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Hierarchical controls of endophyte-mediated drought tolerance: ecological, physiological, and molecular

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Hierarchical controls of endophyte-mediated drought tolerance: ecological, physiological, and molecular

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Hierarchical controls of endophyte-mediated drought tolerance: ecological, physiological, and molecular

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Symbiotic interactions influence many community and ecosystem processes, via their role in nutrient cycling, water acquisition, and pathogen protection. Symbiotic associations can range from antagonisms to mutualisms and depend on multiple levels of control: ecological controls driving species distributions, the physiological interaction of host and symbiont, and molecular regulation of symbiotic interactions. Using horizontally-transmitted endophytes and their associations with C4 grasses, I examined the ecological, physiological and molecular drivers of symbiotic interactions. Horizontally transmitted fungal endophytes reside within the tissues of nearly all studied plants and can alter plant physiology in response to drought. Most climate models predict an increase in the frequency and severity of drought in upcoming decades. If the drivers of endophyte-mediated drought tolerance can be used to predict the outcomes of plant-endophyte interactions more generally, it may have large ecological and economic implications.

Throughout my dissertation, I characterized the ecological, physiological and molecular controls of plant fungal symbioses. To identify the ecological drivers of endophyte distributions, I characterized the plant and endophyte communities across a precipitation gradient, finding that historical and current climate explained most of the variation in community composition. Biotic factors, including host specificity and host traits, were substantially less predictive than biotic factors. To understand the physiological controls of plant-endophyte interactions, I characterized both functional traits of endophytes in culture and their effect on their plant host in symbiosis. Fungal resource use and stress tolerance were strongly predictive of the outcome of symbioses under stress, but less so under non-stressed conditions. To understand the molecular regulation of plant-fungal symbioses, I identified differentially expressed plant genes under differing environmental conditions. I found that, while certain pathogen and drought response genes correlated with plant response to fungal colonization, overall, beneficial fungi affected expression of a smaller number of genes than antagonistic fungi. Together, the results of these experiments emphasize the potential of multiple levels of regulation (ecological, physiological, and molecular) to regulate the outcome of symbioses.

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Introduction

Symbiotic interactions are characterized as any long-term association between two or more species. These interactions affect many community and ecosystem processes, including nitrogen fixation, resource capture, and disease incidence (Mylona *et al*., 1995; Vance, 2001; Arnold & Herre, 2003). However, symbioses are not necessarily mutualisms; instead the outcomes for hosts and symbionts can range along a continuum from mutually beneficial (mutualism) to mutually harmful (antagonism) (Bronstein, 1994; Johnson *et al*., 1997). While symbiotic interactions are critically important for understanding many community and ecosystem processes, the drivers that determine their outcome are poorly understood. Symbiotic outcomes likely depend on a combination of multiple levels of control, including ecological, physiological, and molecular controls. Ecological controls drive species distributions, determining whether two species overlap and have the potential to form symbioses (Van der Putten *et al*., 2010). Once two species associate, the physiological responses, or traits, of both host and symbiont to each other and environmental conditions determine the outcome of the symbiosis (Johnson et al., 1997). Further regulation of the symbiotic interaction can occur at a molecular level. The differential gene expression of both host and symbiont can affect the strength of association and the direction (Chisholm *et al*., 2006; Dale & Moran, 2006). Thus, predicting the outcome of symbiotic interactions requires a hierarchical approach that considers ecological, physiological, and molecular controls.

Horizontally-transmitted fungal endophytes and their interactions with plant hosts are an ideal study system for characterizing the hierarchical controls of symbiotic interactions. Endophytes are organisms that inhabit plant organs at some point during their life and can colonize plant tissues without causing apparent disease (Petrini, 1991).

Horizontally-transmitted endophytes (also known as 'Class 3' endophytes) differ from the more commonly-studied vertically-transmitted endophytes in that they have a broader, but still largely uncharacterized, host range (Rodriguez *et al*., 2009). Additionally, horizontally-transmitted endophytes (hereafter known as 'endophytes') are thought more likely to demonstrate habitat-adapted benefits on their plant host (Rodriguez et al., 2009). These endophytes reside within the tissues of nearly all studied plants and can alter plant physiology, including leaf conductance, root to shoot ratio, and antioxidant systems (Arnold & Engelbrecht, 2007; Torres *et al*., 2012; Czarnoleski *et al*., 2012). Many endophytes are mutualists that benefit their plant hosts by conferring increased tolerance to a range of abiotic and biotic stresses (Rodriguez et al., 2009). However, endophytic fungi can also behave as commensals or antagonists, depending on current environmental conditions (Carroll, 1988). Molecular regulation of plant-endophyte interactions has been demonstrated in multiple systems and can affect symbiotic outcome and establishment (Young *et al*., 2005; Bailey *et al*., 2006; Paparu *et al*., 2007). Additionally, most horizontally-transmitted endophytes have both free-living and symbiotic lifestyles (Rodriguez et al., 2009) and many are culturable, making them easier to manipulate experimentally.

Many fungal endophytes can improve plant drought tolerance. This has been observed in tropical tree species (Arnold & Engelbrecht, 2007), crops like rice and tomatoes (Rodriguez et al., 2008), and numerous grasses (Rodriguez *et al*., 2008; Ren & Clay, 2009). The majority of fungal endophytes decrease plant water loss, but identifying specific mechanisms governing these interactions has proven difficult. Possible mechanisms include fungal moderation of leaf conductance, reactive oxygen species accumulation, and root to shoot ratios (Rodriguez *et al*., 2008; Ren & Clay, 2009). While fungal effects on water loss could be due to fungal production of secondary compounds

(Rodriguez et al., 2009), fungal effects on plant survival are more difficult to understand. One possibility is that fungi that improve plant survival under drought are able to moderate their resource demands in response to drought to avoid stressing their host (Vannette & Hunter, 2010). If the drivers of endophyte-mediated drought tolerance carry over to plant-endophyte symbioses more generally, understanding these drivers may have large ecological and economic implications. However, changing environmental conditions, including increased drought, are likely to affect the endophyte ranges, physiological responses to stress, and the molecular controls of these symbioses.

Therefore, each chapter of my dissertation focuses on one of these levels of regulation of plant-endophyte symbioses. In Chapter 1, I identified the ecological drivers of endophyte community composition and distributions by characterizing the plant and endophyte communities across a precipitation gradient. For most studied organisms, environmental filtering, along with dispersal, are two of the primary factors structuring communities locally (Leibold *et al*., 2010). Biotic interactions are most frequently considered in terms of competition or predation. However, unlike free-living organisms, endophytes have hosts as an added layer of selection on their spatial distributions. To identify environmental, host variable, spatial, and temporal drivers of endophyte structure, I used a culture-based approach to characterize endophyte community structure in a single plant species (*Panicum hallii*) across a precipitation gradient.

In Chapter 2, I examined the physiological controls of plant-endophyte interactions by screening plant-endophyte pairings to determine their symbiotic outcome and identifying potential drivers: habitat adaptation, phylogenetic relatedness, and functional traits. Initially, the paradigm that fungal endophytes were habitat-adapted suggested that predicting symbiosis outcomes would be relatively simple (Rodriguez et al., 2009). However, selection of horizontally transmitted endophytes can occur during

both their free-living (Evans & Wallenstein, 2012) and symbiotic stages (Higgins *et al*., 2007) such that adaptation of endophytic fungi to their environment may not translate into host effects. Therefore, historic selection and phylogenetic relatedness may be better predictors of symbiotic outcomes. Alternatively, it may be that functional traits, which are more easily tested and screened, act as a better predictor of the outcome of plant-fungal interactions. I screened 35 plant-fungal pairings, focusing on *Panicum hallii*, to determine the symbiotic outcome under drought-stressed and well-watered conditions. Using the 35 screened fungi, I characterized functional traits related to resource use and stress responses, which were chosen because of their potential role in plant growth and physiological responses to drought.

Finally, in Chapter 3, I identified the genes that are likely involved in the molecular regulation of plant-fungal symbioses by identifying differentially expressed plant genes under differing moisture conditions. Little is understood about the relationship between symbiotic outcomes and gene expression in C4 grasses and horizontally-transmitted endophytes. Potential mechanisms of endophyte-mediated drought tolerance include fungal moderation of abscisic acid concentrations (Hao *et al*., 2009), maintenance of cell turgor via the fungal production of osmolytes (Waqas et al., 2015), and increased efficiency of plant stress responses due to fungal colonization (Ren & Clay, 2009), among many others. Of the plant-fungal pairings screened in Chapter 2, I selected 3 beneficial and 3 antagonistic fungal taxa and characterized their effect on gene expression in *Panicum hallii* under low water and high water conditions.

Characterizing the ecological, physiological, and molecular drivers of symbiotic outcomes independently would be a challenging task. Addressing multiple levels of control is made more difficult by the likely interactions between drivers. The experiments described here represent a first step towards addressing the relative importance of

ecological, physiological, and molecular mechanisms in determining the outcome of symbiotic associations.

Chapter 1: Drivers of endophyte community composition and diversity[1]

Introduction

Horizontally-transmitted, Class 3, foliar fungal endophytes (hereafter, 'endophytes') are present in all terrestrial plants examined to date (Carroll, 1988; Rodriguez *et al*., 2009). Although these heterotrophic fungi use photosynthate carbon from the plant (Siegel *et al*., 1987; White & Torres, 2010), many endophytes appear to be commensals or mutualists (Carroll, 1988; Eaton *et al*., 2011). For example, some endophytic fungi ameliorate plant physiological and growth responses to abiotic or biotic stressors, including salt (Rodriguez *et al*., 2008), heat (Redman *et al*., 2002), drought (Giauque & Hawkes, 2013), herbivory (Arnold & Lewis, 2005), and pathogens (Arnold & Herre, 2003). Despite their ubiquity and ecological significance, our understanding of endophyte ecology and evolution remains limited. Improved knowledge of endophyte distributional patterns will allow us to identify potential underlying drivers and will ultimately provide the basis for scaling their ecological impacts.

Unlike free-living organisms, symbionts have hosts as an additional layer of constraint in their spatial distributions. Fungal endophytes rely on their host plant for protection and resources (Rodriguez *et al*., 2009), and some endophytes are unique to divergent host clades (Higgins *et al*., 2007), suggesting that host distribution and abundance has some control over endophyte distribution. However, because there is little evidence for endophyte host specificity, particularly among closely related plant species (Higgins *et al*., 2011; Del Olmo-Ruiz & Arnold, 2014; Higgins *et al*., 2014), important host plant characteristics might relate more to potential incidence (abundance or density) or potential carbon provision (plant size) than to host identity per se. Nevertheless, as with free-living organisms (Legendre *et al*., 2005), the spatial distributions of symbiotic endophytes are also governed by non-host factors. Endophyte communities vary across

[1] Giauque H, Hawkes CV. 2016. Historical and current climate drive spatial and temporal patterns in fungal endophyte diversity. Fungal Ecology 20: 108–114.
Giauque planned, executed, and wrote up project. Hawkes supervised project.

large latitudinal gradients (Arnold & Lutzoni, 2007)and across habitats (Loro *et al*., 2012) likely resulting from changes in abiotic and biotic components of the sites. For example, endophyte communities in single host plants were primarily controlled by environmental gradients in both central Texas savanna and the Mauna Loa volcano in Hawaii (Zimmerman & Vitousek, 2012).

Less is known about temporal variation in endophytes, but extrapolating from the drivers of spatial variation suggests that endophytes should track host and climatic factors over time. Within a single year, endophytes can vary seasonally due to increasing plant productivity and other phenological traits (Ghimire *et al*., 2010; Ek-Ramos *et al*., 2013). For example, endophyte diversity in *Panicum virgatum* peaked with maximum plant biomass and decreased rapidly with plant senescence (Ghimire *et al*., 2010). Given observed differences in endophytes between wetter and drier sites (Zimmerman & Vitousek, 2012; Loro *et al*., 2012), it is likely that weather variation and associated environmental stress across years will also play a role either through direct effects on the fungi or a response of fungi to the host plant condition (Garrett *et al*., 2006). In contrast, in regions such as wet tropical forests where climate varies little between years, interannual differences in endophytes are more likely to be caused by differences in host plant age or life stage (Del Olmo-Ruiz & Arnold, 2014; Higgins *et al*., 2014).

Current weather must be distinguished from the effects of historical climate, because fungal communities should reflect long-term trends more than short-term annual conditions unless dispersal and species sorting occur on the same time scale as weather variation. Although endophytes have not been considered in this context previously, past drought patterns create legacies that constrain the composition of current soil microbial communities (Evans & Wallenstein, 2012); however, this is not always the case (Rousk *et al*., 2013). We expect historical climate effects to predominate when communities assembled by environmental filtering over time are difficult to overcome by either resuscitation of endophyte taxa from local dormant pools (Jones & Lennon, 2010) or immigration from regional species pools across years (Kinkel *et al*., 1989). However, determining the relative importance of these potential drivers of endophyte communities

requires studies to capture a range of local abiotic and biotic factors in the context of climate variability over time.

In a previous study, we determined that endophyte community composition and diversity across sites differed largely as a function of the interaction between historical and current precipitation (Giauque & Hawkes, 2013). We could not parse the effect of rainfall any further, having examined only a single time point, and we did not consider other host plant or vegetation characteristics that might be important for symbionts. Here, we investigated temporal variation in endophytes by measuring both spatial and annual variation in potential biotic and abiotic drivers. Specifically, we examined endophyte richness and community composition over three years and assessed how these patterns reflected historical climate, annual weather, host plant traits, vegetation structure, and spatial distributions. We hypothesized that interannual variability in rainfall and temperature would modify endophyte communities both directly and through changes in host plant traits or vegetation structure. To address these questions, we characterized annual variation in leaf endophytes of the grass *Panicum hallii* Vasey across a steep rainfall gradient over three years. The rainfall gradient allowed us to examine sites with different historical climate conditions, host plant traits, and vegetation structure. By studying endophyte communities at these sites across the three years, we could further separate annual weather variation from historical climate conditions and other factors.

Materials and Methods

Study sites and field sampling

We sampled fungal endophytes from ten sites located across a steep precipitation gradient in Central Texas. Sampling was restricted to the Edwards Plateau to maintain consistent vegetation and soil types: savanna grasslands on shallow, rocky, calcareous Mollisols. The Plateau spans ~400 km from west to east, with mean annual precipitation (MAP) varying from ~400 to 900 mm, mean annual high temperatures (MHT) from ~24 to 28 °C and mean annual low temperatures (MLT) from ~11 to 13 °C, as reported by PRISM Climate Group (Oregon State University, http//prism.oregonstate.edu, created 15

August 2014). Sites were selected based on MAP, availability of host plants, and at least 50% grass cover.

We isolated endophytes from *P. hallii*, due to its abundance across the Edwards Plateau. *Panicum hallii* is a perennial, warm-season grass native to North America. From 2012 to 2014, we sampled plants annually in June in an area of 20 m x 20 m at each site. The month of June was chosen because that is when *P. hallii* typically reaches peak biomass and begins to flower. The same plots were revisited each year, but not the same individual plants. At each site at each sampling date, three tillers were collected from each of four individual *P. hallii* plants. Plants were rinsed in the field with sterile water and stored in plastic bags. Soils were also sampled for gravimetric moisture at each date. Plant leaves were kept on ice for transport back to the lab and refrigerated at 4°C for no more than 48 hours prior to leaf culturing. Additionally, plant characteristics were recorded for each plant, including tiller number, basal area, height, flowering height, and density.

Fungal culturing

Tillers were sectioned into three 2-mm fragments, which were surface sterilized using 0.5% sodium hypochlorite (2 min), 70% ethanol (2 min), and sterile water (30 sec), following Arnold et al. (2000). Sterilized leaf fragments were placed on petri dishes containing 2% potato dextrose agar (PDA) and 50 ppm ampicillin. The use of PDA is common for fungal-growth media because it minimizes the risk of nutrient limitation and yields large numbers of fungal isolates (Ghimire *et al*., 2010; Orlandelli *et al*., 2012; Loro *et al*., 2012). Plates were incubated at room temperature and assessed daily for fungal growth. When hyphae emerged from a leaf fragment, the fungus was transferred to a new PDA plate to obtain pure cultures. Once in pure culture, 1-cm x 2-cm fungal fragments were subsampled for (1) long-term storage of three subsamples in 2 ml of RNase/DNase-free water at room temperature (Burdsall & Dorworth, 1994), and (2) immediate DNA extraction of two subsamples.

Fungal identification

Isolates were initially assigned to morphotypes based on morphological characteristics, as previously described (Arnold *et al*., 2000). Morphotype identity was confirmed by DNA sequencing, including 195 isolates with at least three representatives of each morphotype. DNA was extracted from fungal tissue using a standard phenol-chloroform-isoamyl procedure modified with bead beating (Griffiths *et al*., 2000).

For DNA-based identification, we used both the ITS and LSU regions of rDNA, because these capture both variable and conserved regions to allow for robust alignments (Liu *et al*., 2012; Porter & Golding, 2012). The ITS region (~500-800 bp) was amplified using the primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al*., 1990; Gardes & Bruns, 1993). The D1/D2 region of the LSU (~650 bp) was amplified using the general fungal primers NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') AND NL4 (5' GGTCCGTGTTTCAAGACGG3 3') (O'Donnell, 1993). Each 25-µl PCR reaction contained approximately 10 ng of fungal DNA, 0.75 U *Taq* polymerase, 1x PCR buffer, 2 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} dNTPs, and 0.5 µmol L^{-1} each of primers. Thermal cycling reactions used the following conditions: 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; 1 cycle of 72°C for 5 min. Amplified products were sequenced on an ABI3730XL DNA Analyzer (Applied Biosystems, Carlsbad, California, USA) at the DNA Sequencing Facility at the University of Texas at Austin. Sequences have been deposited into NCBI GenBank under accession numbers KC582560-KC582600 and KP401860-KP401949.

Sequences were trimmed and quality checked as detailed in Arnold and Lutzoni (2007). Large subunit and ITS reads were aligned separately using SATé v2.2.7 (Liu *et al*., 2011) and then combined into a single composite sequence for each isolate (James *et al*., 2006). Composite sequences were clustered into initial operational taxonomic units (OTUs) based on 97% sequence similarity using UCLUST (Edgar, 2010). Representative sequences of each OTU were aligned to an internal guide tree of 112 known *Ascomycota* sequences spanning the ITS and LSU regions, again using SATé. Guide tree sequences

were compiled from the Ribosomal Database Project (Liu *et al*., 2012) including representatives of genera from the best BLAST matches. To define phylogenetic OTUs, we collapsed monophyletic groups at 97% sequence similarity. To identify functional guilds of the fungi (endophyte, saprophyte, pathogen), we submitted the sequences to FunGuild (Nguyen *et al*., 2015) and carried out a literature search for the nearest identified sister taxa. In the literature search, we used the species name of the sister taxa and the keywords 'endophyt*,' 'sapro*,' and 'pathogen*.'

Site characteristics

Spatial location was recorded as latitude, longitude, and elevation using a handheld GPS (GPSMAP 62S; Garmin Ltd., Olathe, KS, USA). For climate variables, historical means (30-year, 1981-2010) and annual values for precipitation, maximum temperature, and minimum temperature were obtained from the PRISM Climate Group (Oregon State University, http//prism.oregonstate.edu, created 15 August 2014). Annual spring rainfall and temperature were summed over 3 months (April – June) each year, whereas year-to-date (YTD) rainfall and temperature were summed over 6 months (January – June). Soil moisture, quantified gravimetrically, was used as an additional indicator of conditions at the time of sampling. Vegetation structure at each site was quantified by determining percent grass, shrub, and bare ground cover using i-Tree Canopy version 6.1 (http://www.itreetools.org/canopy/).

Statistical analyses

To ensure sufficient sampling, we estimated total endophyte species richness based on our sampling effort for every site, date, and plant using the Chao2 estimator in EstimateS v8.2 (Colwell, 2005). To test for differences in endophyte richness across sites and years, we used a linear mixed effects model with site, year, and their interaction as fixed factors and plant nested within site as a random factor. To identify potential drivers of endophyte richness across years, we used stepwise multiple regression. Prior to the regression analyses, Pearson's correlations were used to determine if two variables were correlated; when a pair of variables was correlated at $r^2 > 0.70$ and $P < 0.01$, the variable with less explanatory power was removed. For example, longitude was 95.8% correlated

with MAP and thus only MAP was included in the analysis because individually it was 12.2% more strongly correlated with richness. Additionally, we included only plant and site variables that differed significantly by site, year, or their interaction; results of these analyses are in the supplementary material (Tables 1.3-1.4). After these screens were implemented, the model included the following independent variables: (1) host plant characteristics (tiller number, basal area, height, flowering height, density), (2) site characteristics that varied spatially or temporally: historical climate (mean annual precipitation, mean annual high temperature, mean annual low temperature), annual weather (spring and YTD rainfall, maximum temperature, minimum temperature), and vegetation structure (grass cover, shrub cover, bare ground), and (3) spatial location (latitude, elevation). Variables were log-transformed to ensure a normalized distribution. A subset of the variables from the full model was chosen as the best explanatory model using backwards stepwise selection based on Akaike Information Criteria (Akaike, 1974). Note that we also compared the log-transformed linear models to non-linear models and untransformed data; in all cases, best fit and lowest AIC scores were achieved using linear models with normalized data. All linear models, correlations, and linear and non-linear regressions were performed in SPSS v17.0.0.1, (IBM North America, New York, USA).

To examine temporal and spatial variation in site-level endophyte community composition, we performed nonparametric multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities with 999 permutations using the Adonis function of Vegan in R (Oksanen *et al*., 2015), with site, year, and their interaction, with each factor run last in the model to obtain Type III sums of squares. Pairwise comparisons of all sites were used to determine significant post-hoc differences among sites, with Bonferroni corrections for multiple comparisons.

When community composition differed significantly as a function of either the main effects or the interaction term, we reran the PERMANOVAs to determine the contributions of environmental, biotic, and spatial variables to the observed variation in community composition. These PERMANOVAs were run in parallel to the multiple

regressions for richness, with the same variables included as in the regression models. To determine the order of variables in the PERMANOVA, each variable was initially run individually and significant variables were then included in the full analysis in decreasing order of r^2 (Borcard *et al*., 1992). Because of these multiple comparisons (n=21), PERMANOVA *P*-values were Bonferroni-corrected *(P* < 0.002). Again, variables that did not differ significantly by site, year, or their interaction were removed. Differences in community composition among sites and years were visualized using nonmetric multidimensional scaling (NMS) with Bray-Curtis dissimilarities in PC-ORD v 6.08 (McCune & Mefford, 2011).

To account for phylogenetic beta-diversity, we repeated the PERMANOVAs using unweighted UniFrac distances calculated with GUnifrac v1.0 (Chen *et al*., 2012). To identify phylogenetic patterns in endophyte community structure, we assessed phylogenetic signal at the tips and base of the phylogeny using the standardized effect sizes of mean nearest taxon distance (MNTD) and mean phylogenetic distance (MPD). Analyses were performed using the 'mpd' and 'mntd' functions in Picante v.0.7-2 (Kembel *et al*., 2015).

Results

Site and gradient characteristics

Different weather conditions prevailed in 2012, 2013, and 2014 (Table 1.1, 1.2). For example, across all sites the average total spring rainfall was 333.9, 285.2, and 233.4 mm and average high temperature was 34.93, 33.33, and 32.31°C, respectively. As expected, both historical climate and annual weather differed among the gradient sites (Table 1.3); for example, MAP ranged more than 400 mm from east to west. Host plant characteristics paralleled climate across sites and years (Table 1.4), with larger plants at the wetter end of the gradient and in wetter years compared to smaller plants at the drier end of the gradient and in drier years. Overall vegetation structure varied across sites, with grass cover also tracking the precipitation gradient.

Endophyte richness

A total of 811 fungal isolates were cultured from 1,020 leaf segments and grouped into 66 initial morphotypes, which were identified by DNA-sequencing and assigned to 65 phylogeny-based OTUs (Appendix 2). Our sampling captured an average of 96.8% (± 0.8) of the expected site richness based on the Chao2 estimator (Figure 1.1). Foliar endophyte richness in *P. hallii* varied among sites ($F_{9,90} = 37.258$, $P < 0.001$), ranging from 5 to 36 taxa. Richness was best predicted by MAP ($r^2 = 0.557$) and MHT ($r^2 = 0.120$) ($r^2 = 0.677$, $F_{2,27} = 28.340$, $P < 0.001$) across the gradient, with fewer endophyte taxa found at historically drier and warmer sites (Figure 1.2). Endophyte richness was the same across years ($F_{2,90} = 1.378$, $P = 0.257$), although individual sites did not necessarily maintain the same richness over time ($F_{18,90} = 4.401$, $P < 0.001$).

Endophyte taxa

Of the 65 unique fungal taxa that were isolated, ten different orders were represented, with more than 60% of isolates from *Pleosporales*, *Eurotiales*, and *Hypocreales* (Appendix 2). Most of the isolated fungi were previously identified as endophytes (50/65), but these were also observed in at least one other functional guild (saprophyte or pathogen; Table 1.5). The remaining 15 isolates were previously identified as or were closely related to known pathogens or saprophytes (Table 1.5). None of the isolated endophytes were from the family Clavicipitaceae suggesting that none were vertically transmitted.

Endophyte community composition and phylogenetics

Endophyte community composition differed primarily across sites, although site effects varied somewhat from year to year (Table 1.1, Figure 1.3). In addition, endophyte communities varied across years independent of site effects (Table 1.1, Figure 1.3). The largest amount of endophyte community variation (24.8%) was explained by historical climate conditions and their interactions (Figure 1.4, Table 1.6), with the majority in MAP (17.5%). Annual weather (10.4%) and its interactions with historical climate (1.4%) together had roughly two-thirds of the explanatory power as MAP alone (Figure 1.4, Table 1.6).

