillus species 1	26	3	1	0	0	30
Bacillus species 2	2	0	6	0	2	10
Lysinibacillus species	5	1	1	0	1	8
Bacillus species 3	3	0	3	1	0	7
Bradyrhizobium species	5	0	0	0	0	5
Fictibacillus barbaricus	1	0	3	1	0	5
Bacillus species 4	0	1	2	0	1	4
Achromobacter species	4	0	0	0	0	4
Kosakonia cowanii	4	0	0	0	0	4
Pseudomonas species	1	0	2	0	0	3
Aeromonas hydrophila subspecies *hydrophila*	1	0	1	0	0	2
Alcaligenes faecalis	0	1	0	1	0	2
Stenotrophomonas species	1	0	0	1	0	2
Bacillus species 5	0	0	0	0	2	2
Bacillus drentensis	1	0	0	0	1	2

[a]See notes 1 and 2 in Table 1 for description of putative taxa search parameters. Singletons and classifications from the BLAST analysis using UNITE and SILVA databases are listed in Table S2

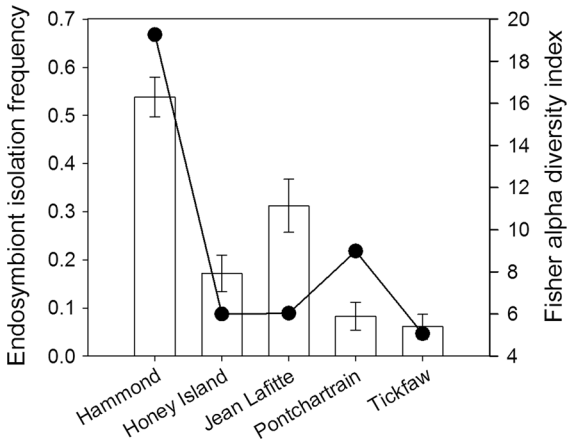

Fig. 3 Root bacterial isolation frequency from baldcypress at five sites in southeastern Louisiana. Bars show mean ± one standard error of bacterial isolation frequency from roots (proportion of root pieces with bacteria). Black circles with lines show the Fisher's alpha diversity index for root bacteria at the sites

Community composition

We observed a difference in root bacterial community composition among sites (ANOSIM global test, R = 0.309, p = 0.001, 999 permutations, Fig. 4). Pairwise comparisons between sites showed that Hammond, the treated site, had a different community composition of root bacteria than Lake Pontchartrain (R = 0.56, p = 0.022), Jean Lafitte (R = 0.39, p = 0.001), and Tickfaw (R = 0.56, p = 0.002). Honey Island and Jean Lafitte communities also differed from each other significantly (R = 0.18, p = 0.032) (Fig. 4). All other pairwise comparisons were not significant (p > 0.05).

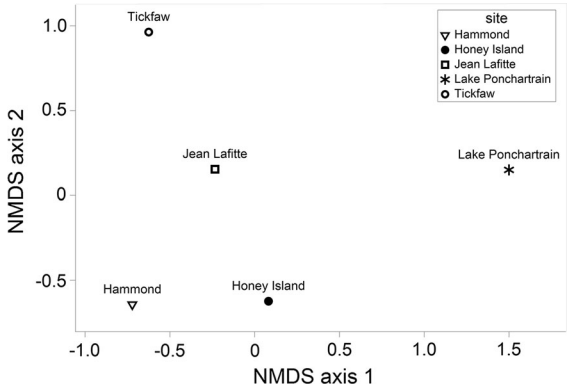

Fig. 4 Similarity of community composition for root bacteria at five sites in southeastern Louisiana, represented by nonmetric multi-dimensional scaling (NMDS) based on a Bray–Curtis similarity matrix. The symbols show the centroid of a cloud for 12–16 trees sampled at each site

Discussion

Baldcypress endophyte communities and wastewater treatment wetlands

We found that endophyte abundance was significantly different for symbiont type (bacteria versus fungi) and location within the plant (roots vs. leaves). At the wastewater treatment site, baldcypress were dominated by root bacterial endophytes, with many fewer fungal endophytes or foliar endophytes. Root bacterial endophytes increased in abundance with increasing distance from the outfall pipe (where there were lower nutrients), while root fungal endophytes decreased in abundance with increasing distance from the outfall pipe.

Previous studies for members of the Cupressaceae and baldcypress have focused on fungal endophytes, or endohyphal bacteria (bacteria living within fungal hyphae). Petrini and Carroll (1981) surveyed four Cupressaceae species in Oregon and found that fungal endophytes were abundant and diverse in leaves, a finding contrary to the results of our study. Endohyphal bacteria were observed in foliar fungal endophytes isolated from six Cupressaceae host species in Arizona and North Carolina (Hoffman and Arnold 2010). They described 29 foliar fungal endophyte taxa, and none overlapped with the putative taxa found in baldcypress leaves of our study (Hoffman and Arnold 2010). Dark septate endophytes and mycorrhizas in baldcypress roots were visualized and enumerated (but not cultured or identified) in Kandalepas et al. (2010). Kandalepas et al. (2010) found that fungal endophytes were abundant in roots of baldcypress, and were negatively correlated with arbuscular mycorrhizal fungi. Notably, no studies have cultured root bacteria from baldcypress or confamilials, and our results demonstrate that bacteria are abundant in root tissues, suggesting that they play an important role in swamp ecosystem function.

Although the function of bacterial endophytes has not been studied previously in baldcypress, several studies have documented the potential for bacterial endophytes to increase the quality of water in

constructed wetlands. Using mesocosm studies, researchers found that plants inoculated with bacterial endophytes increased the speed at which contaminants were cleaned out of water, including metals and pharmaceuticals (Syranidou et al. 2016), crude oil (Rheman et al. 2018) and phenol (Saleem et al. 2018). In trials with sewage effluent, plant-endophyte combinations reduced the total nitrogen, total phosphates, sulfates, and chlorides in the water compared to treatments without endophytes (Ijaz et al. 2015). These mesocosm studies included endophytes that are congeneric with those observed in baldcypress roots from the treatment wetland of our study, including several *Bacillus* and *Pseudomonas* strains (Table 1, Ijaz et al. 2015; Syranidou et al. 2016; Rheman et al. 2018; Saleem et al. 2018). This overlap suggests that further work identifying endophytic bacteria functions and inoculation potential in baldcypress holds promise for improving conditions in wetlands suffering from contamination.

Nutrients

Interactions among endophytes and soil nutrients are complex, and in many cases unknown. A review on root endophyte functions suggested that increased nutrient uptake, especially of phosphorus, was common when the fungi are present (Mandyam and Jumponnen 2005). Fewer studies have looked at how endophytes interact with soil nutrient status. In pine trees, plant growth increased by 50% when fungal endophytes and nitrogen amendments were added, compared to the treatment with nitrogen only (Jumponen et al. 1998). The authors suggested that removing nitrogen limitation allowed the endophytes to exhibit mycorrhizal (nutrient seeking) behavior (Jumponen et al. 1998). Different from our study, previous work in the area of nutrients and root endophytes has occurred in controlled conditions with inoculations of one or a few fungi. The field conditions in our study led to greater levels of endophyte diversity and heterogeneity in environmental conditions, yet we still saw a pattern of abundant fungi in roots where nutrients were in greater concentration, and abundant bacteria in roots where nutrients were in lower concentrations. Since a previous study at this site showed much greater tree growth nearer the outfall pipe (Lundberg et al. 2011), some of the additional growth may be due to an interaction between high nutrients and fungal endophyte abundance.

Ecological changes in wastewater treatment sites

Interestingly, other studies have tracked changes with treatment at this and at other wastewater treatment wetlands in southeastern Louisiana. At the Hammond site where our study was conducted, Hunter et al. (2009) found that nutrients decreased with distance away from the outfall pipe, and Lundberg et al. (2011) recorded correspondingly greater tree growth where nutrients were greater. Despite this, decomposition rates (using litter bag decay rates) did not change with distance from the pipe (Shaffer et al. 2015; Strickland 2015). Also, at the Hammond site, benthic insect community composition was observed to track wetland deterioration and recovery that occurred 8 years previous to the present study (Weller and Bossart 2017). Insect diversity was reduced when the marsh temporarily converted from vegetated to open water, and diversity rebounded with revegetation (Weller and Bossart 2017). At the Amelia swamp in southeastern Louisiana, Day et al. (2006) also recorded greater macroinvertebrate diversity and abundance near the wastewater outfall pipe compared to reference sites. In contrast, our study at Hammond found that all of the roots harbored greater bacterial endophyte abundance and OTU richness at the wastewater treatment wetland compared to sites where baldcypress was growing in an unmanipulated setting (Fig. 3). However, since abundance and richness are generally correlated, our analysis (Fig. S3) suggested that further sampling was needed at the unmanipulated sites before this comparison could be fairly made. For example, three sites—Lake Ponchartrain, Tickfaw and Honey Island had very low numbers of isolates sequenced ($n < 9$ for these sites). Thus, even though we sampled the same number of trees at the various sites, these sites had very low endophyte abundance, making diversity comparisons difficult.