Plants also played a small role in endophyte community composition, reflecting vegetation structure (3.2%) and host plant traits (2.5%). Spatial factors, independent of all other factors, explained only 2.0% (Figure 1.4, Table 1.6). A substantial portion (56.1%) of the variation in endophyte community composition remained unexplained.

Drivers of endophyte communities based on UniFrac distances were similar to Bray-Curtis, but fewer factors were included and 8.5% less variance was explained (Figure 1.3, Table 1.1, 1.7). Most of the phylogenetic variation among communities was related to MAP (11.5%) and an interaction of spring rainfall and MLT (9.4%). In addition, spatial factors explained 8.6% and the interaction of MAP and grass cover explained 6.3%. Phylogenetic clustering did not differ across sites over contemporary (MNTD) or evolutionary (MPD) timescales (Appendix 2).

Discussion

Consistent with our original hypothesis, climate was directly responsible for more than a third of the observed variation in *P. hallii* endophyte communities over 3 years and higher endophyte diversity occurred at sites that were historically wetter with milder temperatures. Studies of endophytes in grasslands have previously recognized climate, particularly precipitation, as a major driver of spatial patterns of endophyte distributions (Zimmerman & Vitousek, 2012; Loro *et al*., 2012; Giauque & Hawkes, 2013). However, here we identified historical precipitation and temperature as the most important factors determining endophyte community richness and composition by differentiating between spatial and temporal patterns in endophyte communities. Compared to historical climate, annual weather explained only half as much of the variation in endophyte community composition, despite having more than twice the number of variables included in the analysis. Other microbial communities can be similarly resistant to experimental changes in rainfall (Cruz-Martinez *et al*., 2009; Evans & Wallenstein, 2012). In our system, local endophyte communities likely reflect species sorting over time (Van der Gucht *et al*., 2007), with sufficient inertia to maintain largely similar communities across years even with up to 100-mm differences in spring rainfall. That inertia may be caused by limited

immigration or resuscitation, or may result from local factors not measured here that parallel historical climate.

Year-to-year variation in endophyte community composition was smaller than differences among sites and there was no year-to year variation in endophyte richness. The low temporal variation in endophyte communities is probably because variation in long-term historical rainfall among sites was 2.4x greater than variation in annual rainfall within sites. Similarly, annual high temperature differences were only slightly lower among sites (1.7°C) than among years (2.3°C). We sampled during a period of extended drought in central Texas: the Palmer Drought Severity Index for the sites included in this study ranged from -2.85 to -1.75 in 2012, -2.58 to -1.78 in 2013, and -2.51 to 1.89 in 2014. If drought stress was more alleviated in some years, we might expect to see a larger role for annual weather. In most other studies, spatial patterns in endophyte communities were related to climate (Zimmerman & Vitousek, 2012; Loro *et al*., 2012; Giauque & Hawkes, 2013). Even less temporal variation (<5%) has been found in other studies that tracked endophytes across multiple years (Del Olmo-Ruiz & Arnold, 2014; Higgins *et al*., 2014), perhaps because these were in tropical ecosystems where weather conditions vary little.

Surprisingly, plants played only a small role in endophyte communities, with plant size and cover explaining 5.7% of the variation in endophyte community composition. These patterns are on par with growing season differences in endophyte communities observed in other studies (Ghimire *et al*., 2010) and may reflect colonization patterns that differ slightly in larger plants or areas with more grass cover (Arnold & Herre, 2003; Ranelli *et al*., 2015). However, density of *P. hallii* had no effect on endophyte community composition, consistent with broad host generalism of horizontally-transmitted endophytes and widespread host availability. It is possible that plants play a larger role, but that we did not measure the specific plant traits to which endophytes respond. For instance, physiological traits such as photosynthetic rates may more directly affect endophytes by determining the amount of available carbon (Lam *et al*., 1994; Rasmussen *et al*., 2012).

Although climate factors explained the majority of variation in endophyte community composition, spatial effects cannot be entirely discounted. Previous studies have shown that endophyte community similarity decreases over large geographic distances (Arnold & Lutzoni, 2007) and the spatial structure of endophytes in tropical forest grasses was consistent with dispersal limitation (Higgins *et al*., 2014). Limited spatial effects in our study may be due to the gradient nature of the study system, where the primary climate axis varies directionally east to west in space and thus longitude and MAP were 95.8% correlated. Latitude, however, was not correlated with climate or weather, and this factor did not significantly explain any variation in community composition.

Compositional changes were at least partly concomitant with phylogenetic shifts in endophyte communities based on the comparison of Bray-Curtis and UniFrac distances. However, phylogenetic beta-diversity was less well explained overall (35.8 vs. 56.1%) and the emphasis shifted slightly away from climate factors and towards vegetation-climate interactions and spatial factors. In addition, no phylogenetic structure was observed. To our knowledge, other studies of endophyte distributions rarely examine phylogenetic beta-diversity (Oono *et al*., 2015) and have not compared taxonomic and phylogenetic beta-diversity. Nevertheless, our results are not surprising given the multiple phylogenetic origins of endophytic lifestyle (Massimo *et al*., 2015), sampling limited to one host plant, and isolation of taxa only in the Ascomycota.

Ecologically, more than 75% of fungal taxa in this study had been previously identified as endophytic, and many of the genera (e.g., *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium, Phoma*, *Preussia*) are common endophytes in other studies (Ghimire *et al*., 2010; Kleczewski *et al*., 2012; Massimo *et al*., 2015). Nevertheless, 100% of fungi in this study also were previously identified as saprobes or pathogens. The term endophyte is used to characterize fungi found inside plant tissue in the absence of disease, but their function in symbiosis can range from mutualistic to antagonistic and can include latent pathogens (Siegel *et al*., 1987; Carroll, 1988; Schulz & Boyle, 2005). Ultimately, a full understanding of endophytes will require determination of the contexts

that trigger specific fungal behavior in symbiosis, for example, as with stress in vertically transmitted endophytes (Eaton *et al*., 2011) and whether those are related to their distributions. The few studies to date are equivocal, with evidence for habitat-adapted function in some cases (Rodriguez *et al*., 2008), but not others (Giauque & Hawkes, 2013).

The results of this study have several additional limitations. The variables we measured explained 43.9% of the variance in endophyte community composition, but over half of the variation remained unexplained. Potential contributing factors that were not measured here include plant community composition and soil characteristics such as nutrients, organic matter, and texture. Given that endophytes putatively spend some portion of their life cycle in soil (Schulz & Boyle, 2005), soil properties may act as an important filter for local species pools. In addition, the variation of endophyte communities across the growing season may be much greater than interannual variation (Ghimire *et al*., 2010), such that we may have missed some important drivers of temporal variation. Use of a culture-based approach to characterize endophyte communities can also result in missing unculturable species (Arnold & Lutzoni, 2007). Finally, we selected isolates for sequencing based on morphology, which may mask some cryptic diversity; for instance, one out of 66 morphotypes was split into two taxa after ITS and LSU sequencing of replicate isolates. Although we identified >90% of the estimated culturable endophyte richness at every site, additional plant-level sampling and a DNA-based approach could provide greater resolution.

Climate was the primary driver of endophyte community diversity and composition in this study. The strong effects of historical climate and the relatively small amount of temporal variation may indicate that endophyte communities are resilient to interannual differences in precipitation and temperature, at least of the magnitude observed here. However, small amounts of species turnover did occur in response to annual weather conditions, suggesting that composition could shift more in response to long-term changes in water availability. A better understanding of current endophyte distributions and prediction of future distributions will require further study across

broader climatic regions, measures of both local and regional dispersal patterns, and integration across multiple host plant species.

Chapter 2: Fungal strategies driving outcome of plant-endophyte symbioses

Introduction

Horizontally-transmitted non-*Clavicipitaceous* fungal endophytes (hereafter 'endophytes') reside within the tissues of nearly all terrestrial plants and can mediate plant stress tolerance via effects on plant physiology and gene expression (Arnold & Engelbrecht, 2007; Torres *et al*., 2012; Czarnoleski *et al*., 2012). However, endophytic fungi can range from antagonistic to mutually beneficial (Carroll, 1988) making development of a predictive framework of symbiont outcomes key to both basic understanding and potential applications of endophytic fungi. Here we examine three paradigms for predicting the effects of endophytes on plant hosts: habitat adaptation (Rodriguez *et al*., 2009), evolutionary history (Doebeli & Knowlton, 1998), and ecological or physiological traits (Crowther *et al*., 2014).

Initially, the paradigm that the benefits conferred by fungal endophytes were habitat-adapted suggested that symbiosis outcomes would be a relatively simple function of environmental selection (Rodriguez *et al*., 2009). Habitat-adapted benefits from endophytes are well-documented from sites with extreme temperature and salinity (Rodriguez *et al*., 2008; Redman *et al*., 2011). Nevertheless, adaptation of horizontally transmitted endophytic fungi to the local environment may not translate into host effects of fungi found outside of high-stress habitats because selection can occur during both their free-living (Evans & Wallenstein, 2012) and symbiotic stages (Higgins *et al*., 2007). One potential example is that habitat origin along a steep rainfall gradient controlled endophyte beta diversity, but had no effect on endophyte-mediated drought responses of *Panicum virgatum* (Giauque & Hawkes, 2013).

An alternative to contemporary habitat adaptation is shared evolutionary history, where more closely related fungi behave more similarly in symbiosis than more distantly related fungi. Currently there is no direct evidence supporting a strong phylogenetic link to the outcome of plant-endophyte symbioses. Fungal lineages have shifted between endophytic and pathogenic lifestyles multiple times (Delaye *et al*., 2013) and some

endophytes are considered latent pathogens that switch strategy based on environmental conditions . Phylogenetic constraints have been observed in other fungal symbionts, such as arbuscular mycorrhizal fungi colonizing roots (LaJeunesse, 2001; Powell *et al*., 2009; Sachs *et al*., 2014). It is possible that detection in endophytes is limited by the lack of sufficient functional data for taxa across the fungal phylogeny.

Traits of individual species have long been used to predict the outcome of biotic interactions (Tilman & Haddi, 1992) and recently traits have been used to explain microbial life history, niche, and diversity patterns (Chagnon *et al*., 2013; Krause *et al*., 2014; Aguilar-Trigueros *et al*., 2014; Crowther *et al*., 2014; Winemiller *et al*., 2015). Traits can capture a combination of mechanisms that might not otherwise be detected, but are not necessarily mechanistic themselves. Traits may also reflect both phylogenetic relatedness and habitat adaptation (MacPherson *et al*., 2015; Liu *et al*., 2015), and thus multiple reviews have emphasized the potential value of analyzing all three simultaneously (Losos, 2008; Comas *et al*., 2010; Pavoine *et al*., 2010). However, most studies have separately examined either traits and habitat adaptation (Moeller *et al*., 2013) or traits and phylogenetic relatedness (Maaß *et al*., 2015) as predictors of function, making it difficult to assess the relative importance of each.

We tested the ability of habitat origin, phylogenetic history, and functional traits to serve as a predictive framework for the outcome of plant-endophyte symbioses using functional trait data for 35 endophyte taxa. The fungi were distributed across the Ascomycota phylogeny to allow for robust tests of phylogenetic relationships. To address habitat adaptation, we imposed two environments, low and high water, based on the habitats from which the fungi were isolated. To examine fungal traits, we characterized fungal resource use and stress tolerance that putatively reflect physiological pathways to affect the host plant. We also limited potential host effects by using a single C4 grass species (*Panicum virgatum*). We hypothesized that fungal traits would best predict symbiotic outcomes if those traits reflect fungal functioning in plant; alternatively, function would reflect either habitat or phylogeny if contemporary or historical selection were more important. We expected to find greater support for traits, because our prior

work with a smaller group of fungi did not support a relationship between the outcome of symbiosis and either fungal habitat origin or phylogeny (Giauque and Hawkes 2013).

Materials and Methods

Characterizing symbiosis outcomes for fungal endophytes

From 2011 to 2014, we isolated and identified fungal endophytes from leaf tissue of three native, C4 bunchgrasses (*Panicum hallii, Panicum virgatum,* and *Sorghastrum nutans*) from across central Texas (Giauque and Hawkes 2013, 2016). Briefly, we used both the ITS and LSU regions of rDNA to identify endophyte taxa because these capture both variable and conserved regions to allow for robust alignments. Sequences were trimmed, quality checked, and clustered into operational taxonomic units (OTUs) based on 97% sequence similarity (Giauque & Hawkes, 2016). We then selected 35 endophytes to study their effects on plants; the fungi were chosen to provide a phylogenetically broad range of taxa. Further details of endophyte collection and identification are available in Appendix 1.

To identify endophyte effects on plants, we grew sterile *P. virgatum* seedlings at 25% gravimetric soil moisture until all had reached a standard height (~4 cm) at which point each seedling was inoculated with one of the 35 fungal isolates or with no fungus (as a sterile control). After allowing 1 week of fungal colonization, we imposed water treatments and characterized plant growth under low- and high-water conditions (3% and 15% gravimetric soil moisture). There were a total of six trials to test all 35 taxa. Each treatment and control combination was replicated five times for a total of 410 plants. To estimate evaporative water losses, a further ten replicates without plants were included with five replicates for each moisture treatment in each trial. We measured whole-plant water loss, and plant height at three-day intervals (days 1, 4, 7, 10, 13, 16, 19). Additionally, plant wilting (number of wilt free days) and survival (days until first tiller death) were assessed daily, with tiller death defined by a complete lack of green tissue on one or more tillers. Whole-plant water loss was measured as the loss of water by weight from each planting box at each time point, adjusted for average water loss from plant-free controls during the same time period (Meurs & Stranghellini, 1992). The average rate of

water loss was calculated over time (g water d^{-1}). Growth rate was calculated based on the change in plant height over time (cm d^{-1}). On day 19, shoots and roots were harvested, dried, and weighed. Whole-plant transpiration efficiency (TE) was calculated as total plant biomass divided by total water loss (mg ml^{-1}).

Quantifying standardized fungal effects on plant moisture responses using log response ratios

The effects of endophyte and soil moisture treatments on each plant responses were quantified using the log-response ratio (LRR). Log-response ratios give a standardized measurement of change relative to a control and as such allow us to analyze data across multiple trials (Hedges *et al*., 1999). For all plant responses, we calculated the LRRs for both main effects and the interaction:

$$LRR_{symbiont} = \left(\ln \overline{Y}_{h,f} + \ln \overline{Y}_{l,f}\right) - \left(\ln \overline{Y}_{h,c} + \ln \overline{Y}_{l,c}\right)$$
$$LRR_{water} = \left(\ln \overline{Y}_{h,f} + \ln \overline{Y}_{h,c}\right) - \left(\ln \overline{Y}_{l,f} - \ln \overline{Y}_{l,c}\right)$$
$$LRR_{interaction} = \left(\ln \overline{Y}_{l,f} - \ln \overline{Y}_{l,c}\right) - \left(\ln \overline{Y}_{h,f} - \ln \overline{Y}_{h,c}\right)$$

where $\overline{Y}$ is the sample mean for l = low-water treatment, h = high-water treatment, f = fungus, and c = control. The interaction term is particularly useful because it quantifies the endophyte benefit/cost relative to control under stressed vs. non-stressed conditions. Therefore, a positive value of $LRR_{interaction}$ indicates that a plant received a greater benefit (or lesser detriment) from a fungal symbiont compared to fungus-free controls under low soil moisture compared to high soil moisture. When the $LRR_{interaction}$ was significant in statistical analysis, we further partitioned this effect size into the two component terms in parentheses to compare the contributions of fungal effects on plants in low and high water conditions (Hawkes & Sullivan, 2001). A single variance estimate was also calculated for each response:

$$v = \frac{s_{h,f}^2}{n_{h,f}\overline{Y}_{h,f}^2} + \frac{s_{l,f}^2}{n_{l,f}\overline{Y}_{l,f}^2} + \frac{s_{h,c}^2}{n_{h,c}\overline{Y}_{h,c}^2} + \frac{s_{l,c}^2}{n_{l,c}\overline{Y}_{l,c}^2}$$

where s is the standard deviation of the mean and n is the sample size for each treatment group (Hedges *et al*., 1999). We used v to calculate 95% confidence intervals for each measured plant response, both for the LRR terms and the interaction components. Additional information on effect size calculations is included in Appendix 1.

Phylogenetic tree inference

To generate the fungal phylogeny, we pruned untested fungal taxa from our previously published phylogeny (Giauque & Hawkes, 2016). Briefly, the phylogeny was built using LSU and ITS reads that were aligned separately using SATé v2.2.7 (Liu *et al*., 2011) and then combined into a single composite sequence for each tested isolate (James *et al*., 2006). The chosen representative sequence of each OTU was aligned to an internal guide tree of 112 known *Ascomycota* sequences spanning the ITS and LSU regions, again using SATé. Guide tree sequences were compiled from the Ribosomal Database Project (Liu *et al*., 2012) in addition to representatives of genera from the best BLAST matches.

Habitat origin characteristics

To test for habitat effects on plant-endophyte interactions, fungal sites of origin were characterized based on historical climate means as described in Giauque and Hawkes (2016). Briefly, we recorded mean annual (precipitation, maximum temperature, and minimum temperature based on the 30-year record from 1981 to 2010 (PRISM Climate Group, Oregon State University, http//prism.oregonstate.edu, created 15 August 2014). We also noted the C4 grass host species from which the fungi were isolated at each site (*Panicum hallii, Panicum virgatum*, *Sorghastrum nutans*).

Fungal functional trait characterization

To understand the role of fungal traits in the outcome of plant-endophyte interactions, we screened fungal traits in culture related to resource use and stress responses, which were chosen because of their potential role in plant growth and physiological responses to drought. Traits for fungal resource use were (1) fungal growth rate and biomass in liquid 1x M9 media (Miller, 1972) supplemented with 20% glucose, (2) substrate use profiles for 95 substrates using Biolog FF MicroPlates (Biolog, Hayward, California, USA), and (3) carbon-degrading enzyme activity for starch (α-1,4-glucosidase) and cellulose (β-1,4-glucosidase and cellobiohydrolase) using fluorometric methods (Saiya-Cork *et al*., 2002). Fungal stress response traits were (1) osmotic sensitivity calculated as the slope of fungal growth on a gradient of NaCl (0-12 g/L) in 1x M9 liquid media (Bell & Gonzalez, 2009), (2) osmotic threshold calculated as the

maximum concentration of NaCl at which the fungus could grow, and (3) production of the enzyme 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase that cleaves the ethylene precursor ACC using a colorimetric assay (Li *et al*., 2011). More detailed methods for trait measurements are included in Appendix 1.

Statistics

Plant responses to fungal colonization and drought

We used linear mixed effects models to examine each measured plant response as a function of fungus, water treatment, and their interaction, which were considered fixed effects; trial was also included as a random effect. Linear mixed effects models were run using the lmer function in the lme4 package of R (Bates *et al*., 2016) and *P*-values were calculated for fixed factors using Kenward-Roger's approximations and for random factors using likelihood ratio tests, as performed by the lmertest package of R (Kuznetsova *et al*., 2016). Plant responses were total biomass, growth rate, water loss, TE, wilt-free days, and days to first tiller death. We also calculated LRRs for each plant response as defined above; the LRRs were considered significant if their 95% confidence intervals did not overlap zero.

Delineating symbiosis outcomes

To identify symbiosis outcomes while accounting for phylogenetic relatedness, we used phylogenetic principle component analysis (PPCA) to reduce the LRRs for multiple plant responses (biomass, growth rate, physiology, survival) to a small number of derived variables that summarize the original information (Lepš & Šmilauer, 2003). This allows us to identify groups of fungi with similar overall effects on the plant. The PPCAs were performed separately for each LRR main effect and their interaction term, and were run using the phyl.pca function in the phytools package in R (Revell, 2015).

To identify groups of fungi with similar symbiosis outcomes under drought, we used K-means clustering (Hartigan & Wong, 1979) on PPCA scores for the LRR interaction term using the kmeans function in the stats package in R (R Core Team, 2015). K-means clustering identifies significant grouping by dividing *X* points occurring in *N* dimensions into *K* clusters to minimize the sum of squares, meaning that movement

of a point from one cluster to another will not reduce the within-cluster sum of squares. Clusters or groups were considered significantly different if 95% confidence ellipses did not overlap; these unique clusters are interpreted as different symbiosis outcomes. We focused on the $LRR_{interaction}$ term to focus on the net effect of the fungus under low water conditions relative to high water.

To characterize the relationship between the symbiosis outcome clusters and underlying plant responses, we used multinomial logistic regression with the multinom function in the nnet package in R (Ripley & Venables, 2016). Each PPCA cluster was analyzed as a function of the plant LRRs (total biomass, growth rate, water loss, TE, wilt-free days, days to first tiller death). Because plant responses and clusters are not independent, we could not identify which plant responses differed by cluster using an ANOVA-based approach. Instead, multinomial logistic regressions predicted the most likely symbiotic outcome for any plant-fungal pairing based on measured plant responses. For further visualization, we calculated eigenvalues for measured plant responses using the envfit function in the Vegan package (Oksanen *et al*., 2015) and superimposed these on the PPCA as vectors.

Identifying the drivers of symbiotic outcomes

To determine the role of fungal traits, habitat origin, and phylogeny in driving symbiotic outcomes, we used four approaches: (1) we examined whether plant responses to fungal symbionts were phylogenetically conserved, (2) we tested fungal traits and habitat origin as a function of the categorical symbiotic outcome groups identified in the PPCA, (3) we used phylogenetic regression to examine the linear relationships between fungal traits, habitat origin characteristics, and the LRR effect sizes of symbiotic outcomes while controlling for phylogeny, and (4) we partitioned the variance in LRRs due to fungal traits, habitat, and phylogenetic relatedness. Each of these is described in more detail below. All analyses were performed for both main effects and interaction terms. Going forward, whenever an analysis produced a significant result based on the interaction term, that analysis was also performed on both components of the interaction term in order to parse out the effect of the low water and high water treatments.

To test for phylogenetic conservation of fungal effects on the plant host, we calculated Bloomberg's K and Pagel's λ for each plant response trait (biomass, growth rate, water loss, TE, wilt-free days, days to tiller death and tiller survival) and for the composite plant responses represented by the PPCA axes (Münkemüller *et al*., 2012). These indices were computed using the phylosig function of the phytools package in R (Revell, 2015).

Fungal traits and habitat origin were tested as a function of the identified PPCA symbiosis outcome clusters using MANOVA. When the MANOVA was significant, we analyzed individual fungal traits using ANOVAs. When fungal traits or habitat origin characteristics differed significantly among symbiosis outcome groups, posthocs were performed with Tukey tests. We used a Bonferroni-corrected $\alpha < 0.004$ for multiple comparisons.

To better understand the relationships between the LRR effect sizes of each individual plant response, fungal traits, and habitat characteristics while controlling for phylogeny, we used phylogenetic generalized least squares (PGLS) (Orme *et al*., 2015). Bonferroni corrections were used for multiple comparisons ($\alpha < 0.007$). Independent trait variables were only included that were not significantly correlated (< 90%) and did not display multicollinearity based on variance inflation factors using the vif function in the car R package (Fox *et al*., 2015). Linear models consistently produced the highest R^2 compared to tested non-linear methods (log-transformed and Pareto optimality; data not shown).

Finally, to examine the relative contributions of habitat origin, phylogeny, and fungal traits on symbiosis outcomes, we partitioned the variation in plant responses using the varpart function in Vegan with Euclidean distances (Oksanen *et al*., 2015). The previous analyses (MANOVA, linear regressions) merely accounted for phylogeny while determining the relationship between fungal traits, habitat origin characteristics, and plant responses. Variance partitioning quantifies the relative contributions of each factor and their interactions (Borcard *et al*., 1992). The plant response matrices of each set of LRRs (main effects and interaction term) were analyzed individually as a function of fungal

phylogenetic dissimilarity using UniFrac distances, fungal traits, habitat origin characteristics, and their interactions.

Results

Plant responses to fungal colonization and drought

Overall, fungal colonization increased plant biomass, decreased plant water loss, and increased plant TE (Table 2.1, Figure 2.1). Drought stress decreased biomass, growth rate, water loss, wilt-free days, and days to tiller death while increasing TE. The interaction of fungal colonization and drought stress increased biomass and TE while decreasing water loss (Figure 2.1). However, individual fungal isolates varied widely in their effect on the plant (Figure 2.2). Based on PPCA, the effects of fungal symbionts and the interaction of fungus and water treatments were related to their effects on plant TE vs. water loss, as well as plant survival (Figure 2.3, 2.4).

Symbiosis outcomes

Based on k-means clustering of PPCA scores for $LRR_{interaction}$ (Figure 2.3), there were four distinct groups of symbiotic outcomes (I-IV) that differed based on plant responses (Table 2.2). Group I ('Water-Use Mutualists') decreased total plant water loss but increased plant TE (Table 2.2). Group II ('Survival Mutualists') increased survival by delaying the first tiller death via more and less efficient water use (Table 2.2). In contrast, Group III ('Survival Antagonists') induced faster plant tiller death and increased TE without reducing water loss meaning plants grew more despite the stress (Table 2.2). Group IV ('Mixed Mutualist') induced plant responses that combined the positive aspects of both previous mutualist clusters but with smaller effect sizes: plants had decreased water loss and increased TE at less than 25% of that found for Group I fungi and delayed tiller death at 44% of the effect of Group II fungi (Table 2.2). The switch from water use mutualist to survival mutualist to antagonist is clear in the multinomial regressions, where the probability of one strategy drops to zero as the probability of another strategy increases (Figure 2.5A-F). The three mutualist strategies are each characterized by different probability profiles across the plant response traits that correspond to their definitions. For example, Group I water-use mutualists were associated with low plant

water loss, high TE, fast wilting, intermediate biomass, low growth rate, and intermediate survival. In contrast, Group II survival mutualists were associated with high plant survival, high water loss, low TE, and low biomass, with little differentiation based on plant growth rate or wilting. Group IV mutualists were associated with slightly increased survival and TE, slow wilting, decreased growth, and little differentiation based on biomass and water loss (Figure 2.5A-F). These patterns were identified based on the $LRR_{interaction}$ term, but closely mirror symbiont outcomes under low water ($LRR_{low\ water}$) conditions (Figure 2.6A-F), demonstrating that fungal effects are greater under drought than well-watered conditions.