Wastewater treatment site compared to non-treated sites

We found a difference in the composition of root bacterial communities among sites, and the wastewater treated site's community was unique from some non-treated sites. The treated site shared more taxa

with geographically distant compared to closer sites (e.g. Hammond and Jean Lafitte, in Fig. 4, S2), indicating that environmental conditions may play a role in endophyte community assemblage. A greater OTU richness and abundance of root bacteria was observed in the treatment site compared to other sites. Since the abundance values are proportions, these can be compared reliably among sites. The OTU richness and diversity comparisons, however, need to be interpreted with caution since many of our sites had very low abundances of endophytes (Fig. S3). Although the empirical observation of OTU richness ($q = 0$ in Fig. S3) makes the sample at Hammond appear more diverse than the other sites, the overlapping 95% confidence intervals for $q > 0.5$ illustrate that there is not a significant difference in species diversity among sites. Indeed, the large confidence intervals around diversity values at Tickfaw and Lake Ponchartrain show that much more endophytes would need to be sampled at those sites before comparisons can be made reliably. Differences among the sites also could be due to the comparatively young age of the treatment site's trees, as it has been shown that symbiont communities can vary with plant age (Marques et al. 2014). The fact that the reference trees within the treatment wetland did not differ much from trees in the treatment area hints that the differences may have less to do with treatment and more to do with age of trees. According to previous literature, however, tropical plants have seen increases in the diversity of symbiont communities with plant age rather than decreases (Husband et al. 2002a, b).

Endophyte function

Bacterial and fungal endophytes have been shown to promote plant growth and reduce environmental stress (Friesen et al. 2011; Ali et al. 2014). For example, some of the endophytes that were cultured in our study (Tables 1, S1) are in the genera *Bacillus*, *Burkholderia*, and *Pseudomonas* and have been shown to increase plant growth after inoculation (Newsham 2011; Chowdhury et al. 2015; Ijaz et al. 2015; Syranidou et al. 2016; Rheman et al. 2018; Saleem et al. 2018). The possibility remains, however, that not all endophytes are neutral or beneficial to plants (Saikkonen et al. 1998; Rodriguez et al. 2009). Although our study only cultured from apparently healthy roots, some of the endophytes may have negative effects (Mandyam and Jumpponen 2005; Mayerhofer et al. 2013). Whether any of our isolates were pathogenic to baldcypress roots requires further study.

Conclusions

We showed that wastewater treatment may have an impact on the microbiomes of wetland plants. Based on results comparing untreated sites to a wastewater treatment site, the introduction of secondarily-treated wastewater increased the endophyte abundance and richness in baldcypress. Additionally, the endophyte community composition of the wastewater treatment wetland was significantly different from non-treatment sites. These shifts may have consequences for treatment sites, but it is yet difficult to know the implications of a changed microbiome without further experimental work.

Acknowledgements For help in the lab and field we thank Mae Berlow, Kim Mighell, Peter Tellez, Kathalina Tran, Josh Lerner, Casey Gu, James Sebes, Emma Tower, Elaine Broussard, George Washburn, and Julie Whitbeck. Collections that occurred at Jean Lafitte National Historical Park and Preserve were authorized by permit # JELA-2014-SCI-0012.

Funding Funds were provided by the School of Science and Engineering at Tulane University to S. Van Bael, as well as a Louisiana Board of Regents SURE (EPSCOR) award to J. Janowsky and a Tulane Honors College award to J. Janowsky.

References

Ali S, Charles TC, Glick BR (2014) Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. Plant Physiol Biochem 80:160–167. https://doi.org/10.1016/J.Plaphy.2014.04.003

Arnold AE, Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology 88:541–549. https://doi.org/10.1890/05-1459

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecol Lett 3:267–274. https://doi.org/10.1046/j.1461-0248.2000.00159.x

Bodker JE, Turner RE, Tweel A, Schulz C, Swarzenski C (2015) Nutrient-enhanced decomposition of plant biomass in a freshwater wetland. Aquat Bot 127:44–52. https://doi.org/10.1016/j.aquabot.2015.08.001

Boesch DF, Josselyn MN, Mehta AJ, Morris JT, Nuttle WK, Simenstad CA, Swift DJ (1994) Scientific assessment of coastal wetland loss, restoration and management in Louisiana. J Coastal Res 20:1–103

Bokulich NA et al (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:90

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: architecture and applications. BMC Bioinform 10:421

Caporaso JG et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335

Chao A, Jost L (2012) Coverage-based rarefaction and extrapolation: standardizing samples by completeness rather than size. Ecology 93:2533–2547

Chao A, Jost L (2015) Estimating diversity and entropy profiles via discovery rates of new species. Methods Ecol Evol 6:873–882

Chao A, Ma KH, Hsieh TC (2016) iNEXT (iNterpolation and EXTrapolation) Online: Software for Interpolation and Extrapolation of Species Diversity

Chowdhury SP, Hartmann A, Gao X, Borriss R (2015) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42–a review. Front Microbiol 6:780

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Austral Ecol 18:117–143

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial, 6th edn. PRIMER Ltd., Plymouth

Couvillion BR et al (2011) Land area change in coastal Louisiana from 1932 to 2010. US Geological Survey, Reston

Day JW, Shaffer GP, Britsch LD, Reed DJ, Hawes SR, Cahoon D (2000) Pattern and process of land loss in the Mississippi Delta: a spatial and temporal analysis of wetland habitat change. Estuaries 23:425–438. https://doi.org/10.2307/1353136

Day JW et al (2006) Effects of long-term municipal effluent discharge on the nutrient dynamics, productivity, and benthic community structure of a tidal freshwater forested wetland in Louisiana. Ecol Eng 27:242–257. https://doi.org/10.1016/j.ecoleng.2006.03.004

Day JW et al (2007) Restoration of the Mississippi delta: lessons from hurricanes Katrina and Rita. Science 315:1679–1684. https://doi.org/10.1126/science.1137030

Eriksson L, Hermens JL, Johansson E, Verhaar HJ, Wold S (1995) Multivariate analysis of aquatic toxicity data with PLS. Aquat Sci 57:217–241

Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E (2011) Microbially mediated plant functional traits. Annu Rev Ecol Evol Syst 42:23–46

Gibson A, Harper J (1985) Nitrate effect on nodulation of soybean by *Bradyrhizobium japonicum*. Crop Sci 25:497–501

Glöckner FO et al (2017) 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol 261:169–176

Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413:297

Hoffman MT, Arnold AE (2010) Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. Appl Environ Microbiol 76:4063–4075. https://doi.org/10.1128/Aem.02928-09

Hunter R, Lane R, Day J, Lindsey J, Day J, Hunter M (2009) Nutrient removal and loading rate analysis of Louisiana forested wetlands assimilating treated municipal effluent. Environ Manage 44:865–873. https://doi.org/10.1007/s00267-009-9348-y

Hunter RG et al (2018) Using natural wetlands for municipal effluent assimilation: a half-century of experience for the Mississippi River Delta and surrounding environs. In: Nagabhatla N, Metcalfe CD (eds) Multifunctional wetlands. Springer, Cham, pp 15–81

Husband R, Herre EA, Turner SL, Gallery R, Young JPW (2002a) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Mol Ecol 11:2669–2678. https://doi.org/10.1046/j.1365-294X.2002.01647.x

Husband R, Herre EA, Young JPW (2002b) Temporal variation in the arbuscular mycorrhizal communities colonising seedlings in a tropical forest. FEMS Microbiol Ecol 42:131–136. https://doi.org/10.1111/j.1574-6941.2002.tb01002.x

Ijaz A, Shabir G, Khan QM, Afzal M (2015) Enhanced remediation of sewage effluent by endophyte-assisted floating treatment wetlands. Ecol Eng 84:58–66

Imsande J (1986) Inhibition of nodule development in soybean by nitrate or reduced nitrogen. J Exp Bot 37:348–355

Jumpponen A, Mattson KG, Trappe JM (1998) Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. Mycorrhiza 7:261–265

Kandalepas D (2012) Effects of coastal dynamics on colonization of Louisiana wetland plants by fungal endophytes. Louisiana State University, Baton Rouge

Kandalepas D, Stevens KJ, Shaffer GP, Platt WJ (2010) How abundant are root-colonizing fungi in Southeastern Louisiana's degraded marshes? Wetlands 30:189–199

Kandalepas D, Blum MJ, Van Bael SA (2015) Shifts in symbiotic endophyte communities of a foundational salt marsh grass following oil exposure from the Deepwater Horizon oil spill. PLoS ONE 10:e0122378. https://doi.org/10.1371/journal.pone.0122378

Kimbrough ER, Berlow ML, Van Bael SA (2019) Water level and salinity drive community structure of culturable baldcypress (*Taxodiumdistichum*) Endophytes in Southern Louisiana. Wetlands 39:329–335