Phylogenetic conservation of fungal effects on the plant host

Fungal phylogenetic history did not predict any plant responses to the treatments (Figure 2.7). Specifically, all measured plant responses had Blomberg's K values < 0.5 and Pagel's λ values < 1 with *P*-values > 0.05 (Table 2.3).

Fungal traits and habitat origin as a function of symbiotic outcomes

Fungal traits (fungal osmotic stress responses, growth, resource use, cellulase production, ACC deaminase production) were not phylogenetically conserved (Figure 2.8), but differed significantly among fungal taxa (Figure 2.9). Based on the MANOVAs, fungal traits and habitat origin characteristics differed by symbiont outcomes only when considering the $LRR_{interaction}$ term and the component $LRR_{low\text{-}water}$ terms (Table 2.4). However, in posthoc tests no measured individual fungal traits or habitat origin characteristics differed among symbiotic outcomes clusters under any LRR term (Table 2.5).

Phylogenetic regression between fungal/habitat traits and individual plant responses

Based on correlations between fungal traits/habitat and PPCA axes, fungal traits were a better predictor of symbiosis outcomes than characteristics of the original habitat (Table 2.6). Fungal traits related to osmotic stress tolerance significantly predicted symbiotic outcome, with osmotic sensitivity explaining more than a third ($r^2 = 0.379$) of the effect of fungal treatment on plant responses ($LRR_{symbiont}$). Similarly, plant response to water treatment (LRR_{water}) was explained by fungal osmotic sensitivity and osmotic

threshold (r^2 = 0.764). The overall effect of fungal colonization under low water relative to high water ($LRR_{interaction}$) was not significantly explained by any fungal trait or habitat origin, although there were trends related to fungal osmotic sensitivity (P = 0.005), osmotic threshold (P = 0.009), and CBH (P = 0.004).

Based on phylogenetic regression, fungal traits and habitat origin characteristics explained 17.8 to 49.2% of specific plant responses (Table 2.7, 2.8). Fungal carboxylic acid use was the only predictor of plant $LRR_{interaction\text{-}TE}$ (r^2 = 0.263), $LRR_{interaction\text{-}wilt}$ (r^2 = 0.226), and $LRR_{water\text{-}wilt}$ (r^2 = 0.300), and contributed to $LRR_{symbiont\text{-}wilt}$ with fungal amine use (r^2 = 0.290); in all cases increasing use of carboxylic acids led to declining plant responses. Fungal CBH activity predicted the plant biomass response for both $LRR_{symbiont}$ (r^2 = 0.221) and $LRR_{interaction}$ (r^2 = 0.239), but the relationship was negative in the former and positive in the latter reflecting a dependence on water treatment. Fungal osmotic sensitivity and fungal biomass were both positively related to tiller death for both $LRR_{symbiont}$ (r^2 = 0.467) and LRR_{water} (r^2 = 0.492). Fungal osmotic threshold explained nearly half of the variation in $LRR_{interaction\text{-}growth\ rate}$ (r^2 = 0.483), with less tolerant fungi causing slower growth. Mean annual precipitation was the only habitat origin characteristic related to any plant responses, with a negative relationship between MAP and $LRR_{water\text{-}biomass}$ (r^2 = 0.178), as well as a negative relationship between both MAP and CBH and $LRR_{water\text{-}growth\ rate}$ (r^2 = 0.363). When considering the breakdown of $LRR_{interaction}$ into its low and high water components, fungal trait relationships were typically driven by one condition and not the other (Table 2.8). For example, plant wilt was only related to fungal carboxylic acid use in the low water treatment (r^2 = 0.363); in high water conditions fungal osmotic sensitivity predicted wilt (r^2 = 0.197). In other cases, the trait relationship changed direction, such as for CBH and total plant biomass, which were positively related in the overall $LRR_{interaction}$, but negatively related in $LRR_{high\ water}$. Fungal traits and habitat origin characteristics were not significantly related to plant water loss, except when considering only $LRR_{high\ water}$, where carboxylic acid use was positively related to water loss (r^2 = 0.255).

Variance in LRRs due to fungal traits, habitat, and phylogenetic relatedness

Altogether, fungal phylogeny, traits, and habitat origin characteristics explained 56.0% of the variation in $LRR_{symbiont}$ (Figure 2.10), reflecting contributions of fungal traits (6%) and fungal trait interactions with phylogenetic dissimilarity (13%) and habitat origin (6%). LRR_{water} was driven entirely by the two-way intersections between fungal traits, habitat origin characteristics, and phylogeny (r^2= 0.38; Figure 2.10). For $LRR_{interaction,}$ fungal traits were the most important predictor of the outcome of symbioses (r^2= 0.15; Figure 2.10).

Discussion

We identified a continuum of four symbiont strategies that describe the outcome of plant-endophyte interactions under drought. Essentially, the fungi were plant mutualists (Groups I, II, IV) or antagonists (Group III). The mutualist strategies improved plant drought resistance through several different mechanisms, consistent with what we know from previous work on microbial symbionts (Worchel *et al*., 2012). Group I fungi enhanced plant drought resistance by doubling plant TE under drought, whereas Group II fungi increased plant survival by 20% while having no effect on plant water use. Group IV fungi combined both of these strategies, increasing plant TE by 54% and survival by 11%. In contrast, antagonist fungi (Group III) increased plant growth without substantially affecting water loss, resulting in 9% lower survival relative to sterile controls and 33% lower survival under drought compared to well-watered conditions.

Despite the identification of a range of mutualist to antagonist strategies based on the interaction of fungus and water treatments, fungal benefits are likely to be somewhat plastic. Here we observed context-dependence in how plants responded to fungi under dry vs. well-watered conditions, with endophytes demonstrating a range of mutualist and antagonistic strategies under dry conditions but only providing weak benefits under well-watered conditions. In other systems, plant-fungal symbioses also vary depending on abiotic conditions (Chamberlain *et al*., 2014) as well as factors such as plant life history (Heschel & Riginos, 2005). This can make development of a predictive framework difficult, which is clear when we consider that only 31% of the overall variation in plant

responses to the interaction of fungus and water treatments was predicted by measured fungal traits, habitat characteristics, and phylogeny.

Nevertheless, fungal traits in culture were the most successful predictor of fungal effects on the plant host under all conditions. Traits related to osmotic stress tolerance and resource use explained up to 50% of individual plant responses, 6% of the overall plant response to fungal treatment, and 15% of the overall plant response to the combination of fungus and water treatments (data not shown). However, these relationships were only detectable when considering the data continuously, rather than as categorical strategies, suggesting that discrete assignments of symbiont outcomes may not be useful in a predictive framework. Moreover, trait interactions with habitat origin and phylogeny were equally or more important than traits alone. The two- and three-way interactions among traits, phylogeny, and habitat, based on variance partitioning, explained 24% of the variation in plant responses to fungal colonization, 38% of the variation in plant responses to water treatment, and 16% of the interaction between fungus and water treatments. These results provide support for both habitat adaptation (Moeller *et al*., 2013; Maaß *et al*., 2015) and phylogenetic relatedness (Lugo *et al*., 2014; Amend *et al*., 2016) in predicting fungal function, but these factors were insufficient to independently predict symbiotic outcomes in the plants.

Symbiont resource use, as reflected by enzyme production, substrate use, and biomass, has long been thought to play a role in determining the outcome of plant-fungus interactions (Delaye *et al*., 2013; Aguilar-Trigueros *et al*., 2014). Unsurprisingly, plant biomass ($LRR_{symbiont\text{-}biomass}$) decreased with increasing fungal production of a cellulose-degrading enzyme (Table 2.7), a trait shared with many pathogens (Acosta-Rodríguez *et al*., 2005). Directly relating fungal substrate use in culture to plant responses is more complex. Fungal isolates that grew better on carboxylic acid compounds were associated with 30% faster plant wilting ($LRR_{symbiont\text{-}days\ to\ first\ wilt}$). At least one carboxylic acid-containing compound is a suspected mechanism of endophyte-mediated drought tolerance: 1-aminocyclopropane-1-carboxylic acid (Schäfer *et al*., 2009; Zuccaro *et al*., 2011; Khatabi *et al*., 2012). Thus, taxa that disproportionately utilize carboxylic acids

may adversely affect the plants ability to moderate its own drought responses. However, increased fungal biomass in culture was correlated with increased plant survival, suggesting that higher fungal resource use does not necessarily equate to adverse effects on the plant host. One potential explanation is that fungi that grow more quickly on nutrient rich media rely more on passive uptake of nutrients, rather than extracellular enzymes, as is characteristic of many mutualist fungi (Chibucos & Tyler, 2009).

Fungi are known for their ability to tolerate desiccation and often dominate soil microbial communities in drier ecosystems (Harris, 1981; Gordon *et al*., 2008; Barnard *et al*., 2013). Here, fungi that better tolerated osmotic stress were associated with increased plant tolerance to drought. The fungi may produce osmolytes in response to drought stress, many of which may be compatible with the host plant effort to maintain cell turgor (Yancey, 2005; Seki *et al*., 2007; Hamilton & Bauerle, 2012). Alternatively, fungi produce antioxidants in response to desiccation (Gorbushina *et al*., 2008; Sterflinger *et al*., 2012), which is consistent with previous work identifying antioxidant effects on plant reactive oxygen species concentrations as a primary mechanism of endophyte-mediated drought tolerance (Rodriguez *et al*., 2008; Sherameti *et al*., 2008). In addition, we expected that traits such as growth and osmotic stress tolerance would trade-off (Grime, 1977; Chagnon *et al*., 2013), but found no such tradeoff. In fact, there was a weak positive relationship between fungal biomass and osmotic stress tolerance (data not shown).

Independently, fungal habitat characteristics explained very little of plant responses to fungal colonization regardless of water treatment. Mean annual precipitation was the only measured habitat characteristic that was related to any plant response, predicting ~30% of changes in plant growth rate due to water treatment. Some fungal taxa isolated from drier climates may be adapted to local conditions, such that their effects on plant growth are optimized for the lower soil moistures from which they were isolated (Conover *et al*., 2009). Although the distributions of endophytic fungal taxa can reflect environmental filtering based on rainfall history (Zimmerman & Vitousek, 2012; Giauque & Hawkes, 2016), such environmental selection does not directly translate to the

outcome of plant-fungal symbiosis. However, conspecific endophytes differentially affected plants across geographically distant regions (Antunes *et al*., 2010), suggesting that selection for functional divergence may require more divergent environments. Rainfall in the current study may be a weak habitat filter or there may be a disconnect between the filter and function in symbiosis. Alternatively, habitat-adapted benefits of endophytes may be unlikely outside of high stress environments (Giauque & Hawkes, 2013).

Phylogeny had even less predictive power for plant responses, given that neither symbiotic outcomes nor fungal traits were phylogenetically conserved. Although phylogenetic conservation of traits in plants and animals is well-documented (Blomberg *et al*., 2003; Fritz & Purvis, 2010; Rafferty & Ives, 2013; Erickson *et al*., 2014), examining phylogenetic conservation of traits in microbes has been limited by the lack of trait data for many unculturable taxa. Additionally, measured traits and symbiotic outcomes could in reality be combinations of many physiological traits (Wainwright, 2007), encoded by suites of genes that are not tightly linked and less likely to undergo phylogenetically-patterned selection. Similarly, analysis of functional traits within fungal genomes found that fungal morphology was a stronger predictor of traits than phylogenetic relatedness (Treseder & Lennon, 2015). However, other studies analyzing as few as 27 fungal taxa in a single order (Powell *et al*., 2009) and as many as 1000 taxa (Amend *et al*., 2016) have detected conservation of fungal traits related to symbiont effects on their plant host and to ecosystem functioning. We did not directly detect a relationship between fungal phylogeny and traits for our 35 taxa in 5 orders and 19 families, probably because of the interactions among drivers.

Our predictive framework has several limitations. First, symbionts can switch between pathogenic and mutualist strategies on relatively short time scales (Sachs *et al*., 2011; 2014) and we do not know the full spectrum of function in each of the fungi tested here. Second, studying fungi in isolation is clearly an oversimplification of plant-fungal interactions when any given plant might be colonized by 10-30 endophytic fungal taxa (Ghimire *et al*., 2010; Márquez *et al*., 2012; Giauque & Hawkes, 2016). Finally, we do

not fully understand the mechanistic relationship between fungal traits in culture and their physiological impact on the plant. Traits in culture do not necessarily reflect what is happening in the plant (Fajardo López *et al*., 2008) and attempting to directly relate the two may result in spurious correlations without any biological mechanism.

By demonstrating that easily screened fungal traits can be strongly predictive of their function in the plant, we developed the beginnings of a framework that could eventually be used to predict plant-microbial relationships across a wide array of symbioses. Fungal traits were by far the most predictive, explaining nearly half of the variation in some individual plant responses. However, based on the interactions observed among predictor variables, a robust mechanistic understanding of endophyte-mediated drought will require a three-pronged approach incorporating fungal traits, habitat origin, and phylogenetic history.

Chapter 3: Using host transcriptomes to demonstrate the mechanism of endophyte-mediated drought tolerance

Introduction

Because water availability is a primary controller of plant growth and survival (Knapp & Smith, 2001; Laurenroth & OE, 2003), the ability of plants to resist drought can have large impacts on primary production, diversity, and distributions (Tilman & Haddi, 1992; Knapp *et al*., 2002; Archaux & Wolters, 2006; Craine *et al*., 2012). Plant drought resistance is likely to become even more critical given future climate predictions for widespread increases in drought frequency and intensity (Seager *et al*., 2007; Pachauri *et al*., 2014). Horizontally-transmitted fungal endophytes (hereafter 'endophytes') live within the tissues of a wide range of terrestrial plants and can alter plant physiology and productivity (Kleczewski *et al*., 2012), particularly in stressful environments (Arnold and Engelbrecht 2007, Torres et al. 2012, Czarnoleski et al. 2012). Endophytes represent a potential novel pathway for plant drought management, but progress in this area will require an understanding the mechanisms underlying endophyte-mediated drought resistance.

Endophyte-mediated drought tolerance has been demonstrated in multiple species, including tomatoes (Rodriguez *et al*., 2008; Azad & Kaminskyj, 2015), C3 grasses (Hubbard *et al*., 2013; Afkhami *et al*., 2014), and C4 grasses (Afkhami *et al*., 2014). In general, drought-stressed host plants colonized by mutualistic endophytes have increased biomass production, reduced stomatal conductance, and decreased whole-plant water loss relative to plants without endophytes (Elmi and West, 1995; Kannadan and Rudgers, 2008; Rodriguez et al., 2008; Kane, 2011). Not all endophytes are mutualists, however, and colonization by some endophytes results in reduced biomass, increased rate of leaf water loss, and decreased survival (Kleczewski *et al*., 2012). Although fungal effects on plant production and physiology are somewhat well-characterized, the genetic mechanisms regulating these effects remain largely undiscovered.

In many host plant species, endophyte colonization of stressed plants affects specific suites of plant genes putatively associated with observed changes in plant

growth, physiology, and survival. Examples in response to drought stress include abscisic acid synthesis genes in *Gingko biloba* (Hao *et al*., 2009), nine genes associated with drought stress in *Arabidopsis thaliana*, including dehydration signaling and drought response genes (Sherameti *et al*., 2008), and 19 drought-responsive ESTs in *Theobroma cacao* including rubisco small subunit, cellulose synthase, and pathogen response genes (Bae *et al*., 2009). Additionally, fungal colonization frequently triggers plant stress responses, leaving the plant primed to deal with environmental stress (Ren & Clay, 2009). However, little is known about how foliar fungal endophytes affect gene expression in warm-season grasses. Further potential genetic mechanisms of endophyte mediated drought tolerance are included in Table 3.1. If changes in plant gene expression can be correlated with whether a fungus acts as a mutualist or an antagonist (Redman *et al*., 1999; Eaton *et al*., 2011), then the two groups likely have unique mechanisms for interacting with the plant host.

Identification of differentially expressed genes that drive symbiotic interactions could allow selection of symbionts with specific functions or alteration of symbiotic interactions through gene targeting. Based on the plant-fungal trials discussed in Chapter 2, we selected six fungal endophytes with a range of positive to negative effects on plant survival, water loss, and growth under drought (Table 1.5). The six fungi were paired with *Panicum hallii* plants and used to identify potential genetic mechanisms of endophyte-mediated drought tolerance. We predicted that genes involved in plant growth and stress responses would be differentially expressed based on the identity of the fungal symbiont. We focused on: (1) growth genes involved in photosynthesis, abscisic acid production, and auxin production and (2) stress response genes involved in ethylene production, osmolyte secretion, and ROS concentrations, as well as general drought and pathogen response genes (Table 3.1). Specifically, we predicted that plants colonized by beneficial fungi would upregulate plant growth and stress response genes under drought as a way of mitigating drought stress. We predicted that antagonistic fungi would act as another stress on the plant, triggering expression of pathogen response genes regardless of water status.

Methods

Plant and fungal material

Our study focused on an inbred line of *P. hallii* var. *hallii* (HAL2), initially derived from a natural collection made at the Lady Bird Johnson Wildflower Center (Austin, TX; 30.19° N, 97.87° W) as described in Lowry et al. (2015). Although our previous work focused on fungal effects on drought responses in *P. virgatum* (Giauque & Hawkes, 2013), we used the diploid *P. hallii* here because it is a simpler genetic model (Meyer *et al*., 2012) than tetra- or octoploid *P. virgatum*. *Panicum hallii* seeds were surface sterilized in 70% ethanol for 90s and 0.5% bleach for 60s. Seeds were placed on damp, sterile filter paper and allowed to germinate before being transferred to a pot containing a 50:50 mixture of sterile sand and soil. Seedlings were planted in June 2014 and were immediately transferred to a sterile chamber in a greenhouse at ambient light and temperatures ranging from 27 to 35°C (day vs. night). Plants were maintained at a constant gravimetric soil moisture (15%), which was assessed every 3 days. Pot positions were randomized every 3 days to minimize the effects of greenhouse heterogeneity. After 16 weeks, plants had reached adult size and were transferred to a growth chamber for plant-fungal drought assays.

Fungal isolates were chosen based on their effect on *P. virgatum* seedlings under drought, as described in Chapter 2. Isolates were chosen to include fungi that benefited the plant under drought (Isolates 22, 44, 61) and fungi that had an adverse effect on plants under drought (Isolates 5, 18, and 47). More information about each isolate is available in Table 1.5. All fungal taxa were isolated from samples collected along a 400-km precipitation gradient in central Texas, as described in Giauque & Hawkes (2013). To inoculate the plants with target endophytes, the fungi were cultured in liquid potato dextrose agar and diluted in sterile water as needed to obtain 10^5 hyphal fragments ml^{-1}. Each plant received 1 ml of inoculum by pipetting directly onto at least half of adult tillers (Rodriguez *et al*., 2008). Control plants were mock-inoculated with the same volume of sterile water. We allowed 1 week for fungi to colonize the plant (Rodriguez *et al*., 2008), after which we imposed two moisture treatments (low and high). For the low

treatment, half the pots were allowed to dry down to 5% moisture; the remaining pots were kept at 25% moisture. These soil moisture treatments were selected based on previous work which found that 5% soil moisture was sufficient to induce drought stress, decreasing plant growth by up to 50% (Giauque & Hawkes, 2013). After 21 days, 1 tiller from each of the three replicates from each treatment was harvested for RNA-seq. Another three replicates were allowed to grow for another 21 days prior to harvesting for measuring fungal colonization rates.

RNA isolation, RNA-seq library construction and sequencing of plant and fungal samples

Tissue samples were ground in liquid nitrogen using sterile, RNAse-Zap treated beads (Ambion, Austin, TX). Isolation of RNA from plant tissues was performed using a TRIzol (Life Technologies, Carlsbad, CA), chloroform, and isoamyl alcohol extraction (Rio *et al*., 2010). The integrity of extracted RNA was analyzed by resolving 1 μg RNA in a 1%-formaldehyde denaturing agarose gel. RNA-seq library samples were prepared using a modified version of the TAG-seq protocol (Meyer *et al*., 2011), briefly described here. An aliquot of 10 μg total RNA was fragmented to the desired size range (200-500bp) by incubating at 95°C for 8 minutes, and the fragment size was confirmed using a 2% agarose gel. The remaining 9 μl were incubated with 1 μl of 10 μM oligonucleotide 3ILL-30TV (5' ACGTGTGCTCTTCCGATCTAATTTTTTTTTTTTTTTTTTTTTTTTTTV 3') at 65°C for 3 min. Each 20 μl reverse-transcription reaction contained 10 μl of the previous RNA and 3ILL-30TV reaction as well as 1 μl H_2O, 1 μl 10mM dNTPs, 2 μl 0.1M DTT, 4 μl 5X first-strand buffer (Invitrogen, Carlsbad, California, USA), 1 μl 10 μM S-ILL-swMW (5' ACCCCATGGGGCTACACGACGCTCTTCCGATCTNNMWGGG 3'), and 1 μl SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA). The reaction was then incubated for 1 hour at 42°C and then at 65°C for 5 min to inactivate the reverse transcription.

To amplify the newly synthesized cDNA, we prepared the following reaction: 30 μl template, 46 μl H_2O, 10 μl 2.5 mM dNTPs, 10 μl 10X PCR buffer (Clontech, Palo Alto, CA, USA), 10 μM 5ILL oligonucleotide (5'CTACACGACGCTCTTCCGATC T

3'), 10 μM 3ILL-30TV oligonucleotide, and 2 μl of the Titanium Taq polymerase (Clontech, Palo Alto, CA, USA). The following thermocycler reactions were used to amplify the cDNA: 1 cycle of 5 min at 95°C; 15 cycles of 40 sec at 95°C, 1 min at 63°C, and 1 min at 72°C. Amplification was confirmed on a 2% agarose gel. To purify the cDNA, we used the NucleoFast PCR Clean-up protocol (Macherey-Nagel, Germany), according to the manufacturer's instructions.

We diluted the purified cDNA to 5 ng/μl and prepared the following reaction for adaptor extension: 10 μl purified cDNA, 1 μl barcode oligonucleotide, 27 μl H_2O, 5 μl 2.5 mM dNTPs, 5 μl 10x PCR buffer (Clontech, Palo Alto, CA, USA), 1 μl 10 μM TruSeq Mpx oligo (5' AATGATACGGCGACCACCGAAAAATACACTCTTTC CCTACACGACGCTCTTCCGAT 3'), and 1 μl Titanium Taq polymerase (Clontech, Palo Alto, CA, USA). The following thermocylcer conditions were used to extend the adaptors: 1 cycle of 5 min at 95°C; 4 cycles of 40 sec at 95°C, 1 min at 63°C, and 1 min at 72°C. To select the desired fragment sizes (350-450 bp), we loaded the entire sample (50 μl) to a 2% agarose gel and cut out the desired portion of the cDNA smear with a razor blade. We extracted the cDNA from the gel slice using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, California, USA), according to manufacturer's instructions. Forty prepared, barcoded libraries were quantified using Qubit (Invitrogen, Carlsbad, California, USA) and split into two pools. The Genome Sequencing and Analysis Facility at the University of Texas at Austin loaded each pool onto a single lane of an Illumina HiSeq 2500 analyzer, generating 101 bp fragments.

Bioinformatic analysis of RNA-seq data

Sequencing returned between 8.2 x 10^6 and 6.1 x 10^7 reads per sample (Table 3.2). Due to insufficient reads (< 1 x 10^6 reads in 2 of 6 samples), Isolate 22 was discarded. Statistics for each remaining sample were obtained using FastQC software (version 0.9.2) (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Sequences were trimmed (90 bp) and quality-filtered with a threshold of a Phred quality score of 30 using

the FASTX Toolkit v.0.013 (http://hannonlab.cshl.edu/fastx-toolkit). Sequences were aligned to the *Panicum hallii* var FIL2 reference currently available through the DOE Joint Genome Institute (http://phytozome.jgi.doe.gov/) using BWA-MEM (version 0.7.5) (Li & Durbin, 2010) and aligned reads were counted using HT-seq count in union mode (Anders *et al*., 2015).

Assembled transcripts were annotated with gene names based on BLASTX comparisons with the UniProt database (version 2016_02; e-value ≤ 10^{-4}), and with Gene Ontology (GO) terms based on GO annotation of UniProt records (www.geneontology.org). Additionally, because only 41.6% of transcripts were assigned a GO term, we also used the best *Arabidopsis thaliana* (Arabi-defline) gene match (http://phytozome.jgi.doe.gov/), assigned using blast+ 2.2.26 (Camacho *et al*., 2009). This allowed us to assign function to an additional 9,407 transcripts (25.3%).

Statistics

Univariate ANOVAs were used to examine whole-plant biomass, height growth rate, rate of plant water loss, days to first wilt, and days to first death as a function of soil moisture treatment (low, high), endophyte colonization (Control, Isolates 5, 18, 44, 47, 61), and their interaction. Soil moisture treatment was a fixed factor while endophyte identity was included as a random factor. The significance cutoff was set at $\alpha = 0.001$ based on Bonferroni correction. When fixed factors were significant, posthoc Tukey tests were used to examine differences among endophyte taxa.

Differential expression was inferred by analyzing raw counts using the DESeq2 package and raw counts were normalized across all samples using the DESeq2 (Love *et al*., 2014) in R (R Core Team, 2015), which infers differential expression after accounting for library size variation and mean-variance structure. We examined differences in individual gene expression based on fungal identity, water treatment, and their interaction. We corrected *P*-values based on the false discovery rate (FDR) using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). To evaluate transcriptional responses of individual genes to fungal colonization in the context of drought stress, we compared the log2-fold change in gene expression (fungus relative to

sterile control) among treatments. Differences in gene expression were visualized using volcano plots and euler diagrams generated in eulerAPE v3 (Micallef & Rodgers, 2014).

To integrate the overall effects of fungus and water treatments on plant gene expression, we used a principal components analysis (PCA) as implemented using the prcomp function in the stats package of R (R Core Team, 2015). We used multiple linear regression of each plant response against individual PCA axes to relate overall gene expression patterns to plant physiology, performed using the lm function in the stats package of R (R Core Team). Linear models consistently produced the highest r^2 compared to tested non-linear methods (log-transformed and Pareto optimality; data not shown). We set the significance cutoff at $\alpha = 0.01$ using a Bonferroni correction to account for multiple comparisons.

To examine the details of gene expression responses to fungus and water treatments we focused on (1) target genes based on previous work and (2) non-target genes with no prior support. Target genes were based on known effects of fungal endophytes on specific plant physiological pathways (Table 3.1) and included only genes that were differentially expressed based on fungal colonization vs. fungus-free controls. Non-target genes consisted of genes with either no known function, no known effect on plant growth or stress responses, or genes that were not differentially expressed based on fungal colonization. We focused on target genes known to be involved in regulation of plant growth (abscisic acid, photosynthesis, and more) and responses to osmotic, heat, desiccation, and pathogen stresses (ethylene, osmolytes, antioxidants, and more), as well as genes identified as critical to *P. hallii* drought responses based on transcriptome analysis (dehydrin, aquaporin, and LEA-family proteins as particularly critical to plant drought responses; Lovell *et al.*, in review). Target genes were annotated as described above and results were visualized using heatmaps. We used linear regressions to test the relationship between the expression of differentially expressed target genes and plant responses, based on regularized logarithm transformed expression data (Love *et al.*, 2014) and whole-plant biomass, height growth rate, rate of plant water loss, days to first

wilt, and days to first tiller death. The significance cutoff was set at $\alpha = 0.01$ using a Bonferroni correction to account for multiple comparisons.

To identify relationships between non-target genes and plant responses, we used the maximal information coefficient (MIC) as implemented in the Minerva package in R (Albanese *et al*., 2012). Maximal information coefficient is a type of maximal information-based nonparametric exploration (MINE) statistic that captures a wide range of associations between gene expression and physiology and produces a score that roughly equals the coefficient of determination ($r2$) of the data relative to the regression function (Reshef *et al*., 2011). Significance of these relationships was evaluated using pre-computed *P*-values from MINE (Reshef *et al*., 2011), which were FDR-corrected using the Benjamini-Hochberg procedure as implemented using the p.adjust function in the stats package of R (R Core Team). Nonlinear relationships (Pearson's $|r| < 0.8$) were visualized using local polynomial regression as implemented using the locpoly function in the locpol package in R (Cabrera, 2012).

Results

Plant responses to fungal colonization and water treatment

Fungal colonization and water treatment affected plant growth rate, water loss, biomass, days to first wilt, and days to first tiller death (Figure 3.1). In dry soils, plants wilted 1.5 times faster ($F_{1,60} = 89.867$, $P < 0.001$) and tillers died between 2 and 10 days sooner ($F_{1,60} = 58.911$, $P < 0.001$) on average compared to plants in wet soils. The magnitude of wilt and mortality responses depended on fungal identity (days to first wilt: $F_{5,60} = 9.636$, $P < 0.001$; days to tiller death: $F_{5,60} = 3.870$, $P = 0.004$), but moisture responses were the same across all fungi (days to first wilt: $F_{5,60} = 1.755$, $P = 0.136$; days to tiller death: $F_{5,60} = 3.036$ $P = 0.016$). Isolates 44 and 61 increased days until first wilt and tiller death under low water conditions; in contrast, isolates 5, 18, and 47 had no effect on plant survival, but instead resulted in faster plant wilting (18 and 47), or had no significant effect on wilting (5). Plant growth rate ($F_{5,60} = 15.460$, $P < 0.001$) and water loss ($F_{5,60} = 69.820$, $P < 0.001$) depended on the interaction of fungus with moisture treatment: in response to drought, individual fungi reduced plant growth rate by 0 to

35.2% and altered water loss by -43.1 to 41.1% (Figure 3.1). Again, isolates 18 and 47 adversely affected plant growth under drought whereas isolates 5, 44, and 61 increased plant growth under drought. Total plant biomass was unaffected by water treatment ($F_{1,60}$ = 0.343, P = 0.560), fungus ($F_{5,60}$ = 0.204, P = 0.959), or their interaction ($F_{5,60}$ = 0.131, P = 0.985). Fungal abundance based on percent fungal colonization differed by fungal identity ($F_{7,32}$ = 2.976, P = 0.016), but not water treatment ($F_{1,32}$ = 1.833 P = 0.185). However, the differences in fungal abundance based on fungal identity did not relate to any measured plant response (data not shown)

Expression profiling of drought and fungal colonization using RNA-seq

Between 8.2 x 10^6 and 6.1 x 10^7 reads were sequenced from each treatment (n = 36) and at least 3.7 x 10^6 reads mapped to the *P. hallii* genome (Table 3.2). Plant gene expression varied significantly among the fungal isolates (Figure 3.2), with a range of 606 genes in isolate 5 (1.6% of the total transcriptome) to 5,763 genes in isolate 47 (15.5% of the total transcriptome). For all fungi except isolate 5, there was differential expression based on water treatment. More unique genes were found in high moisture soils (>1% to 9.4% of the transcriptome; Figure 3.2K-L), and there was only >1% to 2.5% overlap of genes between high and low moisture. When comparing all fungal treatments, no shared genes were differentially expressed under low moisture and only 30 were shared under high moisture (Figure 3.3A). However, some fungi were more alike than others in shared gene expression: isolates 47 and 18 shared over 1200 differentially expressed genes in both moisture treatments, with 5.3% in high and 3.9% in low moisture conditions; these two isolates also cluster in the PCA (Figure 3.4A). Shared gene expression among other fungal pairs ranged from >1% to 2.0%, and these also clustered together in the PCA (Figure 3.4A).

Functional annotation of target and non-target genes and relationships to plant growth and physiology

We identified multiple molecular processes affected by fungal colonization of plants under drought (Figure 3.5). Regardless of target gene function, isolates 18 and 47 had the greatest effects on gene expression, with isolate 18 leading to differential

expression of > 50% of genes related to plant metabolism, translation and stress responses relative to fungus-free control plants. Isolate 5 had moderate effects on plant gene expression (~10% of genes related to metabolism, translation, and stress were differentially expressed), and isolates 44 and 61 had minimal effects on either plant stress responses or translation (< 10% of transcripts were differentially expressed). However, both isolates 44 and 61 had major effects on non-target genes associated with certain metabolic processes. Isolate 61 led to the upregulation of all genes involved in alcohol metabolism (n = 7), while isolate 44 increased expression of glycine catabolism and photosynthesis related genes (Figure 3.5).

Only plant growth rate was significantly driven by variation in overall target plant gene expression as expressed by PCA Axis 1 (r^2 = 0.275, P < 0.001; Figure 3.4B). Principal components analysis scores were unable to predict any other plant response (data not shown). We found a total of 93 differentially expressed genes of interest (Table 3.3, Figure 3.6), which were involved in pathogen response (22.1%), ethylene regulation (17.9%), abscisic acid regulation (16.8%), and photosynthesis (16.8%). Of those 93 genes, only two had a significant relationship with any plant response. Expression of 'drought sensitive protein' F02921 and 'pathogenesis related protein' J00932 were positively correlated with plant growth rate (r^2 = 0.513 P < 0.001, Figure 3.44C).

Based on MIC analysis (Bonferroni-adjusted P < 2.712 x 10^{-6}, Table 3.4), we identified an additional 686 non-target genes that were significantly associated with one or more plant responses regardless of differential expression based on fungal colonization vs. fungus-free controls (Table 3.4). Of the 686 genes, 130 had no known function based on Gene Ontology (GO) terms or homology with *A. thaliana* transcripts. The remaining 556 genes fell into three broad categories: (1) general cellular processes (e.g., metabolism, cell division, transcription, translation), (2) plant growth (e.g. photosynthesis, abscisic acid, auxin), and (3) stress responses (e.g. ethylene, cell death, desiccation) There was no detectable relationship between functional gene categories and plant responses.

The majority of differentially expressed non-target genes were correlated with plant growth rate (467) and water loss (210), whereas only 2 genes were related to plant biomass, 4 genes to tiller death, and 3 genes to plant wilting (Table 3.4). Most of the relationships were non-linear (Table 3.4, Figure 3.7), with only plant growth rate demonstrating linear relationships with expression of 101 genes (Figure 3.7E-F). All other plant responses had solely non-linear relationships with gene expression (Figure 3.7A-D and G-H). For example, both days until first wilt and tiller death peak when expression is at its lowest point for the gene C02094 (a mitogen-activated protein kinase). Relationships between plant water loss and growth and gene expression are more complex. Although both plant responses can be explained by linear (or nearly so) relationships with gene expression (Figures 3.7C-D and 3.7E-F), many transcripts appear to have threshold responses (Figures 3.7C-D and 3.7G-H). For example, water loss decreases rapidly with increased expression of stress response protein E00291 (Figure 3.7D) and plant growth is highest at high expression of an auxin response factor (D00365, Figure 3.7F). For transcripts that were not expressed in a number of samples, local polynomial regressions could not be calculated; this excluded all transcripts associated with plant biomass, 1 transcript associated with days until first wilt, and 2 transcripts associated with days until tiller death.

Discussion

By comparing the effects of multiple endophytic fungal isolates under wet and dry conditions, we discovered patterns of gene expression directly related to the outcome of symbiosis. The isolates (18, 47) that stimulated the most differential gene expression (~3%) in the plant under drought were antagonists that caused faster wilt, lower survival, slower growth, and more water loss in host plants; essentially these were either worse or no different from control plants with no fungus. In contrast, the most beneficial fungi (44, 61) resulted in the fewest differentially expressed genes (<1%) when the plant was drought-stressed. The fungal isolate (5) that had mixed effects on the plant had intermediate levels of gene expression and also fell between the two other groups in plant functional responses. Based on genome analyses, there are conflicting results as to

whether mutualists have higher (Karpinets *et al*., 2014) or lower biosynthetic capability (Kohler *et al*., 2015), which likely affects the ability of the fungus to moderate plant gene expression. One possible explanation is that mutualist fungi can "fly under the radar," triggering little in the way of plant response, whereas antagonistic endophytes may be detected as pathogens by the host.

Despite the clear differences between mutualist and antagonist fungi, a large proportion of plant gene expression was fungus-specific. The two mutualists shared only 12 common genes that were differentially expressed under drought stress (between 4% and 6% of total affected genes). Between the two antagonists there were a larger proportion of shared genes, with 1,493 common differentially expressed genes (42% and 82% of total affected genes). Therefore, similar symbiotic outcomes may have very different molecular mechanisms, and beneficial mutualisms in particular may take many different forms. This is consistent with previous work, in which 2- to 18-fold differences in gene expression were found among plants colonized by closely-related *Trichoderma* congeners (Bailey *et al*., 2006). Similarly, studies of plant gene expression in response to pathogen infection have found that fewer than 50% of differentially expressed genes are affected by multiple pathogens (De Vos *et al*., 2005; Adie *et al*., 2007). Ultimately, molecular regulation of plant-endophyte interactions may involve species-specific crosstalk (Bailey *et al*., 2006), making it difficult to generalize about molecular mechanisms governing their symbiotic associations.

Fungal-mediated gene expression patterns were also stress-dependent. There was substantial variation in fungal effects on gene expression between wet and dry soil conditions for all but isolate 5. The mutualistic isolates 44 and 61 induced differential expression of 56% and 94% fewer plant genes under low water conditions relative to high water conditions. Similarly, the antagonistic isolates 18 and 47 decreased effects on plant expression by 10% and 63% from wet to dry treatments, despite overall higher plant gene expression compared to mutualists. One possibility is that endophytic fungi are less active under stressful conditions, since abundance based on percent fungal colonization of plant leaves did not differ by water treatment. Few studies have characterized effects

of both antagonists and mutualists on plant gene expression simultaneously. However, analysis of the magnitude of plant physiological responses to fungal colonization found that beneficial effects were smaller than antagonistic effects (+0.5 to -0.9) (Jumpponen, 2015). If mutualists generally have a relatively small effect on plant gene expression, that may explain why adverse effects on plant physiology due to antagonist or pathogen infection generally dwarf the positive effects of mutualist colonization.

To develop gene-based methods of plant drought management based on fungal endophytes requires identification of specific target genes or pathways. Our targeted gene approach focused on a set of known genes and physiological pathways involved in plant growth, drought responses, cell death, and pathogen response, but these were not the primary predictors of plant responses to fungal colonization under drought. When focused solely on target genes, we found only 2 genes related to drought stress and pathogen response that were highly predictive of plant growth (51%). However, 686 non-target genes successfully predicted one or more plant responses. Only 40 non-target genes could be functionally annotated as plant growth or stress response genes, whereas nearly 500 genes were involved in general cellular processes (DNA binding, cell structure, translation, etc.) and 130 had no known function. Previous research has found that plant-associated fungi lead to differential expression of many stress responses genes under drought (Moy *et al*., 2004; Porcel *et al*., 2006; Ruiz-Lozano *et al*., 2006; Sherameti *et al*., 2008), so their relative lack here suggests that either annotation of plant stress response genes is insufficient (Luhua *et al*., 2013) or that endophytes somehow avoid triggering plant stress responses (Kloppholz *et al*., 2011). Important physiological responses to stress are often regulated downstream of gene expression (Chaves *et al*., 2003). If endophytes affect plant physiology through post-transcriptional or post-translational events, characterizing the plant and fungal proteome may be necessary to fully understand endophyte-mediated drought tolerance.

Although whole-transcriptome shotgun sequencing can be extremely useful in the characterization of potential target genes, the identification of specific mechanisms of endophyte-mediated drought tolerance remains elusive. Annotation of relevant genes in

P. hallii is largely insufficient for identifying specific functions of differentially expressed genes. Even in the most well-characterized plant genetic model, *A. thaliana,* more than 30% of transcripts have no known function (Lamesch *et al*., 2012; Luhua *et al*., 2013) making assignment of even comparative function difficult. Perhaps most critically, true mechanistic understanding of plant-endophyte relationships requires the characterization of fungal gene expression in the plant. Studies characterizing plant and fungal pathogen gene expression have been able to characterize potential crosstalk between host and fungus (Kawahara *et al*., 2012). For example, the upregulation of transcripts encoding glycosyl hydrolases, cutinases and LysM domain-containing proteins in the rice blast fungus, *Magnaporthe oryzae*, led to upregulation of pathogenesis-related and phytoalexin biosynthetic genes in rice (Kawahara *et al*., 2012). Finally, understanding broader patterns of gene expression in endophytic fungi will require examination of more than five fungal isolates.

Fungal endophytes have long been known to affect plant gene expression in response to stress (Bailey *et al*., 2006; Sherameti *et al*., 2008; Meijía *et al*., 2014). Here we further demonstrate that there are clear differences in plant gene expression related to fungal endophyte strategy (antagonist vs. mutualist), and we successfully identified multiple gene expression patterns that predicted the outcome of plant-endophyte interactions under drought. This study marks an important first step to true mechanistic understanding of endophyte-mediated drought tolerance. More research will be needed to identify whether there are broadly shared genes or pathways among fungal mutualists conferring plant drought benefits.

Tables

Table 1.1 PERMANOVA results for endophyte community composition based on Bray-Curtis dissimilarities and UniFrac distances.

		Bray-Curtis				**Unifrac**			
	df	**Mean Squares**	**Pseudo F**	***P***	R^2	**Mean Squares**	**Pseudo F**	***P***	R^2
Site	9	1.751	8.210	<0.001	0.338	0.700	0.700	<0.001	0.266
Year	2	1.444	6.773	<0.001	0.062	0.417	0.417	<0.001	0.035
Site x Year	18	0.484	2.272	<0.001	0.187	0.242	0.242	<0.001	0.184
Total					0.587				0.485

Table 1.2 Site characteristics. Latitude, longitude, and elevation were collected via handheld GPS. Historical climate means (30-year, 1981-2010) and spring values for precipitation, maximum temperature (T_{high}), and minimum temperature (T_{low}) were obtained from the PRISM Climate Group. Spring weather is provided for 2012, 2013, and 2014 (listed top to bottom) for each site.

Site #	Site ID	Lat	Lon	Elevation (m)	Thirty-Year Normals (1980 – 2010)			Spring (April to June)		
					MAP (mm)	MLT (°C)	MLT (°C)	Rain (mm)	T_{low} (°C)	T_{high} (°C)
								151.52	11.32	34.84
1	SCASNA	29.693	-101.318	416	442.33	13.87	27.46	102.20	8.22	36.97
								138.82	7.42	34.83
								134.96	7.26	33.75
2	DRISNA	29.930	-100.929	426	513.63	12.70	26.68	157.98	5.46	36.11
								159.93	10.00	34.13
								195.27	6.34	33.08
3	KCASNA	29.615	-100.451	577	602.66	13.04	26.47	179.48	9.46	34.61
								205.92	6.92	32.02
								220.22	5.71	36.42
4	CASECO	30.153	-99.987	675	661.63	13.29	26.69	52.38	9.60	32.31
								162.56	4.84	31.45
								219.30	3.42	32.45
5	KERWMA	30.071	-99.506	645	721.57	10.52	24.72	213.81	3.18	30.62
								245.94	7.53	34.07
								256.64	8.06	33.94
6	KENECO	30.052	-99.394	645	754.66	10.53	24.75	237.53	3.57	32.52
								220.98	3.92	30.94
								257.00	3.94	34.06
7	INGECO	30.322	-98.449	409	812.19	12.51	25.88	229.85	4.42	31.96
								280.39	8.47	34.42
								274.77	4.61	34.34
8	COLECO	30.329	-98.439	388	814.17	12.51	25.84	256.64	5.94	34.06
								299.31	10.98	31.96
								256.64	3.72	33.29
9	GRAECO	29.913	-99.243	628	841.24	10.62	24.72	274.77	4.61	30.72
								285.56	10.98	32.06
								298.21	12.59	35.12
10	LBJWFC	30.184	-97.876	238	886.79	13.59	26.25	332.39	6.78	32.40
								262.23	7.44	34.91

Table 1.3 Repeated measures ANOVA results for historical climate, annual weather, and vegetation structure by site (df = 1), year (df = 2), and their interaction (df = 2). Site was a between-subjects effect and year was a within-subjects effect.

Variables	Site		Year		Site x Year	
	F	***P***	***F***	***P***	***F***	***P***
Historical climate						
MAP	225.79	<0.001	2.053	0.184	1.655	0.230
MLT	358.48	<0.001	1.281	0.287	1.256	0.291
MHT	781.06	<0.001	0.755	0.418	0.858	0.379
Annual weather						
YTD rainfall	131.84	<0.001	0.476	0.536	0.095	0.765
YTD LT	2.61	0.141	55.581	<0.001	50.456	<0.001
Spring rainfall	106.82	<0.001	8.912	0.011	15.566	0.003
Spring low temp	223.25	<0.001	113.859	<0.001	201.387	<0.001
Spring high temp	95.731	<0.001	32.227	<0.001	88.161	<0.001
Soil moisture	119.91	<0.001	12.558	0.002	15.962	0.003
Vegetation structure						
Grass cover	216.17	<0.001	0.148	0.838	0.160	0.699
Shrub cover	568.44	<0.001	0.724	0.482	0.442	0.523
Bare ground cover	15.86	0.003	0.115	0.806	0.474	0.509

Table 1.4 Linear mixed model results for host plant characteristics by site, year, and their interaction.

Variables	Site			Year			Site x Year		
	df	***F***	***P***	**df**	***F***	***P***	**df**	***F***	***P***
Tiller	9	8.047	<0.001	2	8.765	<0.001	18	3.279	<0.001
Height	9	31.172	<0.001	2	31.172	<0.001	18	16.694	<0.001
Flowering height	9	16.784	<0.001	2	4.381	0.015	18	4.583	<0.001
Basal area	9	5.688	<0.001	2	6.694	0.002	18	2.373	0.004
Density	9	20.318	<0.001	2	5.196	0.007	18	3.114	<0.001

Table 1.5 Known ecological functions of all unique fungal isolates. OTU based on location along phylogeny, as determined in Giauque & Hawkes (2016). 'Symbiont Strategy' refers to cluster assignment based on LRRsymbiont. Fungi screened for effect on plant gene expression (Chapter 3) are marked with an asterisk. LSU and ITS accessions refer to the OTUs in this study. Previously known functions (E = endophyte, S = saprophyte, and P = pathogen) indicated using grey shading for FunGuild assignments and numbers to refer to published references (see 'Table References').

OTU	Symbiont Strategy	Nearest Sister Genus	Sister Accession	LSU Accession	ITS Accession	E	S	P
1	III	*Biscogniauxia*	LN714525	KP401935	KP401934			1
2		*Sordariomycetes*	JQ760984	KP401939	KP401938	2		
3		*Sordariomycetes*	JQ760360	KP401937	KP401936	2		
4	III	*Sordariomycetes*	JQ761854	KP401941	KP401940	2		
5	III*	*Cercospora*	GU214657	KP401903	KP401902		3	4
6		*Davidiella*	EU167591	KP401901	KP401900	5	6	6
7	I	*Cladosporium*	AY251074	KC582568	KP401862	7	8	
8		*Cladosporium*	KC311516	KC582572	KP401885		9,10	9,10
9	I	*Phoma*	KC248542	KC582588	KP401867		11	11
10	III	*Phoma*	KC311486	KC582591	KP401868	12		13
11	III	*Marssonia*	FJ755256	KC582578	KP401879		14	
12		*Phoma*	KC311476	KP401913	KP401912	15	16	15
13		*Epicoccum*	KC311470	KP401915	KP401914	17	18	19
14		*Epicoccum*	HM047194	KC582574	KP401886			20
15	III	*Cochliobolus*	GQ328851	KC582573	KP401899	21	22	21
16	III	*Cochliobolus*	GQ221854	KC582569	KP401898	21	22	21
17	III	*Cochliobolus*	JN943410	KC582571	KP401884	23		
18	III*	*Cochliobolus*	HE792897	KP401907	KP401906	24	25	26
19	III	*Cochliobolus*	KC311473	KC582570	KP401875	27	25	28
20	II	*Alternaria*	AY154682	KC582560	KP401871	29		30
21		*Alternaria*	AY154683	KP401909	KP401908	31		32
22	II	*Alternaria*	GU183130	KC582563	KP401860		33	
23	II	*Alternaria*	KJ541482	KC582561	KP401872			34
24		*Sporormiella*	HQ130664	KP401935	KP401935	35		
25		*Preussia*	GU183123	KP401905	KP401904	36	37	37
26	III	*Preussia*	HQ130702	KC582592	KP401890	38		
27		*Preussia*	HQ130700	KC582582	KP401869	38		
28		*Byssochlamys*	DQ322218	KC582566	KP401883	39	40	40
29		*Aspergillus*	KC119198	KP401921	KP401920	41		42
30	II	*Aspergillus*	FJ867934	KC582565	KP401873	41		42
31	II	*Aspergillus*	EF669599	KC582579	KP401888	43	44	
32		*Aspergillus*	FJ867942	KP401919	KP401918		45	45
33		*Aspergillus*	FJ867942	KC582564	KP401861		45	45
34		*Penicillium*	AF033476	KP401917	KP401916	46		47
35	II	*Penicillium*	DQ339568	KC582586	KP401877			48
36	II	*Penicillium*	AF033472	KC582584	KP401864	49	50	50
37	II	*Penicillium*	DQ339562	KC582589	KP401880	51	52	52
38	I	*Penicillium*	JN642222	KC582590	KP401865		53	54
39		*Penicillium*	HM469412	KP401923	KP401922	55	56	
40	III	*Penicillium*	HM469420	KC582596	KP401881	57	58	58

Table 1.5 continued

41		*Penicillium*	JF772180	KC582594	KP401895		59	60
42	II	*Penicillium*	HM469414	KC582593	KP401894	61		
43		*Talaromyces*	KJ188700	KC582562	KP401870	62	62	
44	II*	*Penicillium*	HM469418	KC582595	KP401878	63		
45		*Phyllosticta*	DQ377928	KP401911	KP401910	64		65
46		*Sordaria*	AF246293	KC582599	KP401896	66	67	68
47	III*	*Sordaria*	AY681171	KP401931	KP401930	69		
48		*Chaetomium*	AY681171	KP401933	KP401932	70		
49	I	*Chaetomium*	HM365261	KC582567	KP401874	71,72	73	
50	III	*Podospora*	GU183117	KC582585	KP401893		74	
51		*Podospora*	AF443851	KP401949	KP401948	75	75	
52		*Podospora*	EF197082	KC582598	KP401891		76	
53	III	*Gibberella*	GQ168842	KC582576	KP401882	77	77	78
54	II	*Fusarium*	AY188919	KC582575	KP401863	79	80	
55		*Acremonium*	KJ194115	KP401945	KP401944	81		82
56		*Acremonium*	KM215633	KP401925	KP401924	83		
57	III	*Nectria*	HM534892	KP401927	KP401926	84		84
58		*Myrothecium*	AJ302002	KC582583	KP401889	85	86	
59		*Fusarium*	FJ345352	KP401929	KP401928	87	88	88
60	II	*Nigrospora*	GQ428201	KC582581	KP401892	89	90	91
61	I*	*Nigrospora*	GQ221861	KC582577	KP401887	89	90	91
62		*Nigrospora*	GQ221860	KC582580	KP401876	89	90	91
63		*Nigrospora*	GQ328855	KP401947	KP401946	89	90	91
64		*Pestalotiopsis*	JF773655	KP401943	KP401942	92		
65	II	*Pestalotiopsis*	GU183121	KC582587	KP401866	93	94	94

Table 1.6 PERMANOVA results for endophyte community composition based on Bray-Curtis dissimilarity as a function of historical climate, annual weather, host plant traits, vegetation structure, spatial factors, and their interactions. For brevity, only significant terms ($P < 0.002$ with Bonferroni correction) included in the model are listed here.