Kõljalg U et al (2013) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277

Li J-y, Strobel G, Sidhu R, Hess W, Ford EJ (1996) Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*. Microbiology 142:2223–2226

Lundberg CJ, Shaffer GP, Wood WB, Day JW (2011) Growth rates of baldcypress *(Taxodium distichum)* seedlings in a treated effluent assimilation marsh. Ecol Eng 37:549–553. https://doi.org/10.1016/j.ecoleng.2010.11.012

Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Stud Mycol 5:173–189

Marques JM et al (2014) Plant age and genotype affect the bacterial community composition in the tuber rhizosphere of field-grown sweet potato plants. FEMS Microbiol Ecol 88:424–435. https://doi.org/10.1111/1574-6941.12313

Mayerhofer MS, Kernaghan G, Harper KA (2013) The effects of fungal root endophytes on plant growth: a meta-analysis. Mycorrhiza 23:119–128

Mugerwa TM, Saleeba J, McGee P (2013) A variety of melanised root-associated fungi from the Sydney basin form endophytic associations with *Trifolium subterraneum*. Fungal Ecol 6:70–82

Murray JD, Liu C-W, Chen Y, Miller AJ (2016) Nitrogen sensing in legumes. J Exp Bot 68(8):1919–1926

Newsham KK (2011) A meta-analysis of plant responses to dark septate root endophytes. New Phytol 190:783–793. https://doi.org/10.1111/j.1469-8137.2010.03611.x

Pedregosa F et al (2011) Scikit-learn: machine learning in Python. J Mach Learn Res 12:2825–2830

Peskan-Berghofer T et al (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. Physiol Plant 122:465–477. https://doi.org/10.1111/j.1399-3054.2004.00424.x

Petrini O, Carroll G (1981) Endophytic fungi in foliage of some Cupressaceae in Oregon. Can J Bot-Rev Can Bot 59:629–636. https://doi.org/10.1139/b81-089

Porras-Alfaro A, Bayman P (2011) Hidden fungi, emergent properties: endophytes and microbiomes. Annu Rev Phytopathol 49:291–315

Redman RS, Kim YO, Woodward CJDA, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS ONE 6:e14823. https://doi.org/10.1371/journal.pone.0014823

Rehman K, Imran A, Amin I, Afzal M (2018) Inoculation with bacteria in floating treatment wetlands positively modulates the phytoremediation of oil field wastewater. J Hazard Mater 349:242–251

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330. https://doi.org/10.1111/J.1469-8137.2009.02773.X

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29:319–343

Saleem H, Arslan M, Rehman K, Tahseen R, Afzal M (2018) *Phragmites australis*—a helophytic grass—can establish successful partnership with phenol-degrading bacteria in a floating treatment wetland. Saudi J Biol Sci 26(6):1179–1186

Shaffer GP et al (2015) System response, nutria herbivory, and vegetation recovery of a wetland receiving secondarily-treated effluent in coastal Louisiana. Ecol Eng 79:120–131

Streeter J, Wong PP (1988) Inhibition of legume nodule formation and N2 fixation by nitrate. Crit Rev Plant Sci 7:1–23

Strickland ER (2015) Optimizing growth and survival of bald-cypress *(Taxodium distichum)* and water tupelo *(Nyssa aquatica)* destined for restoration projects in nutrient-rich, freshwater areas. M. S. Southeastern Louisiana University, Hanmond

Syranidou E, Christofilopoulos S, Gkavrou G, Thijs S, Weyens N, Vangronsveld J, Kalogerakis N (2016) Exploitation of endophytic bacteria to enhance the phytoremediation potential of the wetland helophyte *Juncus acutus*. Front Microbiol 7:1016

R Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. R Foundation for Statistical Computing, Vienna, Austria

U'ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE (2009) Diversity and evolutionary origins of fungi associated with seeds of a Neotropical pioneer tree: a case study for analysing fungal environmental samples. Mycol Res 113:432–449. https://doi.org/10.1016/j.mycres.2008.11.015

Weishampel PA, Bedford BL (2006) Wetland dicots and monocots differ in colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza 16:495–502. https://doi.org/10.1007/s00572-006-0064-7

Weller M, Bossart J (2017) Insect community diversity tracks degradation and recovery of a wastewater assimilation marsh in southeast Louisiana. Wetlands 37:661–673

Wilson D (1995) Endophyte—the evolution of a term, and clarification of its use and definition. Oikos 73:274–276. https://doi.org/10.2307/3545919