Model	**df**	**Mean Squares**	**Pseudo *F***	***P***	R^2
Historical climate					
MAP	1	8.225	47.087	0.001	0.175
MLT	1	1.087	7.778	0.001	0.029
MHT	1	2.088	11.951	0.001	0.044
Annual weather					
Spring LT	1	1.580	9.044	0.001	0.034
Spring HT	1	0.983	5.626	0.001	0.021
Soil moisture	1	0.641	3.672	0.001	0.014
Spring rainfall x Spring LT	1	0.793	4.542	0.001	0.017
Spring LT x Spring HT	1	0.653	3.737	0.002	0.014
Vegetation structure					
Grass cover	1	0.687	3.396	0.001	0.014
Shrub cover	1	0.836	4.788	0.001	0.018
Plant host					
Height	1	0.434	2.485	0.002	0.009
Flowering height	1	0.747	4.274	0.002	0.016
Spatial factors					
Elevation	1	0.956	5.473	0.001	0.020
Historical climate x Annual weather					
MAP x Spring LT	1	0.653	3.740	0.002	0.014
Explained					0.439
Unexplained					0.561

Table 1.7 PERMANOVA results for endophyte community composition using UniFrac distances as a function of historical climate, annual weather, host plant traits, vegetation structure, spatial factors, and their interactions. For brevity, only significant terms ($P < 0.002$ after Bonferroni correction) included in the model are listed here.

Model	df	Mean Squares	Pseudo *F*	*P*	R^2
Historical climate					
MAP	1	2.707	19.235	0.001	0.115
Spatial factors					
Latitude	1	1.140	8.102	0.001	0.048
Elevation	1	0.910	6.464	0.001	0.038
Historical climate x Annual weather					
Spring rainfall x MLT	1	2.567	14.026	0.001	0.094
Historical climate x Vegetation cover					
MAP x Grass cover	1	1.537	8.818	0.002	0.063
Explained					0.358
Unexplained					0.642

Table 2.1 Linear mixed models for plant responses to fungal colonization, water treatment, their interaction, and trial. Fungal treatment ('Fungus') and soil moisture treatment ('Water'), and their interaction were fixed factors; trial was a random factor. Bonferroni adjustment was used for multiple comparisons ($P < 0.007$).

Plant	Fungus		Water		Fungus*Water		Trial	
Response	*F*	*P*	*F*	*P*	*F*	*P*	$\chi 2$	*P*
Total biomass (mg)	2.406	< 0.001	0.948	0.331	2.029	< 0.001	0.124	0.700
Growth rate (cm day^{-1})	2.793	< 0.001	37.405	< 0.001	1.486	0.041	< 0.001	1.000
Water loss (ml)	9.150	< 0.001	840.290	< 0.001	9.910	< 0.001	7.320	0.008
TE (g ml^{-1})	2.007	< 0.001	5.318	0.022	1.998	< 0.001	< 0.001	1.000
Days to first wilt	2.283	< 0.001	83.812	< 0.001	2.025	< 0.001	7.330	0.008
Days to first tiller death	2.446	0.081	29.112	< 0.001	3.753	< 0.001	0.093	0.800

Table 2.2 Fungal strategies that predict the outcome of plant-fungal interactions based on fungal colonization under low water conditions relative to high water ($LRR_{interaction}$). Strategies were identified based on k-means clustering (Figure 1) and univariate ANOVAs identified plant responses that differ based on fungal strategies with Bonferroni corrections for multiple comparisons ($P < 0.007$). Numbers indicate effect size (LRR) direction and magnitude. Variables that did not differ from zero are left blank. Differences in plant responses among strategies are included in Figure 2.5.

Strategy	I Water Use Mutualist	II Survival Mutualist	III Survival Antagonist	IV Mixed Mutualist
Water loss (ml)	-1.655	0.520		-0.310
TE (g ml^{-1})	2.305	-0.961	0.603	0.577
Days to first tiller death		0.091	-0.378	0.040

Table 2.3 Phylogenetic signal of treatment effects on plant responses. Blomberg's K and Pagel's λ calculated using the 'phylosig' function in the Vegan package of R (Oksanen et al., 2015) for phylogenetic signal associated with fungal treatment ('Fungus'), soil moisture treatment ('Water'), their interaction, and both components of the interaction term ("Low water" and "High water") on plant responses. Using a Bonferroni-corrected $P < 0.006$ to determine significant factors, none of the tested factors were significant.

	Fungus		**Water**		**Interaction**	
	K	**λ**	**K**	**λ**	**K**	**λ**
PPCA Axis1	0.267	0.755	0.280	0.846	0.236	0.010
PPCA Axis 2	0.346	0.423	0.426	0.874	0.398	0.891
Total biomass (mg)	0.271	0.614	0.375	0.173	0.276	0.452
Growth rate (cm d^{-1})	0.446	0.776	0.395	0.779	0.341	0.681
Water loss (ml)	0.435	0.363	0.282	0.364	0.247	0.042
TE (mg ml^{-1})	0.279	0.004	0.270	0.29	0.275	0.682
Days to first wilt	0.243	0.292	0.341	0.864	0.279	0.549
Days to tiller death	0.331	0.289	0.369	0.631	0.303	0.167

Table 2.4 MANOVA for fungal habitat and traits as a function symbiotic outcomes (I – IV). Each fungal habitat and trait variable was analyzed as a function of the symbiotic outcomes for that LRR ($LRR_{interaction} = 4$, $LRR_{low\ water} = 4$, $LRR_{high\ water} = 2$).

Source of Variation	**df**	**Pillai**	**Pseudo F**	**Num df**	**Den df**	**P**
$LRR_{interaction}$	1	0.864	3.636	14	8	0.036
$LRR_{low\ water}$	1	0.957	12.729	14	8	< 0.001
$LRR_{high\ water}$	1	0.568	0.754	14	8	0.692

Table 2.5 Univariate ANOVAs of fungal origin and traits as a function of symbiotic outcomes (I – IV). Each fungal variable (origin, stress traits, and resource traits) were analyzed as a function of the symbiotic outcomes for that LRR ($LRR_{interaction} = 4$, $LRR_{low\ water} = 4$, $LRR_{high\ water} = 2$). We used a Bonferroni adjustment for multiple comparisons ($P < 0.004$).

	Source of Variation					
	$LRR_{interaction}$ (df = 3)			$LRR_{low\ water}$ (df = 3)		
	MS	**Pseudo *F***	***P***	**MS**	**Pseudo *F***	***P***
Fungal origin						
MAP	64573.000	4.813	0.040	32439.000	2.418	0.013
MALT	4.395	2.993	0.098	0.465	0.316	0.580
MAHT	0.001	0.002	0.963	0.864	1.234	0.279
Fungal stress traits						
Osmotic sensitivity	0.390	2.883	0.104	0.751	5.547	0.028
Osmotic threshold	6.768	0.471	0.500	66.723	4.646	0.043
ACC	0.001	1.066	0.313	0.001	3.376	0.080
Fungal resource traits						
Carbohydrates	0.977	0.025	0.876	4.476	0.113	0.740
Amino acids	1.196	0.132	0.720	1.204	0.133	0.719
Amines	0.085	0.028	0.869	0.328	0.108	0.746
Carboxylic acids	0.630	0.079	0.781	3.722	0.468	0.501
Polymers	0.965	0.283	0.600	0.099	0.029	0.866
Biomass (mg)	10.596	0.759	0.393	41.529	2.976	0.099
CBH	7822.000	0.105	0.749	495297.000	6.642	0.017
AG	261.800	0.052	0.822	2938.300	0.579	0.455

Table 2.6 Correlations of fungal traits related to habitat origin, stress response, and resource use to PPCA axes. Correlations were calculated using the 'envfit' function in Vegan (Oksanen et al., 2015). Trait and habitat variables were only included that were not significantly correlated (> 90%) and did not display multicollinearity based on variance inflation factors using the vif function in the car R package (Fox et al., 2015). Trait abbreviations are: T = osmotic threshold, S = osmotic sensitivity, AC = ACC concentrations, C = carbohydrates, AA = amino acids, A = amines, CA = carboxylic acids, P = polymers, B = biomass, CB = cellobiohydrolase, and AG = α-1,4-glucosidase. We used the Bonferroni-corrected $P < 0.004$ to determine significant factors

	Symbiont				Water				Interaction			
	PPC1	**PPC2**	R^2	P	**PPC1**	**PPC2**	R^2	P	**PPC1**	**PPC2**	R^2	P
Fungal origin												
MAP	-0.842	-0.540	0.163	0.060	0.818	0.576	0.262	0.013	0.843	-0.538	0.171	0.046
MALT	0.182	-0.983	0.104	0.204	0.543	-0.840	0.023	0.670	-0.385	-0.923	0.045	0.423
Fungal stress traits												
S	0.439	0.899	0.379	<0.001	-0.842	-0.540	0.416	<0.001	-0.406	0.914	0.312	0.005
T	0.479	0.878	0.194	0.028	-0.634	-0.773	0.348	<0.001	-0.370	0.929	0.282	0.009
AC	-0.586	0.810	0.098	0.177	-0.983	0.185	0.010	0.869	0.431	0.902	0.125	0.113
Fungal resource traits												
A	-0.110	0.994	0.008	0.889	-0.276	-0.961	0.021	0.668	-0.856	0.517	0.034	0.583
CA	0.482	-0.876	0.064	0.351	-0.370	-0.929	0.037	0.548	-0.886	-0.463	0.138	0.104
P	0.908	-0.420	0.063	0.360	-0.922	0.386	0.098	0.176	-0.983	0.183	0.066	0.333
B	-0.119	0.993	0.026	0.642	-0.116	-0.993	0.041	0.514	0.257	0.966	0.092	0.225
CB	-0.442	-0.897	0.201	0.060	0.994	0.109	0.098	0.206	0.199	-0.980	0.357	0.004
AG	-0.975	-0.222	0.028	0.644	0.863	-0.505	0.011	0.838	0.326	-0.945	0.026	0.676

Table 2.7 Phylogenetic generalized least squares (PGLS) regression comparing fungal traits and habitat origin with plant responses. Using the 'pgls' function in the Caper package of R (Orme et al., 2015), we performed PGLS regressions to determine the relationships between fungal traits and habitat variables and individual plant responses of the LRR for fungal treatment ('Fungus'), soil moisture treatment ('Water'), and their interaction. Only significant variables were included (Bonferroni-corrected P-value of $P < 0.007$). Statistics are listed for the full model and individual variables included in the full models. Abbreviations are: ACC = aminocyclopropane-1-carboxylate concentrations CA = carboxylic acids, CBH = cellobiohydrolase, MALT = mean annual low temperature, MAP = mean annual high temperature, OT = osmotic threshold, OS = osmotic sensitivity. The level of significance ($P < 0.007$) was obtained after Bonferroni adjustment for multiple comparisons.

	$LRR_{Symbiont}$				LRR_{Water}				$LRR_{Interaction}$			
	Var	**β**	**T**	***P***	**Var**	**β**	**T**	***P***	**Var**	**β**	**T**	***P***
Total biomass (mg)	CBH	-0.001	-2.933	0.006	MAP	-0.003	-2.896	0.007	CBH	0.001	3.416	0.002
	F	5.847	***P***	0.006	***F***	8.388	***P***	0.007	***F***	11.67	***P***	0.002
	R^2	0.221			R^2	0.178			R^2	0.239		
Growth rate (cm d^{-1})					CBH	-0.001	-2.213	0.003	OT	-0.113	-4.461	0.001
					MAP	-0.004	-2.527	0.007				
					F	7.077	***P***	0.003	***F***	11.60	***P***	0.001
					R^2	0.363			R^2	0.483		
Water loss (ml d^{-1})												
TE									CA	0.233	3.289	0.002
									F	7.067	***P***	0.002
									R^2	0.263		
Days to first wilt	A	0.128	2.767	0.007	CA	-0.070	-3.531	0.001	CA	-0.067	-3.302	0.002
	CA	-0.095	-3.639	0.001								
	F	5.621	***P***	0.003	***F***	8.302	***P***	0.001	***F***	10.91	***P***	0.002
	R^2	0.290			R^2	0.300	-3.531	0.001	R^2	0.226		
Days to tiller death	OS	0.293	3.719	0.001	OS	0.341	2.792	0.007				
	B	0.022	3.040	0.005	B	0.032	2.905	0.007				
	F	14.00	***P***	0.001	***F***	11.97 0	***P***	0.001				
	R^2	0.467			R^2	0.492						

Table 2.8 Phylogenetic generalized least squares (PGLS) regression comparing fungal traits and habitat origin with plant responses. Using the 'pgls' function in the Caper package of R (Orme et al., 2015), we performed PGLS regressions to determine the relationships between fungal traits and habitat variables and individual plant responses of the LRR for both components of the interaction term ("Low water" and "High water"). Plant responses were tested only when the interaction term for those responses was significantly predicted by fungal traits or habitat. Only significant variables were included Statistics are listed for the full model and individual variables included in the full models. Abbreviations are: ACC = aminocyclopropane-1-carboxylate concentrations, CA = carboxylic acids, CBH = cellobiohydrolase, MALT = mean annual low temperature, MAP = mean annual high temperature, OT = osmotic threshold, OS = osmotic sensitivity. The level of significance ($P < 0.007$) was obtained after Bonferroni adjustment for multiple comparisons.

	$LRR_{Low\ water}$				$LRR_{High\ water}$			
	Var	**β**	**T**	**P**	**Var**	**β**	**T**	**P**
Total biomass (mg)	CA	0.055	3.288	0.002	CBH	-0.001	-3.779	0.001
	F	7.073	***P***	0.002	***F***	14.290	***P***	0.001
	R^2	0.263			R^2	0.281		
Growth rate (ml day^{-1})	CBH	0.001	2.714	0.005	OT	0.043	2.895	0.006
	F	8.819	***P***	0.005	***F***	8.381	P	0.006
	R^2	0.187			R^2	0.178		
Water loss (ml d^{-1})					CA	0.070	2.961	0.006
					F	6.823	***P***	0.006
					R^2	0.255		
TE								
Days to first wilt	CA	-0.074	-4.119	0.001	OS	0.350	3.055	0.004
	F	7.465	***P***	0.001	***F***	9.332	***P***	0.004
	R^2	0.363			R^2	0.197		
Days to tiller death		NS	NS	NS		NS	NS	NS

Table 3.1 Predicted changes in gene expression of plants under fungal colonization and drought stress. Based on a literature search, potential mechanisms of endophyte-mediated drought tolerance were identified. Evidence for each mechanism is based on changes in fungal-colonized plant gene expression relative to sterile plant gene expression. To identify genes involved in each potential mechanisms, both GO terms and *Arabidopsis* functions were screened for the listed search terms.

Potential mechanism	Evidence	Rationale	Search terms
Growth			
More efficient transpiration per unit biomass due to regulation of photosynthesis	ALTERED expression of genes involved in photosynthesis	Fungi can affect the molecular regulators of photosynthesis, by a currently unknown mechanism (Ghabooli et al., 2013)	'phyotosynt*', 'photosystem'
Increased stomatal closure due to ABA production	DECREASE or MAINTENANCE in abscisic acid biosynthesis genes	Endophyte infection can increase plant ABA concentrations (Hao et al., 2009)	'ABA', 'abscisic acid'
More efficient growth due to regulation of plant growth hormones	ALTERED expression of genes involved in production of plant growth hormones	Fungi can alter plant concentrations of auxin	'auxin', 'gibberellin', 'cytokinin'
Stress			
Increased stomatal closure due to decrease in ethylene production	DECREASE in ACC synthesis genes or genes involved in conversion to ethylene	Ethylene production affects stomatal closure and several fungal species can manipulate plant production of ethylene (Eaton et al., 2011)	'ethylene'
Maintain cell turgor through secretion of osmolytes	DECREASE or MAINTENANCE in expression of osmolyte synthesis gene	Many endophytes produce secondary compounds which might act as osmolytes within the plant (Waqas et al., 2015)	'osmol*', 'antioxidant'
Decreasing apoptosis by decreasing ROS concentrations	DECREASE in expression of ROS synthesis genes or INCREASE in antioxidant synthesis genes	Endophyte-infected plants have lower concentrations of ROS under stress (Rodriguez et al. 2008)	'ROS', 'reactive oxygen species', 'cell death', 'apoptosis'

Table 3.1 continued

More efficient drought response due to stress of endophyte infection	INCREASE in expression of stress response genes, irrespective of water stress	Endophyte-infected plants have increased drought stress response, but recover more quickly (Ren & Clay, 2009)	'pathogen*', 'stress'

Table 3.2 Summary of mapping results for each fungus and water treatment. The total number of reads generated by TAG-seq for each fungus and water treatment (n = 3) are displayed. Succesfully filtered reads (sequence length = 90 bp, Pred ≥ 30) and mapped reads are also displayed. Accompanying each treatment is the number of differentially expressed genes (DEG) due to the fungus-control treatment contrast.

Fungal ID		Total Reads	Filtered Reads	Plant Mapped	DEG (Fungus – Control)
5	Dry	41,935,064	30,077,425	23,815,796	769
	Wet	16,872,832	13,023,183	9,225,964	1233
18	Dry	11,025,670	8,202,728	5,750,769	2812
	Wet	28,163,585	21,033,432	15,163,900	5205
61	Dry	12,779,020	10,398,750	8,607,264	548
	Wet	8,188,562	6,295,072	3,750,081	6023
44	Dry	31,364,432	24,184,753	19,801,465	769
	Wet	28,797,542	21,907,785	16,855,578	1057
47	Dry	26,296,720	18,224,236	13,027,922	4827
	Wet	60,867,846	40,391,626	33,338,674	7668
Control	Dry	43,336,810	31,464,964	29,355,219	
	Wet	34,211,561	25,118,053	24,352,589	

Table 3.3 Differential expression of target genes under low water conditions. Using gene homology and known *Arabidopsis* genes and GO terms, 15 genes were differentially expressed in at least one fungal treatment relative to the sterile control under drought. Each gene is listed by its gene ID (*Panicum hallii* v2.0, DOE-JGI, http://phytozome.jgi.doe.gov/). Only genes that were significantly ($P < 0.01$) overexpressed (+) or underexpressed (-) in at least one of the fungal treatments relative to the sterile control are included.

	GO Term	GO Description	Function in *A. athaliana*
Abscisic acid regulation/signaling			
A00102			Abscisic acid-responsive (TB2/DP1, HVA22) family protein
A00893			ABA Overly-Sensitive 5
A01392			regulatory components of ABA receptor 3
A02678			GRAM domain-containing protein / ABA-responsive protein-related
A02679			GRAM domain-containing protein / ABA-responsive protein-related
C00916			regulatory components of ABA receptor 3
C02541	GO:0043565	sequence-specific DNA binding	ABA-responsive element binding protein 3
C02743	GO:0003824	catalytic activity	highly ABA-induced PP2C gene 2
C02862	GO:0043565	sequence-specific DNA binding	abscisic acid responsive elements-binding factor 2
E01203	GO:0043565	sequence-specific DNA binding	ABA-responsive element binding protein 3
E03755			abscisic acid (aba)-deficient 4
G01345			GRAM domain-containing protein / ABA-responsive protein-related
G01346			GRAM domain-containing protein / ABA-responsive protein-related
H02538	GO:0003824	catalytic activity	highly ABA-induced PP2C gene 2
Ethylene regulation/signaling			
A00609	GO:0006355	regulation of transcription	ethylene responsive element binding factor 3
A00802	GO:0005515	protein binding	Signal transduction histidine kinase, hybrid-type, ethylene sensor
A02790	GO:0006355	regulation of transcription	ethylene responsive element binding factor 5
A03924	GO:0007165	signal transduction	Signal transduction histidine kinase, hybrid-type, ethylene sensor
B03032	GO:0055114	oxidation-reduction process	ethylene-forming enzyme
B03033	GO:0055114	oxidation-reduction process	ethylene-forming enzyme
B03331			Ethylene-insensitive3-like 3
B04996			Ethylene-insensitive3-like 1
C04838			ethylene-responsive nuclear protein -related
D00398	GO:0006355	transcription	ethylene responsive element binding factor 4

Table 3.3 continued

D02410			Ethylene insensitive 3 family protein
E03923	GO:0006508	proteolysis	ethylene-dependent gravitropism-deficient and yellow-green-like 2
G02338	GO:0006355	regulation of transcription	ethylene responsive element binding factor 4
G02671	GO:0055114	oxidation-reduction process	ethylene-forming enzyme
I01039	GO:0006355	regulation of transcription	ethylene response factor 1
I01897			ethylene-dependent gravitropism-deficient and yellow-green-like 3
I04013	GO:0006355	regulation of transcription	ethylene responsive element binding factor 5
Osmolyte regulation			
J02156	GO:0046872	metal ion binding	homolog of anti-oxidant 1
Stress responses			
A00895			oxidative stress 3
A02739	GO:0005515	protein binding	stress-inducible protein, putative
A02944			oxidative stress 3
B03901	GO:0045454	cell redox homeostasis	chloroplastic drought-induced stress protein of 32 kD
B04732			Stress responsive alpha-beta barrel domain protein
C00416			Stress responsive A/B Barrel Domain
I00080			Stress responsive alpha-beta barrel domain protein
I03439			stress enhanced protein 1
Cell death			
C01689			DCD (Development and Cell Death) domain protein
D02616			DCD (Development and Cell Death) domain protein
D02618			DCD (Development and Cell Death) domain protein
E02743			DCD (Development and Cell Death) domain protein
I01097			DCD (Development and Cell Death) domain protein
I03394			accelerated cell death 2 (ACD2)
I03396			accelerated cell death 2 (ACD2)
Drought responses/signaling			
B00301	GO:0016020	membrane	Early-responsive to dehydration stress protein (ERD4)
C01684	GO:0016020	membrane	ERD (early-responsive to dehydration stress) family protein
C01964			Drought-responsive family protein
E00337			Drought-responsive family protein
F02921	GO:0005515	protein binding	Drought sensitive 1
I02369			dehydrin family protein
I03747	GO:0016020	membrane	early-responsive to dehydration stress protein

Table 3.3 continued

I04604			drought-induced 19
J00522	GO:0009415	response to water	Dehydrin family protein
Photosynthesis			
B00694	GO:0015979	photosynthesis	photosystem II reaction center protein F
B01600	GO:0015979	photosynthesis	photosystem I subunit E-2
B01982	GO:0016491	oxidoreductase activity	photosynthetic electron transfer B
B01983	GO:0050821	protein stabilization	photosystem II reaction center protein H
B01985	GO:0019684	photosynthesis	photosystem II reaction center protein B
B04306	GO:0015979	photosynthesis	photosystem II reaction center PSB29 protein
C01977	GO:0015979	photosynthesis	photosystem I subunit H2
C02344	GO:0015979	photosynthesis	photosystem II reaction center W
C03895	GO:0015979	photosynthesis	photosystem I subunit l
D01719	GO:0015979	photosynthesis	photosystem II reaction center protein K precursor
D01720	GO:0019684	photosynthesis	photosystem II reaction center protein C
E01442	GO:0015979	photosynthesis	photosystem II reaction center W
G02753			acclimation of photosynthesis to environment
I01499	GO:0015979	photosynthesis	photosystem I subunit F
I03523	GO:0015979	photosynthesis	photosystem I subunit D-2
J02198	GO:0015979	photosynthesis	photsystem I subunit I
Pathogen response			
A00277	GO:0003677	DNA binding	pathogenesis related homeodomain protein A
A01323			Pathogenesis-related thaumatin superfamily protein
A01324			Pathogenesis-related thaumatin superfamily protein
B00229			CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein
B01550			Pathogenesis-related thaumatin superfamily protein
B03645			Pathogenesis-related thaumatin superfamily protein
C04429			Pathogenesis-related thaumatin superfamily protein
C04431			Pathogenesis-related thaumatin superfamily protein
D00161			Pathogenesis-related thaumatin superfamily protein
D00399			Pathogenesis-related thaumatin superfamily protein
E01011			Pathogenesis-related thaumatin superfamily protein
E03567			Pathogenesis-related family protein
F00441			Pathogenesis-related thaumatin superfamily protein
G00031			Pathogenesis-related thaumatin superfamily protein
H00549	GO:0050832	defense to fungus	pathogenesis-related 4

Table 3.3 continued

H00552	GO:0050832	defense response to fungus	pathogenesis-related 4
H01323			Pathogenesis-related thaumatin superfamily protein
H01663			pathogenesis-related gene 1
H02073			Disease resistance-responsive (dirigent-like protein) family protein
H02077			Disease resistance-responsive (dirigent-like protein) family protein
J00932			pathogenesis-related gene 1

Table 3.4 Relationships between gene expression and plant responses identified using maximal information coefficient (MIC). Numbers of genes with significant relationships (FDF-corrected $P < 0.01$) for each plant response. Relationships with Pearson's correlation coefficient $|r| \geq 0.80$ classified as linear.

Physiological trait	Linear		Nonlinear	
	Positive	Negative	Positive	Negative
Total biomass (mg)	0	0	1	1
Growth rate (cm/day)	40	61	171	195
Water loss (ml/day)	0	0	112	98
Days until first wilt	0	0	1	2
Days until tiller death	0	0	1	3

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and roots of *Stellera chamaejasme* L. in northwestern China. *Antonie van Leeuwenhoek* **104,** 949–963 (2013).
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Figures

Figure 1.1 Species accumulation curves showing the number of endophyte taxa captured as a function of number of plants sampled. Each line represents an individual site across three years: (a) 2012, (b) 2013, and (c) 2014.

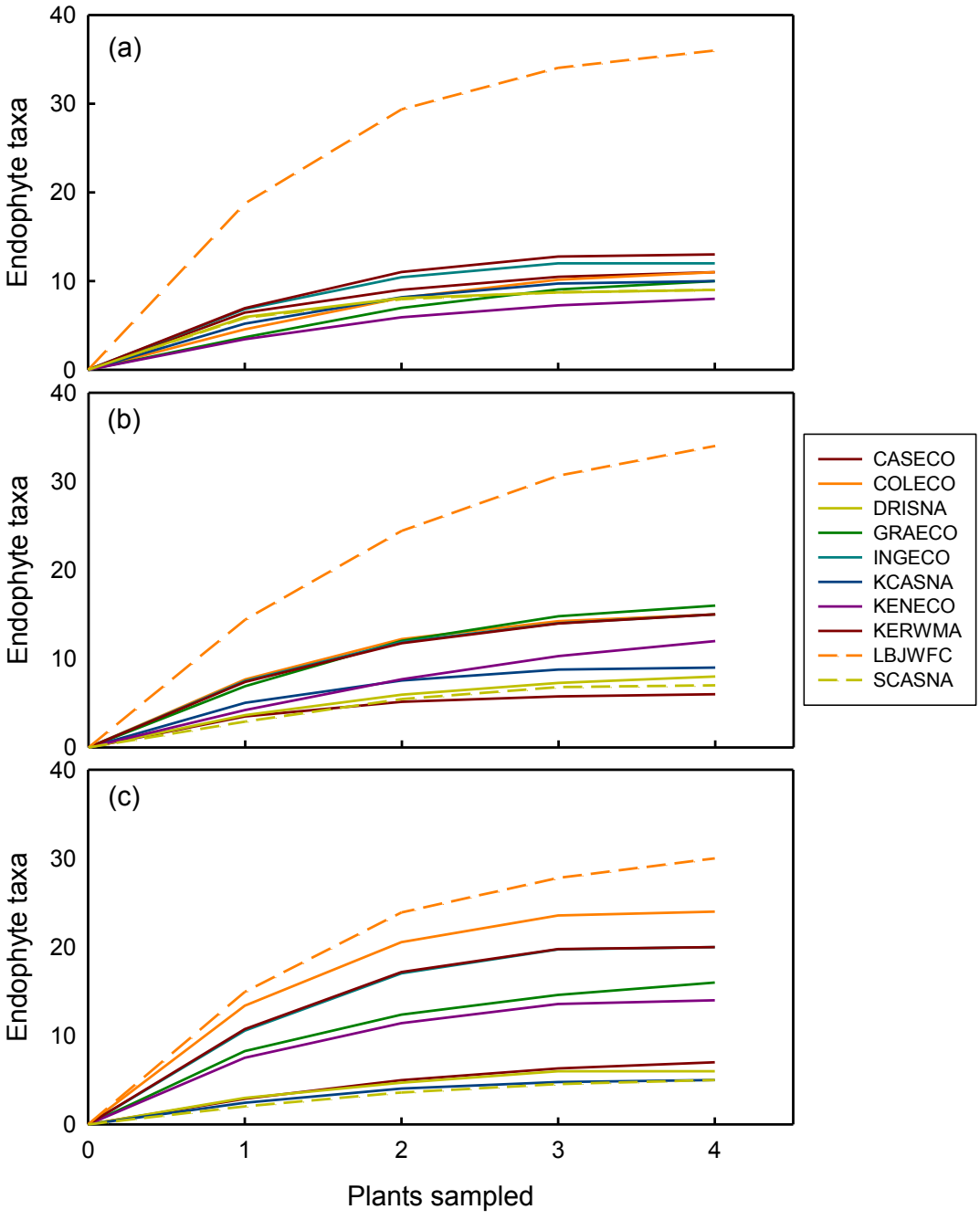


Figure 1.2 Regressions of site-level endophyte richness against mean annual precipitation (MAP, r2 = 0.557, P < 0.001) and mean high temperature (MHT, r2 = 0.120, P < 0.001), which were the two factors identified in stepwise multiple regression analysis.

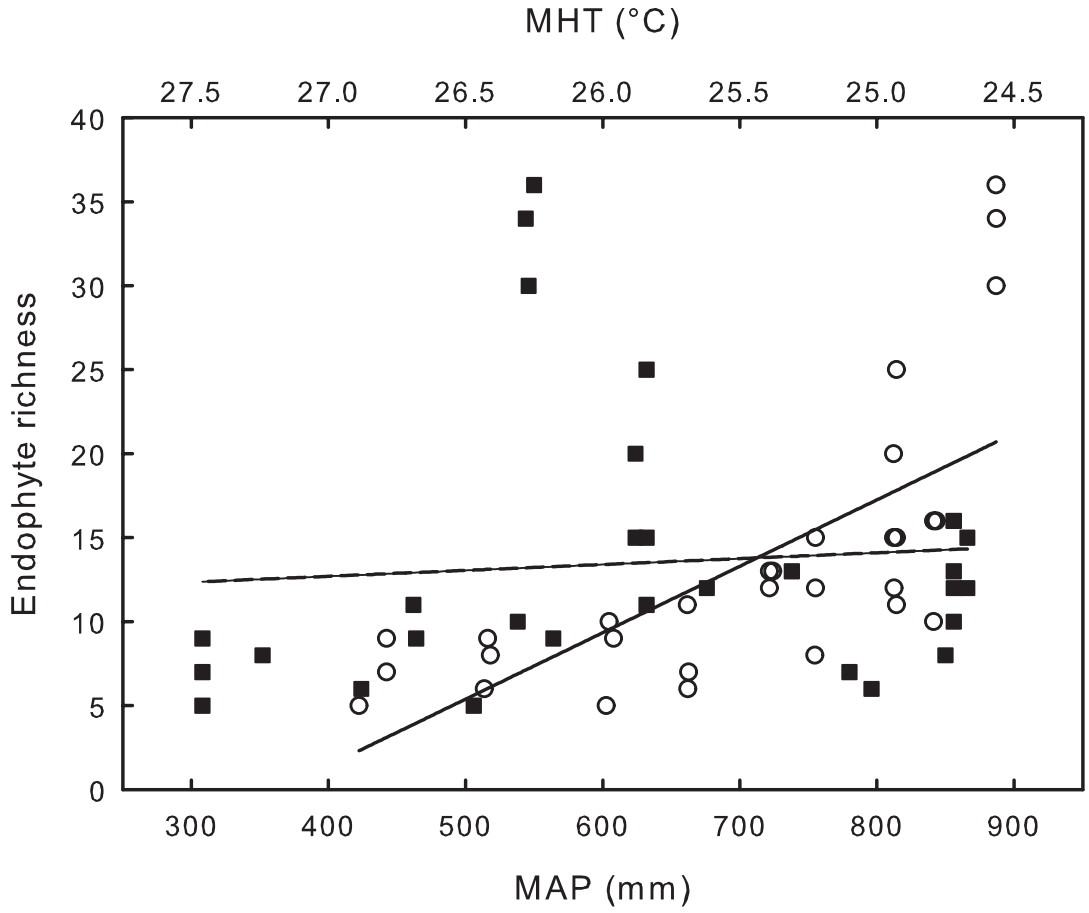


Figure 1.3 Nonmetric multidimensional scaling of endophyte communities by (a, c) site and (b, d) year using (a, b) Bray-Curtis dissimilarities and (c, d) UniFrac distances. Bars are ± 1 SE. Overlaid are vectors for the top three explanatory variables based on the PERMANOVAs, with the length of each vector based on its correlation to the x and y-axes.

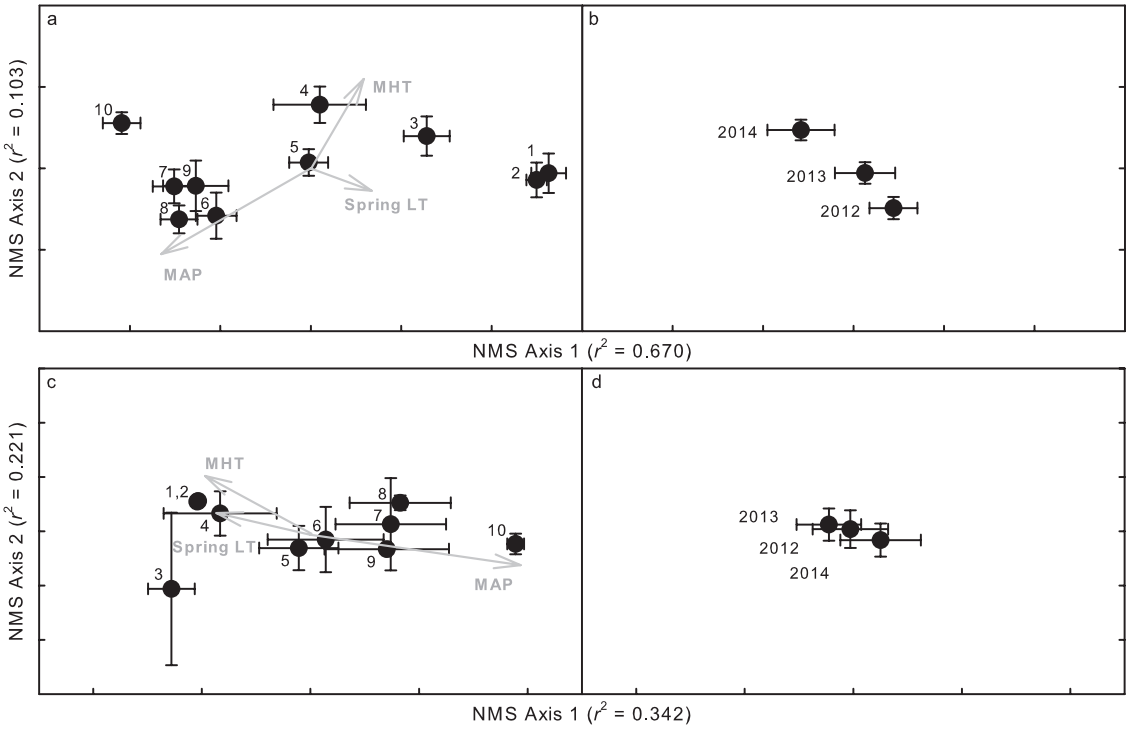


Figure 1.4 Variance decomposition of endophyte community composition with PERMANOVA using Bray-Curtis dissimilarities. Interactions between variables in the same category are stacked with that category and labeled 'Int.' Interactions involving variables in different categories are included in the Interactions category. Note that for interactions, 'Plant' includes both host plant traits and vegetation structure, 'Climate' refers to historical climate variables and 'Weather' refers to annual weather variables. Other abbreviations are as follows: MAP = mean annual precipitation, MHT = mean annual high temperature, MLT = mean annual low temperature, LT = low temperature, HT = high temperature, Moist = soil moisture, Flw = flowering. Detailed PERMANOVA results are in Table S5.

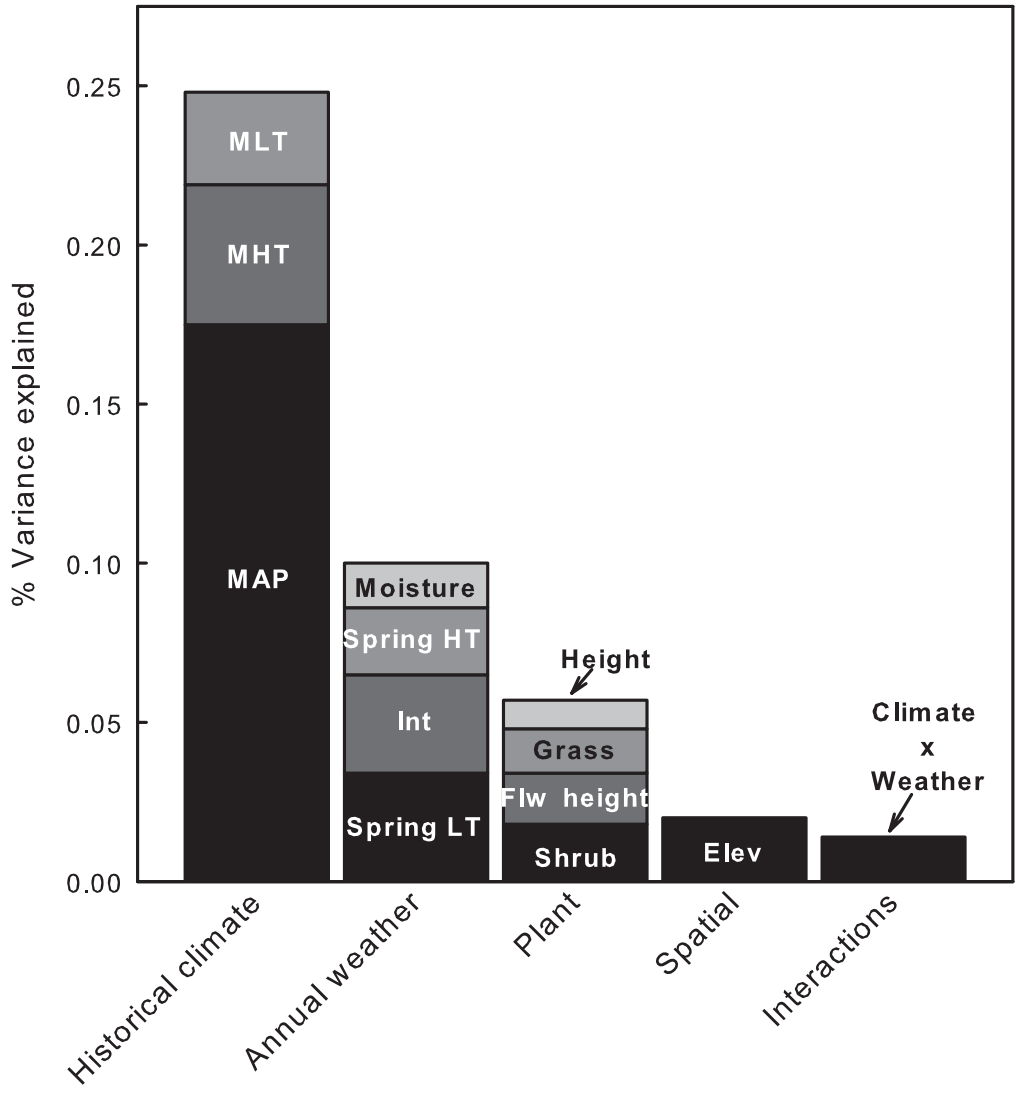


Figure 2.1 Log response ratios for the main effects of fungal colonization, water treatment, and their interaction on plants. Error bars are 95% confidence intervals (CI); LRR values are only considered significantly different from zero when the 95% CI does not overlap zero. For fungal treatment, LRR > 0 and < 0 reflect a larger or smaller effect of fungus on plant responses relative to fungus-free control plants. For moisture treatment, LRR > 0 and < 0 indicate larger or smaller plant responses in low relative to high soil moisture.

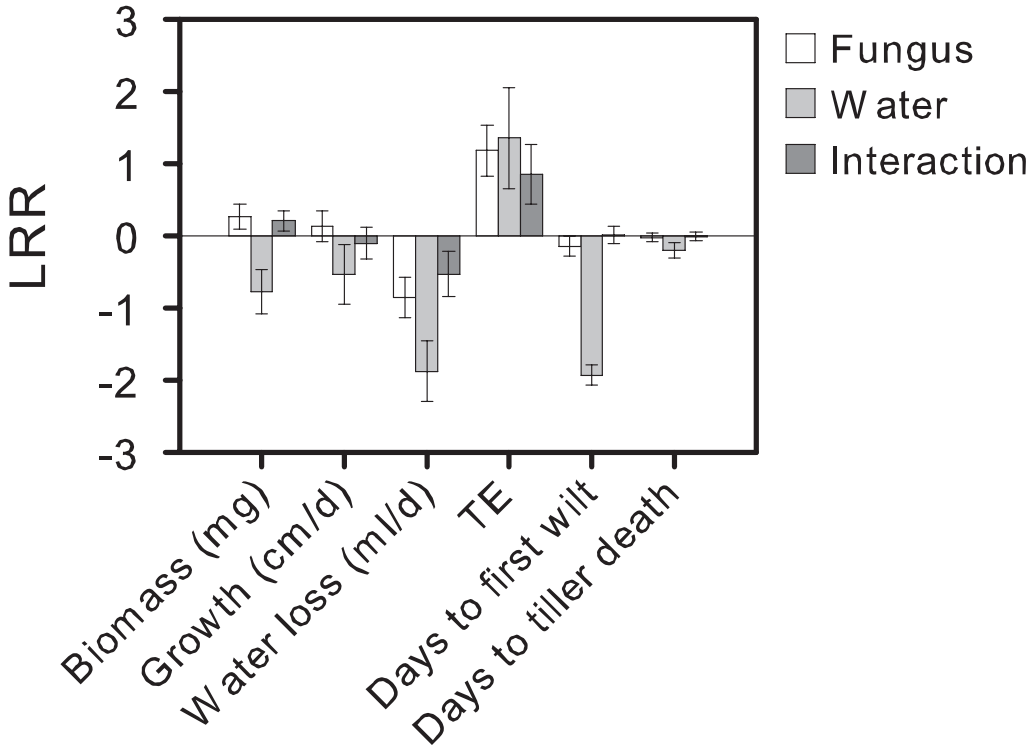


Figure 2.2 LRRs of plant responses for symbiont treatment, water treatment, and each component of the interaction term. Each bar represents a single plant-fungal pair and bars are ordered from left to right on the x-axis based on location on the phylogeny (Figure S5). Error bars are 95% confidence intervals.

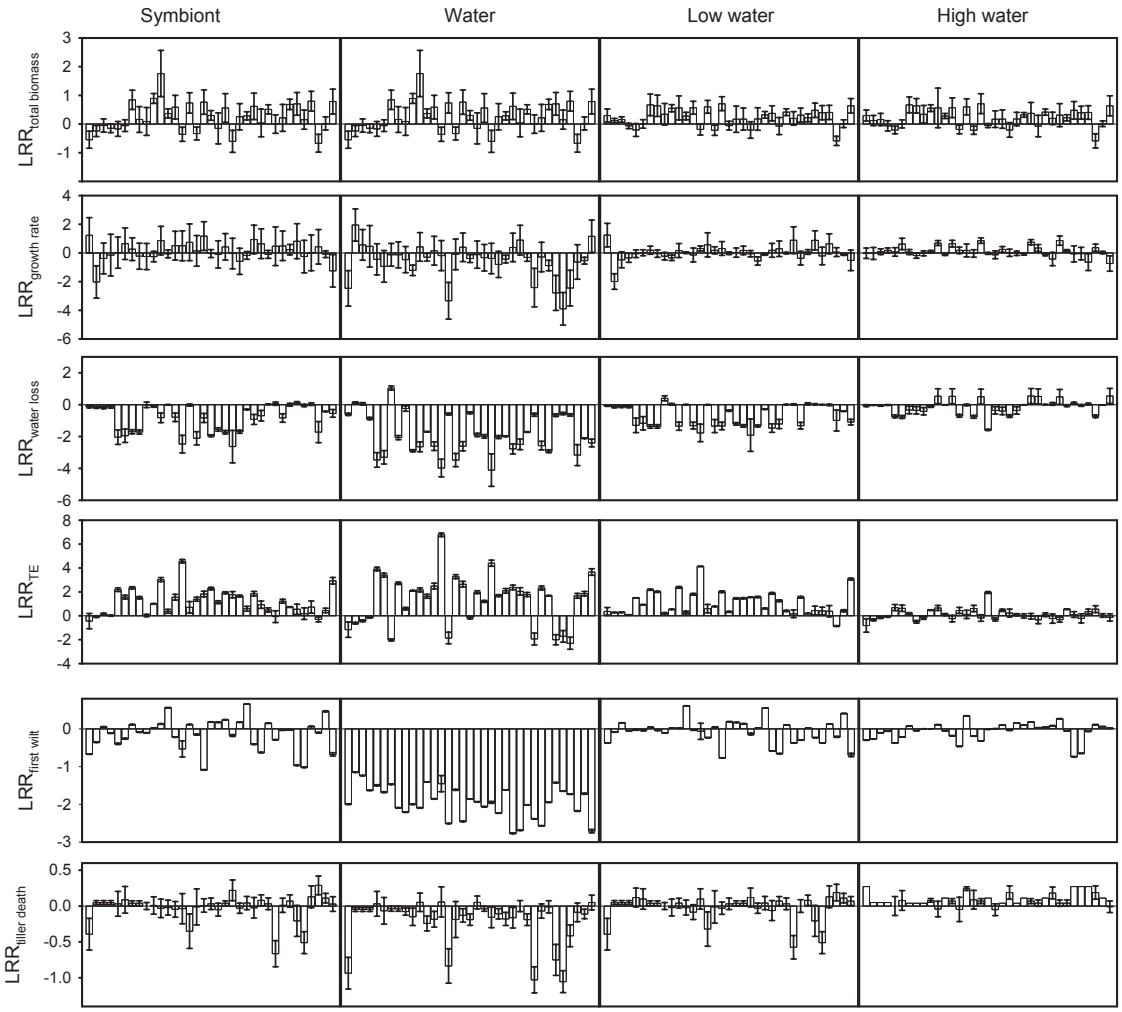


Figure 2.3 Symbiosis outcomes. Phylogenetic PCA (PPCA) for plant responses based on $LRR_{symbiont}$, LRR_{water}, and $LRR_{interaction}$. Each point represents a single plant-fungal treatment. Symbiosis outcome groups from k-means clustering are indicated by Roman numerals, with 95% confidence ellipses colored based on symbiotic strategy (I = blue, II = yellow, III = gray, and IV = purple). Eigenvectors are included for the top 3 plant responses that were correlated ($P < 0.05$) to one or both of the PPC axes. Letters identify specific variables (WL = water loss, TE = transpiration efficiency, DD = days to first tiller death).

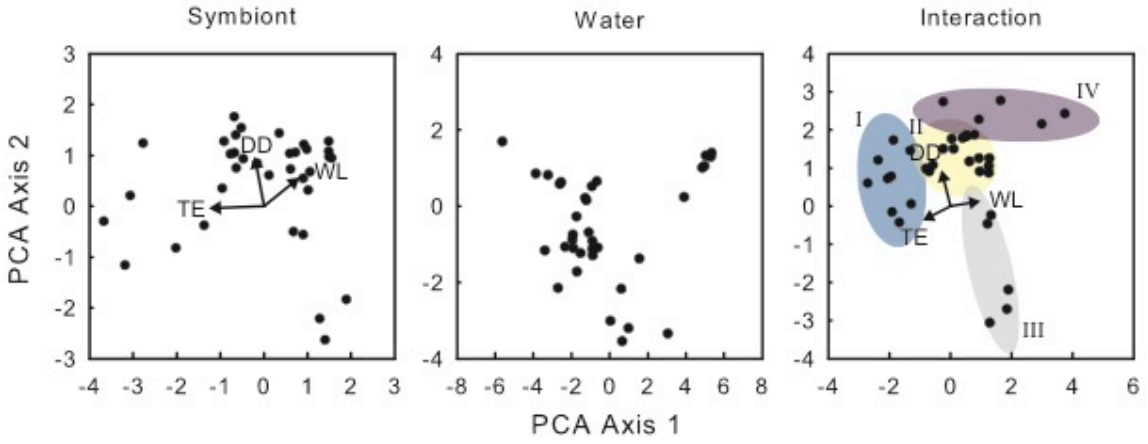


Figure 2.4 Symbiosis outcomes. Phylogenetic PCA (PPCA) for plant responses based on the $LRR_{low\ water}$ and $LRR_{high\ water}$. Each point represents a single plant-fungal pairing. Symbiosis outcome groups from k-means clustering are indicated by Roman numerals, with 95% confidence ellipses colored based on symbiotic strategy (I = blue, II = yellow, III = red, and IV = purple). Eigenvectors are included for top 3 plant responses (top panels) and fungal traits and habitat variables (bottom panels) that were correlated ($P < 0.05$) to one or both of the PPC axes. Letters identify specific variables (R:S = root: shoot ratios, WL = water loss, TE = transpiration efficiency, DD = days to first tiller death).

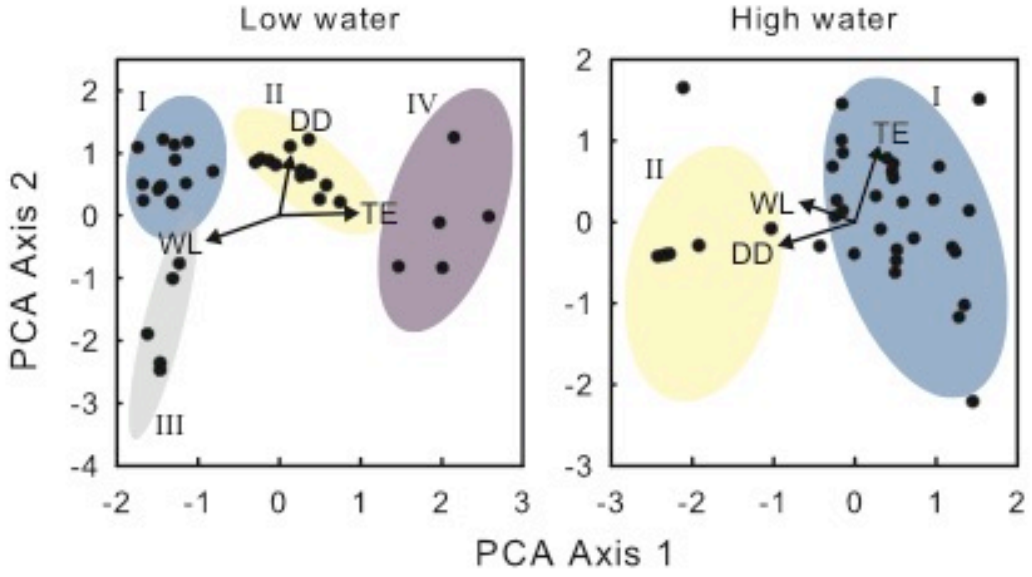


Figure 2.5 Relationships between plant responses and symbiont clusters. Multinomial logistic regressions predict the most likely plant response values for any given symbiotic outcome under low water relative to high water ($LRR_{interaction}$), each indicated by a colored line (I: Water use mutualist = blue, II: Survival mutualist = yellow, III: Survival antagonist = gray, IV: Mixed mutualist = purple).

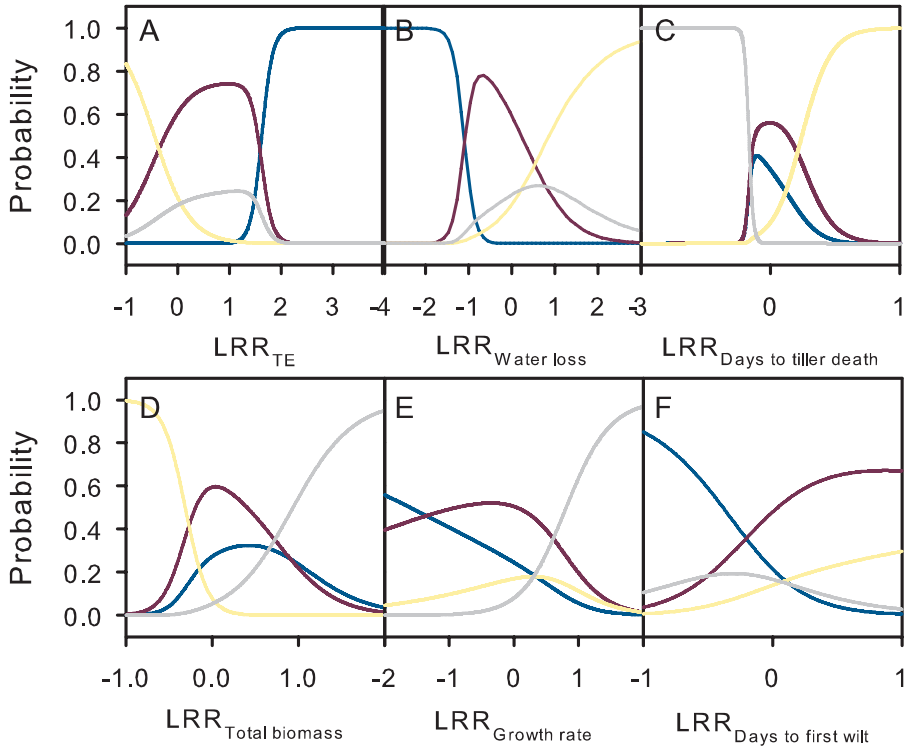


Figure 2.6 Relationships between plant responses and symbiont clusters. Multinomial logistic regressions predict the most likely plant response values for any given symbiotic outcome under low water ($LRR_{low\ water}$), each indicated by a colored line (I: Water use mutualist = blue, II: Survival mutualist = yellow, III: Survival antagonist = gray, IV: Mixed mutualist = purple).

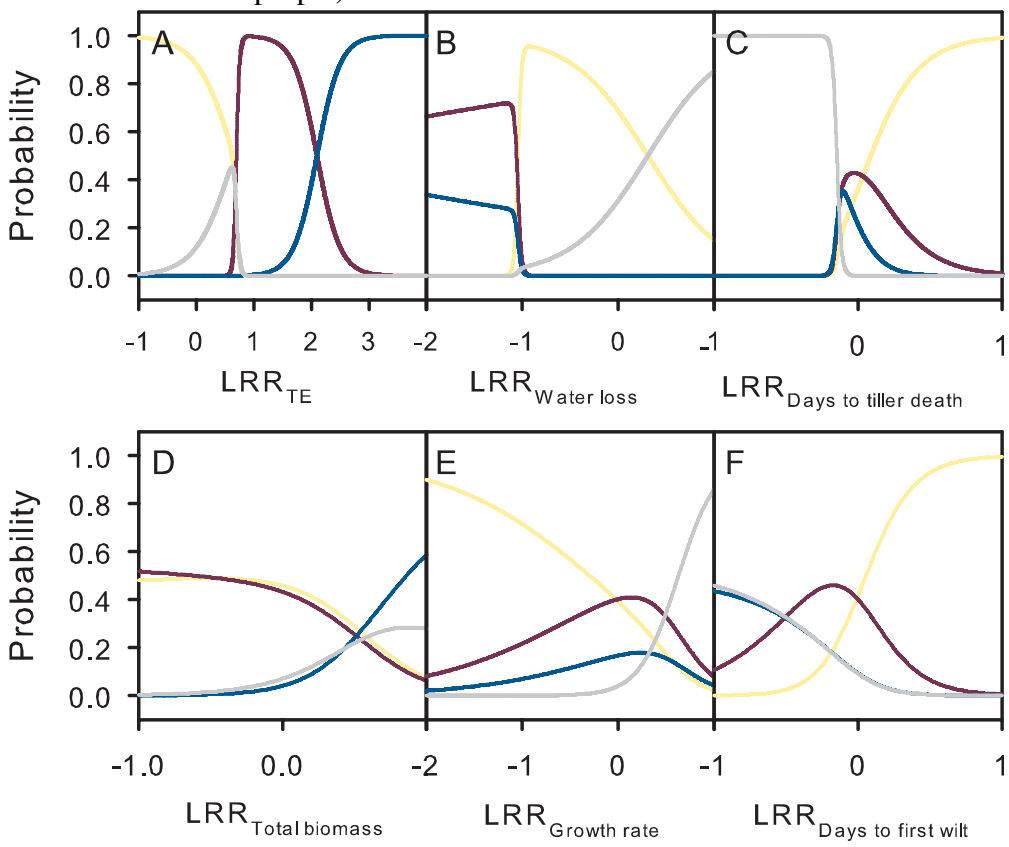


Figure 2.7 Phylogenetic signal of fungal effects on plants. Heatmaps represent the LRR for plant responses (columns) for each fungal isolate arranged phylogenetically (rows). Each LRR is standardized from –1 (red) to 1 (blue). Abbreviations are S = $LRR_{symbiont}$, W = LRR_{water}, I = $LRR_{interaction}$, L=$LRR_{low\ water}$, and H = $LRR_{high\ water}$. Blomberg's K values were < 0.5 and Pagel's λ values were < 0.1 with *P*-values > 0.05 for all LRRs.

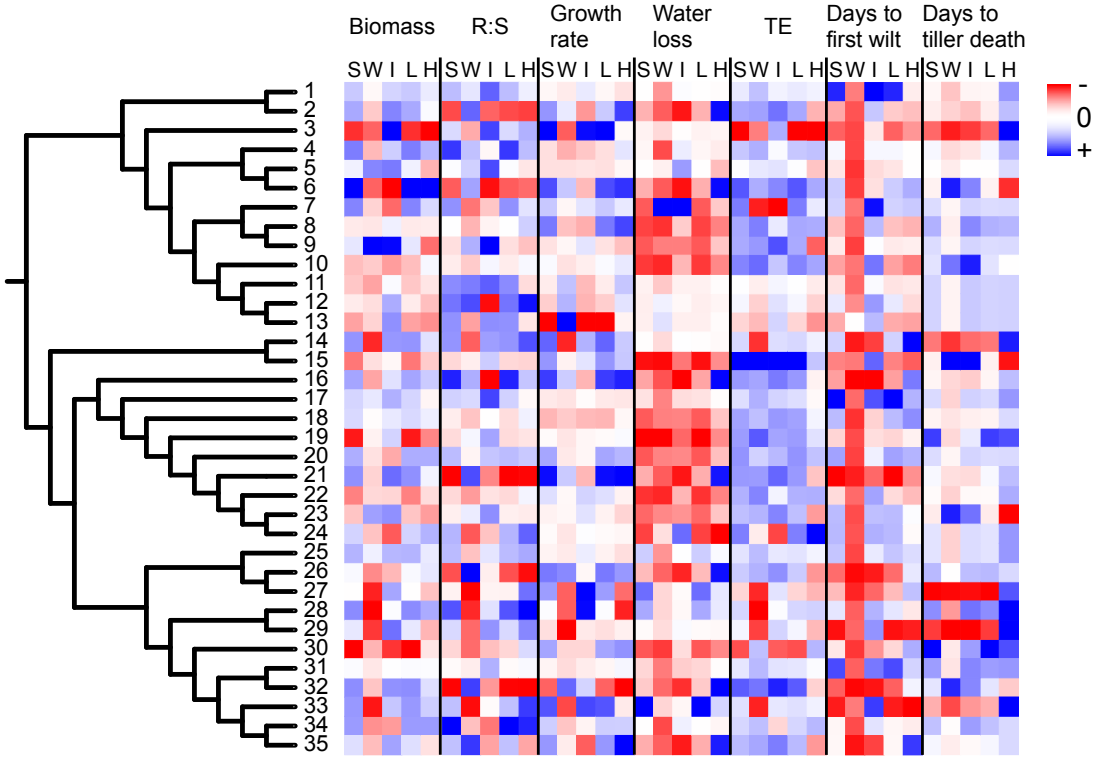


Figure 2.8 Phylogenetic signal of fungal traits. Heatmap of fungal traits (columns) by fungal taxa arranged phylogenetically (rows). Trait data were log-transformed. Trait abbreviations are: T = osmotic threshold, S = osmotic sensitivity, A = ACC concentrations, C = carbohydrates, AA = amino acids, A = amines, CA = carboxylic acids, P = polymers, B = biomass, CB = cellobiohydrolase, and AG = α-1,4-glucosidase, Trait values were standardized to range from -1 (black) to 1 (green) for traits with negative values or 0 (white) to 1 (green) for traits with only positive values. All measured traits had Blomberg's K values < 0.5 and Pagel's λ values < 0.1 with P-values > 0.05.

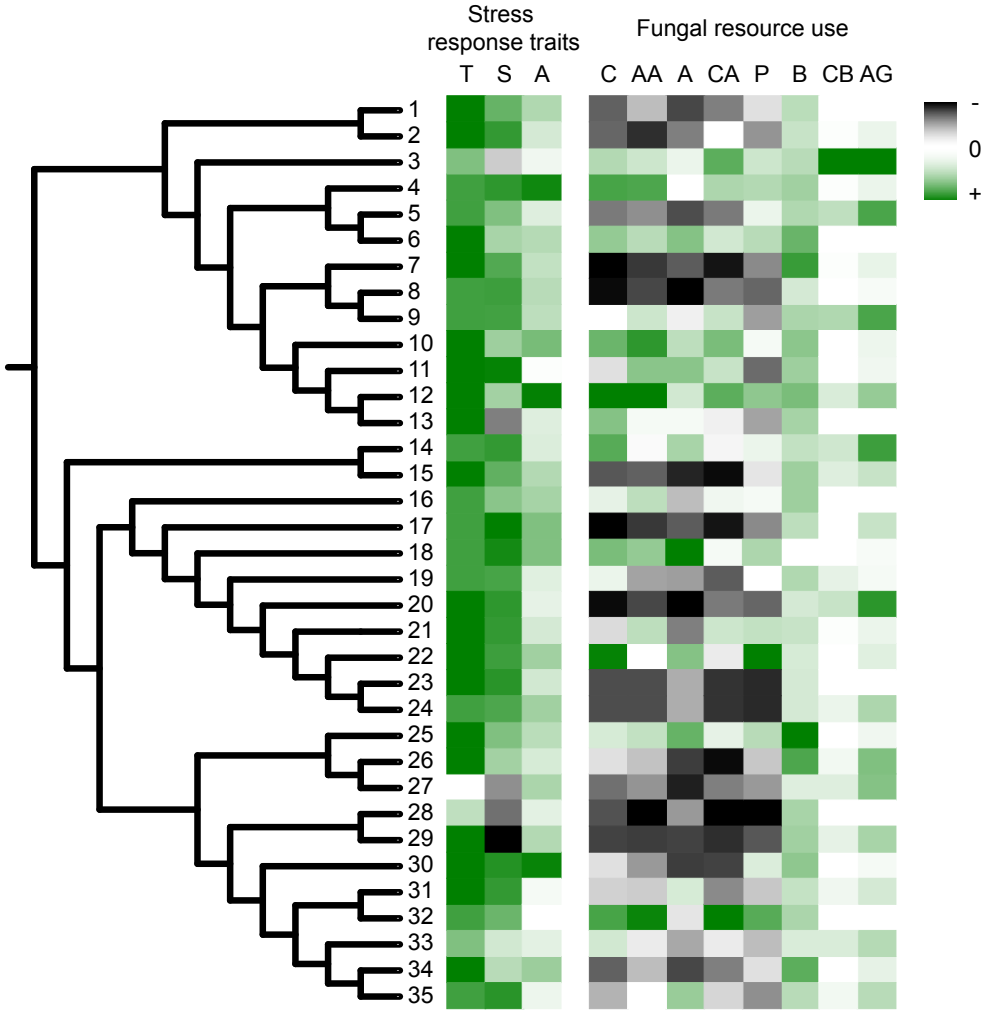


Figure 2.9 Fungal traits. Each bar represents a single fungal isolate and bars are ordered from left to right on the x-axis based on location on the phylogeny (Figure S5). Bars are color-coded based on symbiont outcome (I: Water use mutualist = blue, II: Survival mutualist = yellow, III: Survival antagonist = gray, IV: Mixed mutualist = purple).

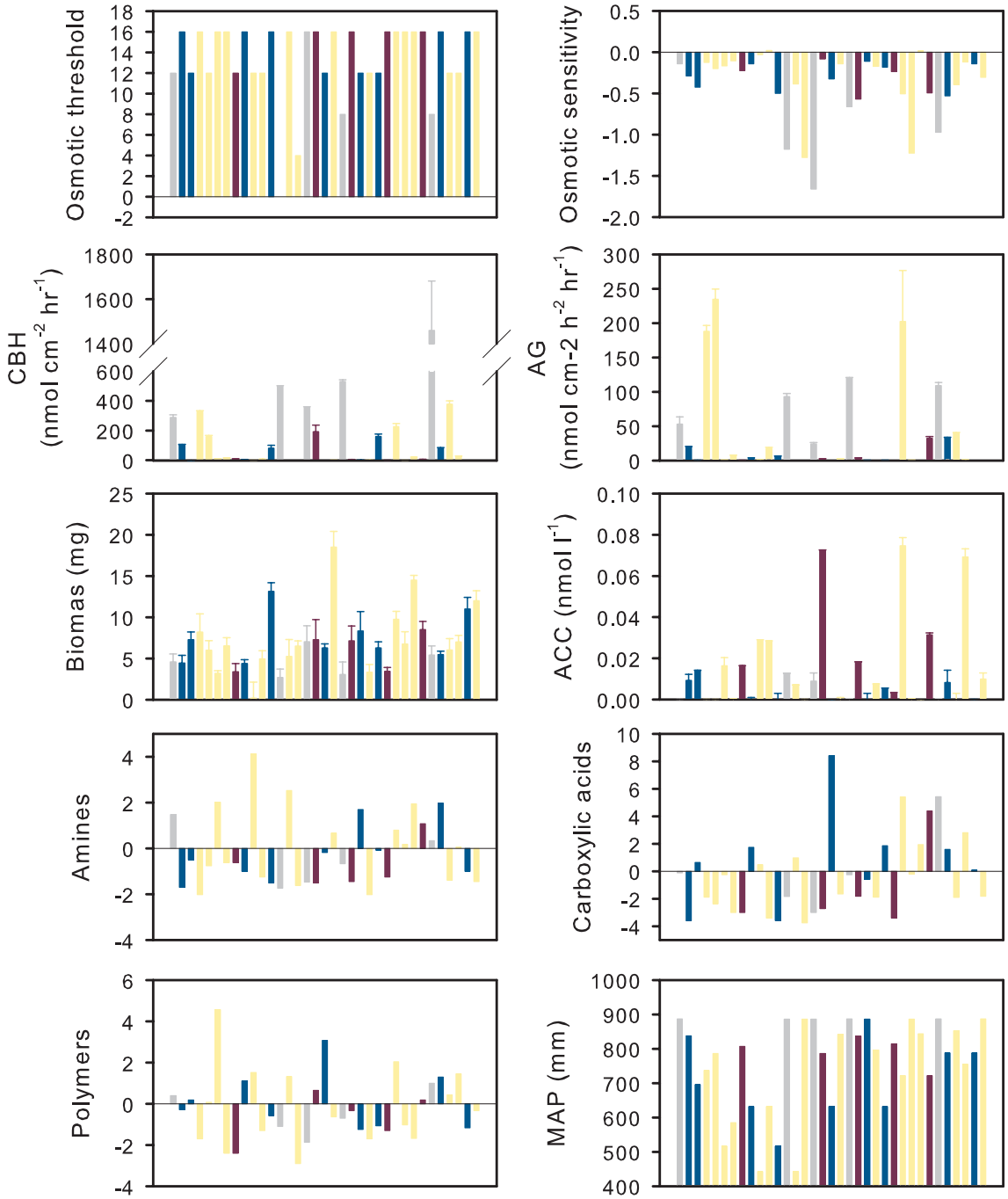


Figure 2.10 Relative importance of fungal phylogeny, traits, and host habitat in predicting the outcome of plant-fungal interactions. Redundancy analyses were performed to determine the contribution of fungal traits (T), habitat origin characteristics (H), phylogenetic distance (P), and their interactions to plant responses based on $LRR_{symbiont}$, LRR_{water}, and $LRR_{interaction}$ ($P < 0.05$). Non-labeled areas are non-significant ($P > 0.05$). Shapes are approximately proportional to the magnitude of the effect size.

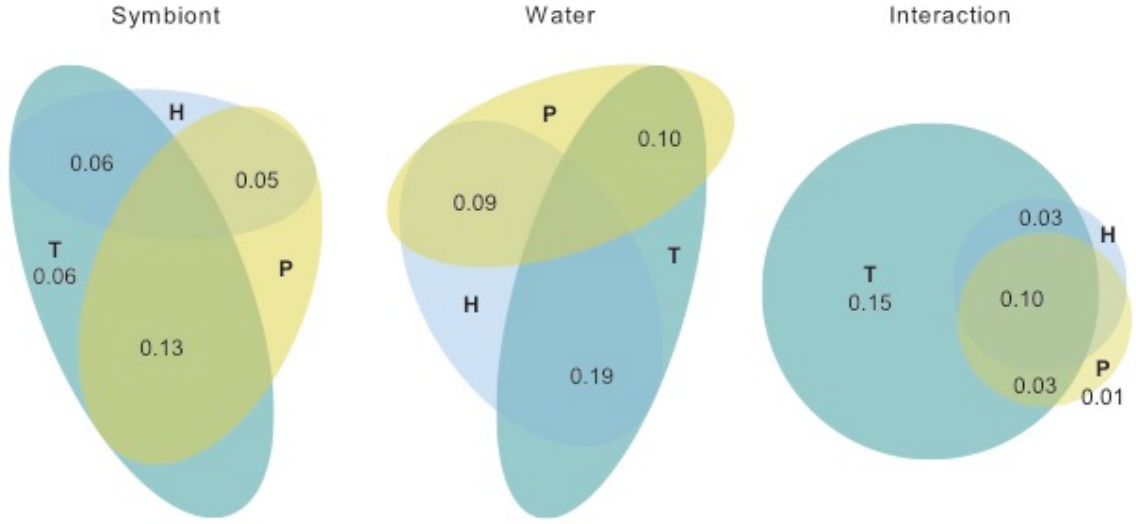


Figure 2.11 Relative importance of fungal phylogeny, traits, and host habitat in predicting the outcome of plant-fungal interactions. Redundancy analyses were performed to determine the contribution of fungal traits (T), environmental variables (H), phylogenetic distance (P), and their interactions to plant responses based on both components of the interaction term ($P < 0.05$). Non-labeled areas are non-significant ($P > 0.05$). Shapes are proportional to the magnitude of the effect size.

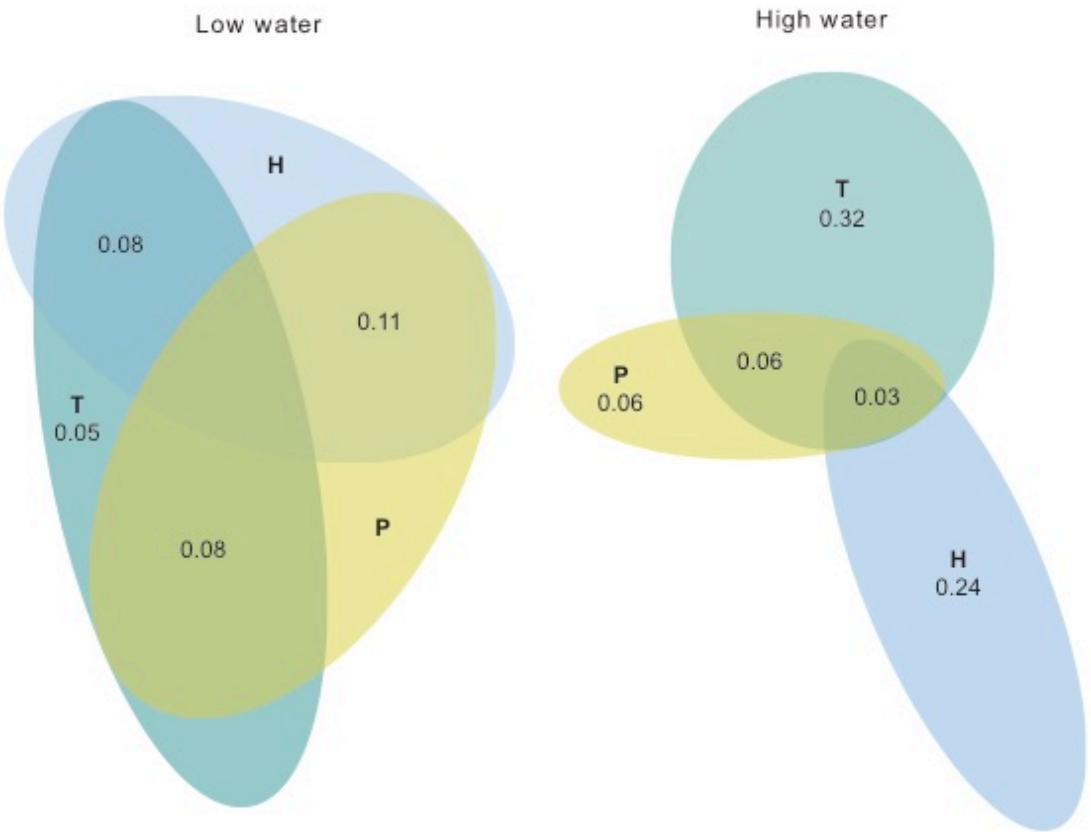


Figure 3.1 Differences in (and whole-plant biomass, height growth rate, rate of plant water loss, days to first wilt, and days to first death at low (5%) and high (255%) soil moisture**.** Individual taxa are designated by different colors: control = black, Isolates: 5 = orange, 18 = green, 44 = dark blue, 47 = yellow, and 61 = light blue. Bars are ±1 SE.

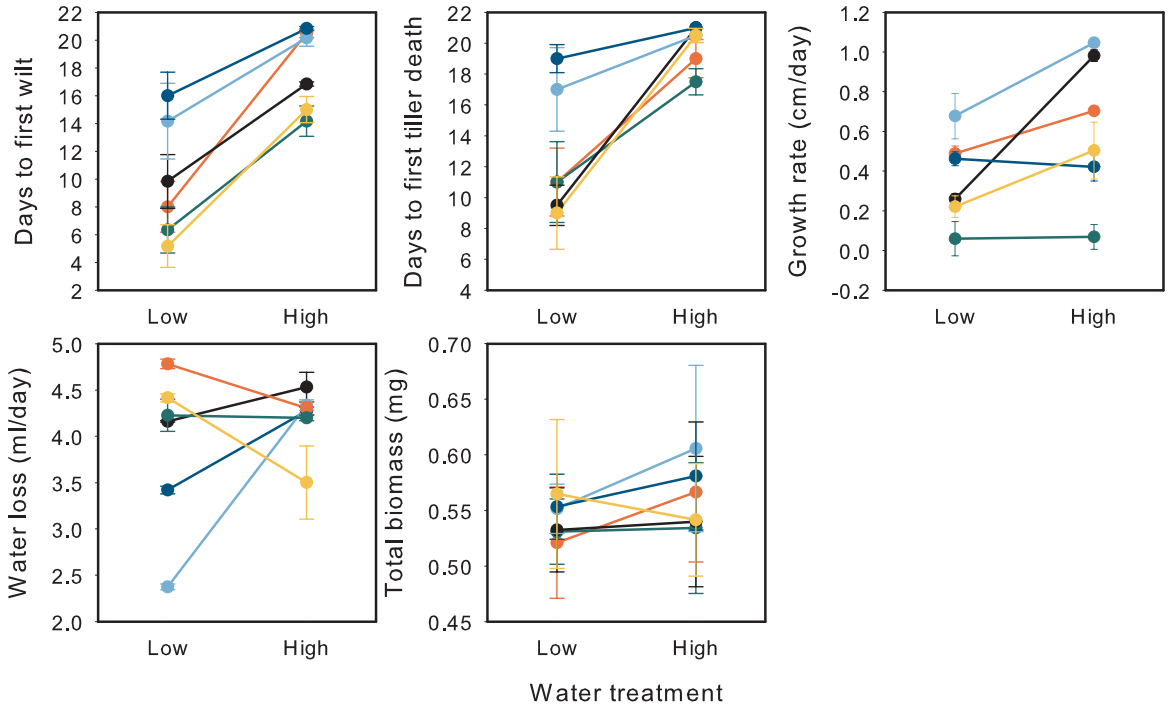


Figure 3.2 Differential gene expression due to fungal colonization by water treatment. Differential expression was characterized via "volcano" plots, where the $\log_2$ fold change of treatment contrasts is plotted on the x- axis and the log10-transformed *P*-value of the associated test is on the y-axis for low water (A, C, E, G, and I) and high water (B, D, F, H, and J). Points were colored by whether the FDR-corrected *P*-value exceeded $\alpha = 0.01$ threshold. The number of significant genes for each of the contrasts were plotted in Euler diagrams (K-O), where circle size is proportional to the number of genes that were significant for each water treatment contrast in each fungal isolate.

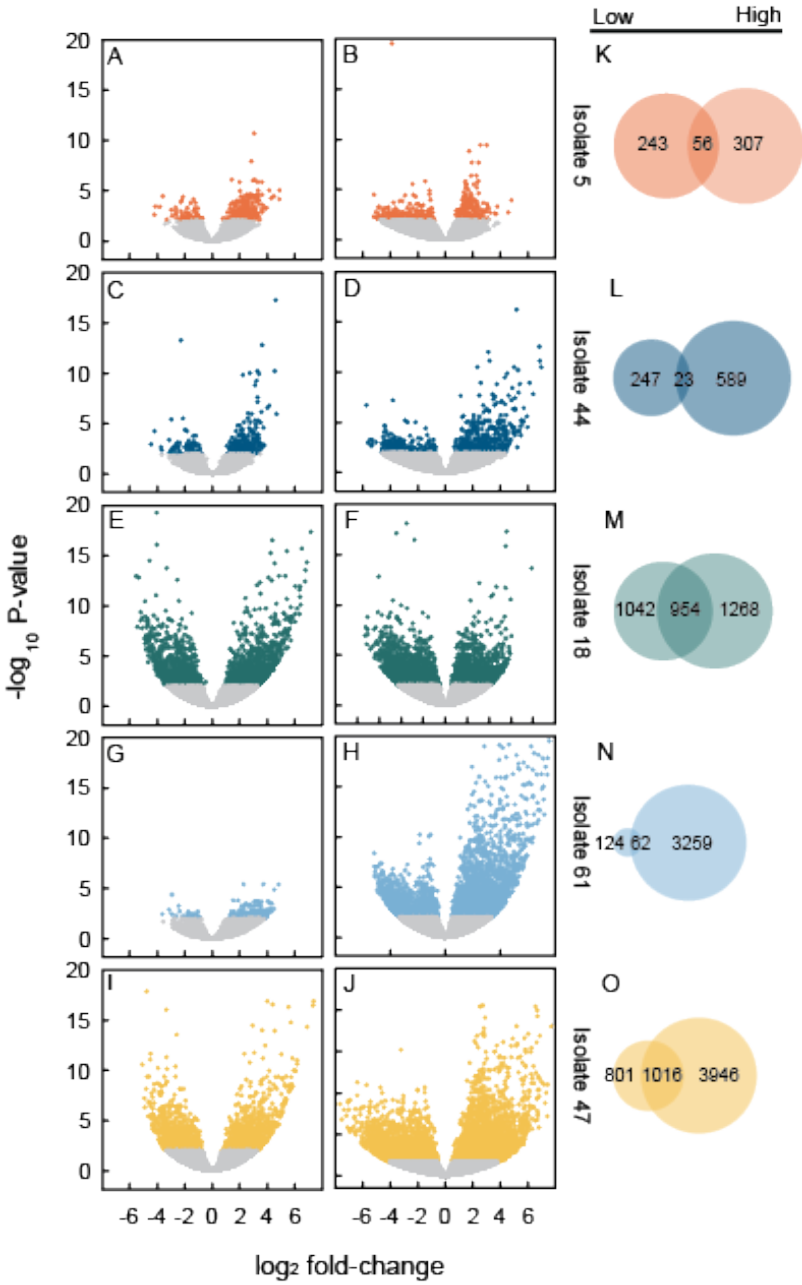


Figure 3.3 Differential gene expression among fungal isolates. Venn diagrams listing the number of genes that were significant for each water treatment (A = low water, B = high water) in each fungal isolate (Isolates: 5 = orange, 18 = green, 44 = dark blue, 47 = yellow, 61 = light blue).

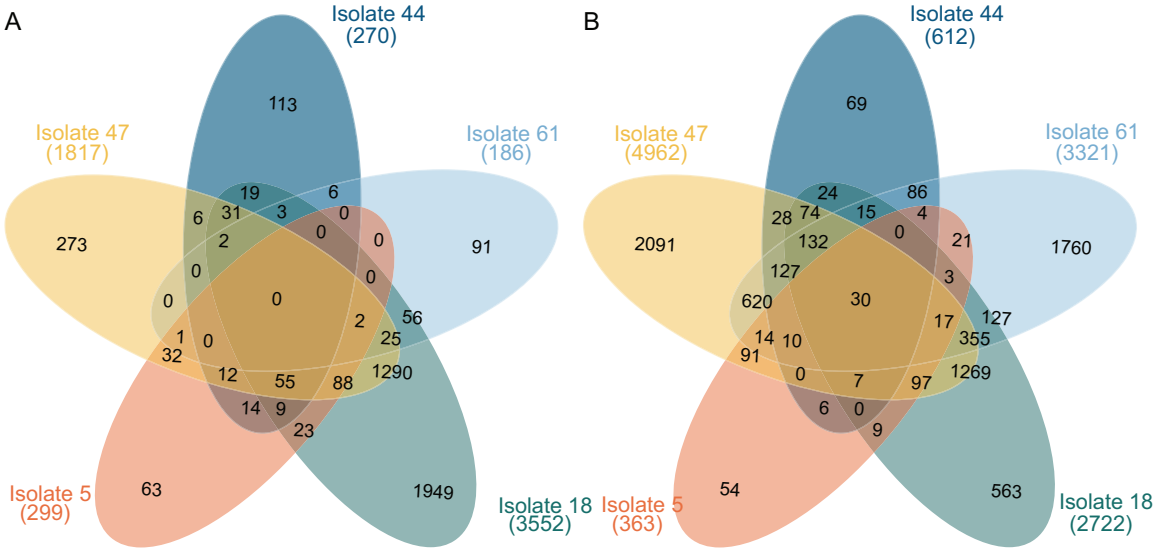


Figure 3.4 Principal components analysis (PCA) plot of overall plant gene expression. (A) PCA on normalized gene expression counts from DESeq2, (B) Linear regression of PCA axes and plant growth rate, and (C) Linear regression of differentially expressed target genes and plant growth rate. Symbols indicate water treatments (▼= low water, ▲ = high water) and fungal treatments (control = black, Isolates: 5 = orange, 18 = green, 44 = dark blue, 47 = yellow, 61 = orange). In (C), open and closed symbols reflect the two genes.

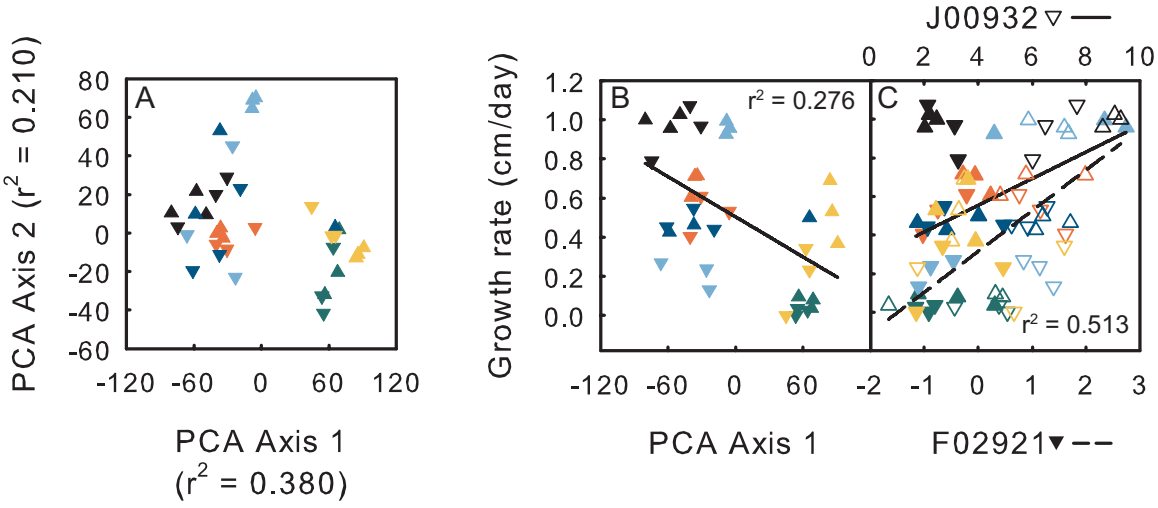


Figure 3.5 Functional categories responding differentially under drought stress to fungal colonization. Functional categories were assigned based on GO term and we focused on genes involved in cellular metabolism (A), transcription and translation (B), and stress responses (C). Within each category the proportions of genes that were significantly up- or down-regulated are coded red and blue respective. Stable genes are coded in gray. Each column reflects differential gene expression from one isolate (PEN = 44, NIG = 61, SOR = 47, CER = 18, COC = 5).

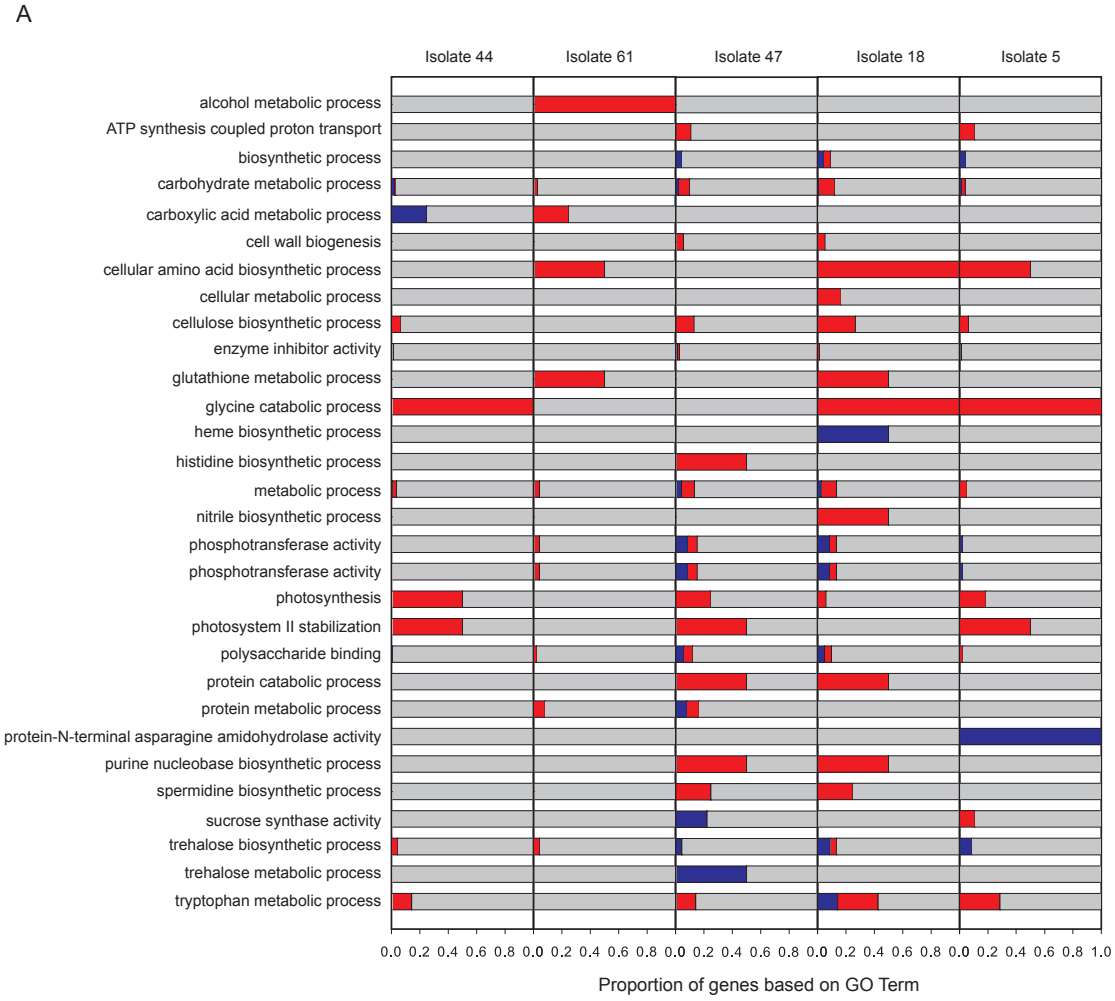
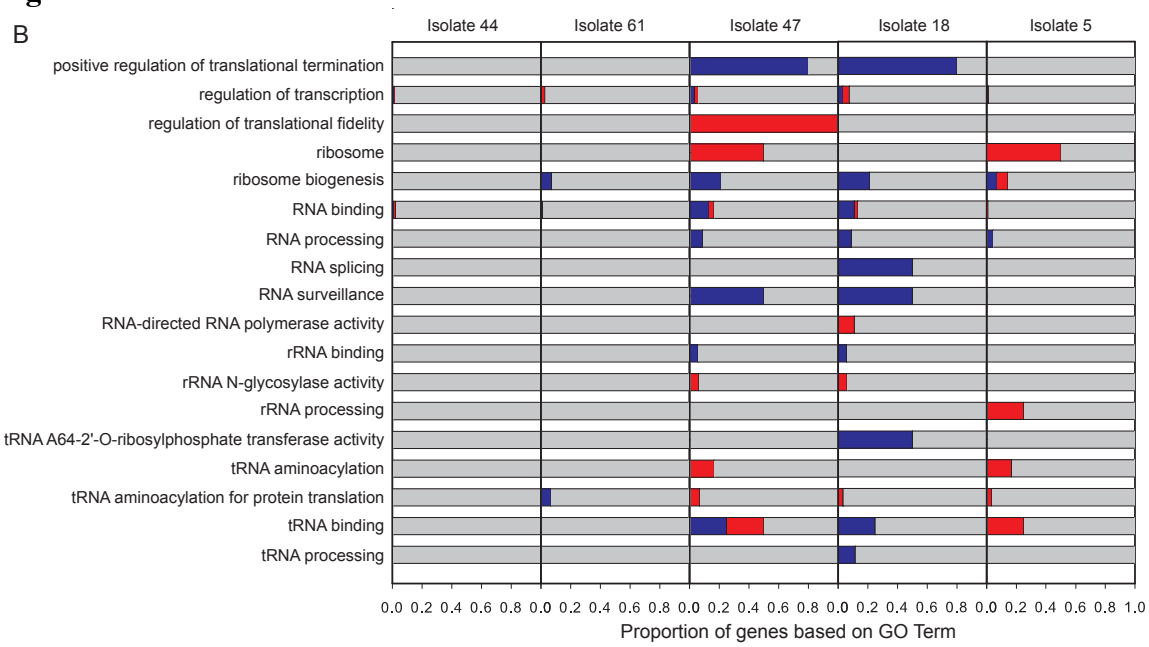


Figure 3.5 continued



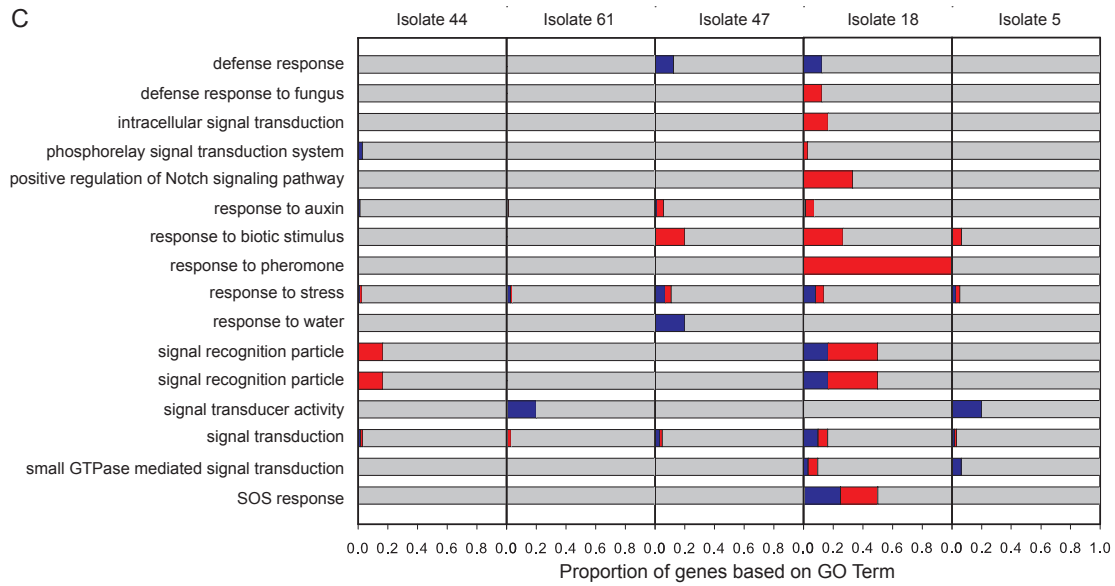


Figure 3.6 Heatmap with cluster dendrogram of differentially expressed target genes. Log2-fold changes show overexpressed (red) and underexpressed (blue) genes (rows) based on fungal colonization (columns) relative to sterile controls under low water (A) and high water (B) conditions. Gene IDs are colored based on gene function. Unsupervised clustering groups genes into similarly expressed clusters.

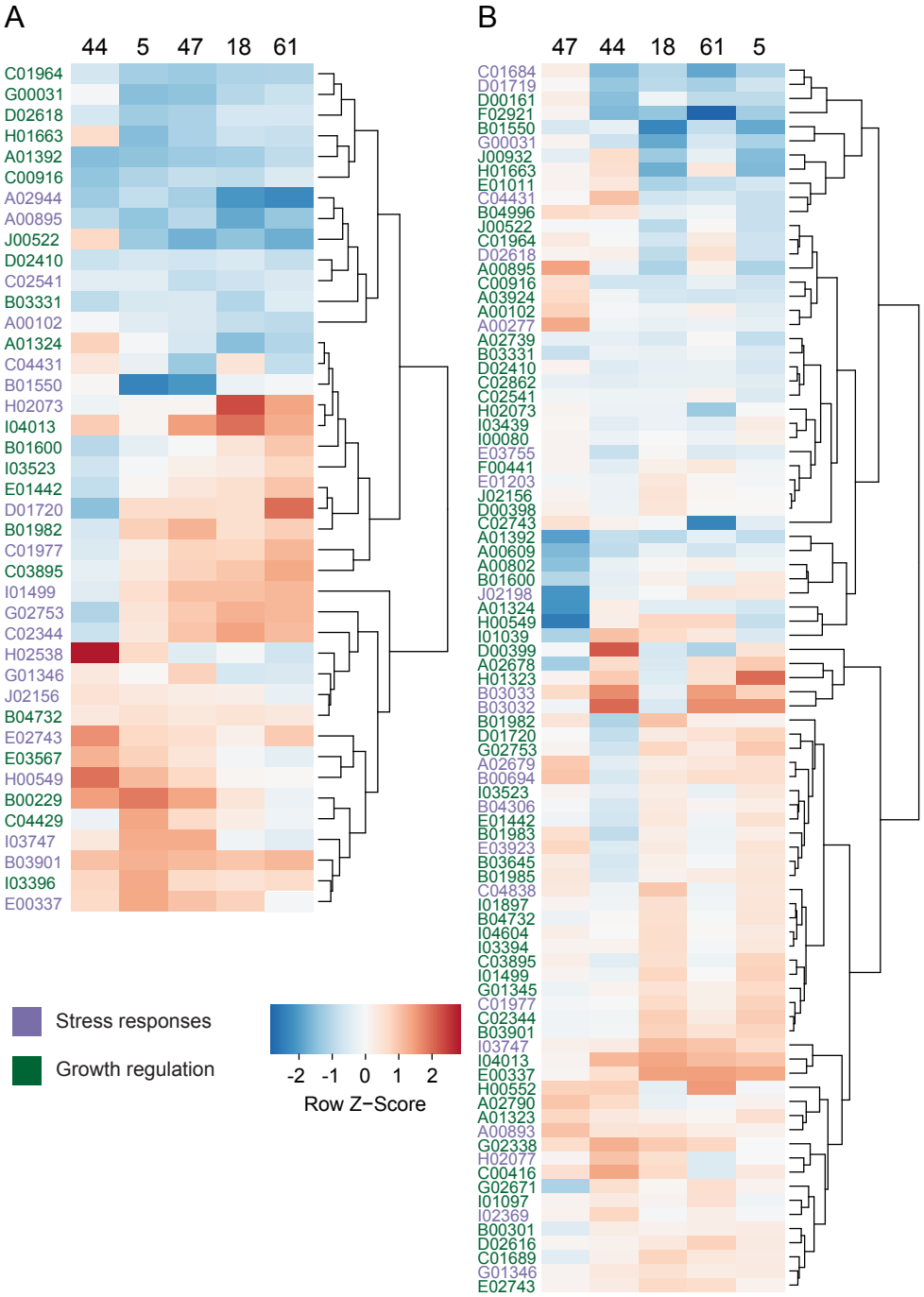
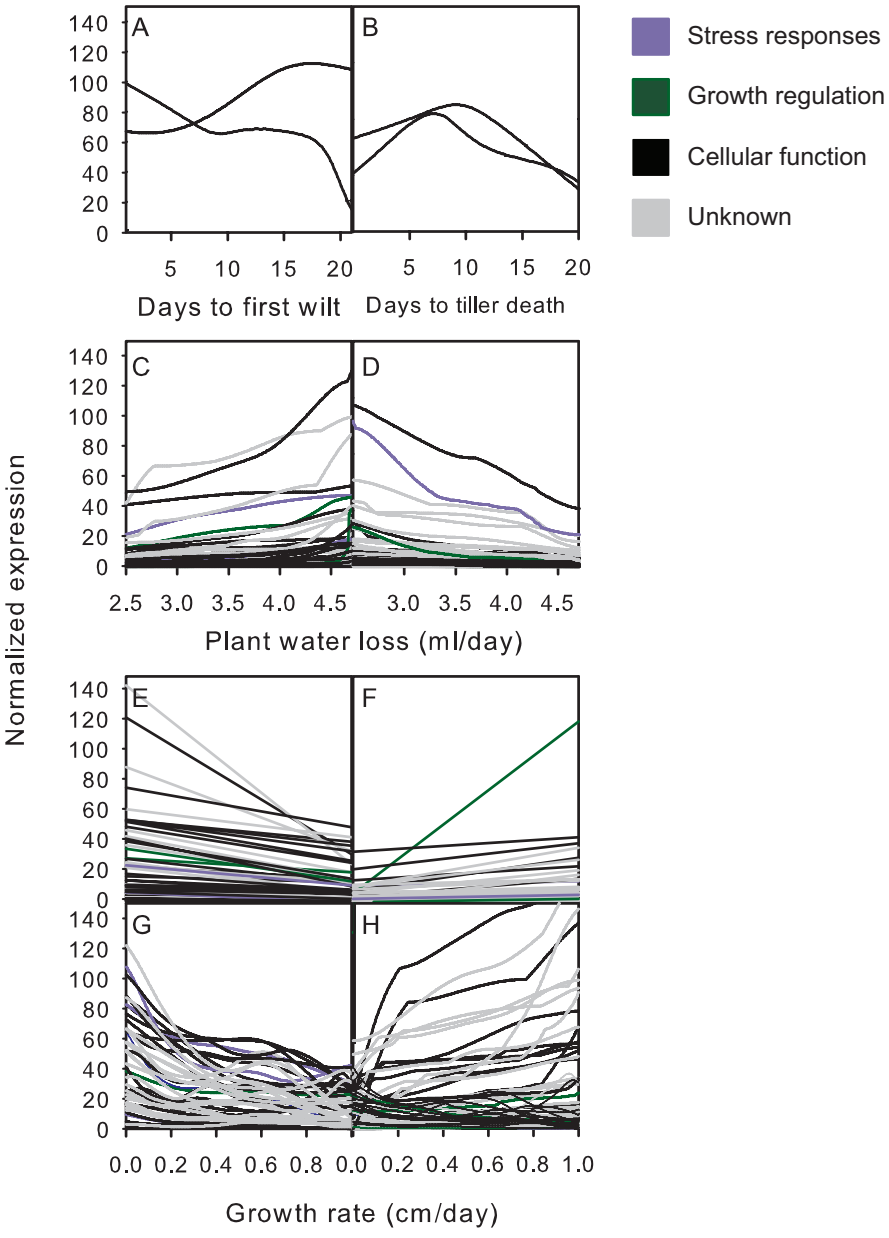


Figure 3.7 Non-linear and linear relationships between gene expression and plant responses. Days to first wilt (A), days to tiller death (B), and water loss (C-D) had only non-linear relationships (FDR-corrected $P < 0.01$, $|r| < 0.8$). Relationships between genes and growth rate were both linear (E-F; FDR-corrected $P < 0.01$, $|r| > 0.8$) and non-linear (G-H). Non-linear relationships were approximated using local polynomial regression. Relationships are color-coded based on known gene function as assigned by GO term or function in *A. thaliana*.



Appendix 1

Characterizing symbiosis outcomes for fungal endophytes

From 2011 to 2014, we isolated fungal endophytes from leaf tissue of two native, C4 bunchgrasses (*Panicum hallii, Panicum virgatum*) across a steep rainfall gradient in central Texas (Giauque and Hawkes 2013, 2016). For the current study, we also included endophytic fungi from one additional native C4 bunchgrass (*Sorghastrum nutans*) sampled from a single location in central Texas. All plant sampling, fungal isolation, and fungal identification occurred as described in Giauque and Hawkes (2013, 2016). Briefly, 3-4 tillers per plant were subsampled for three 2-mm fragments, which were surface sterilized and placed on petri dishes containing 2% potato dextrose agar (PDA). Once in pure culture, 1-cm x 2-cm diameter subsamples were removed for (1) plant-endophyte trials (2) long-term storage in 2 ml of RNase/DNase-free water at room temperature for trait assays (Burdsall, 2003), and (3) immediate DNA extraction (Giauque & Hawkes, 2013). To identify fungal isolates, we used both the ITS and LSU regions of rDNA, as in Giauque & Hawkes (2016), because these capture both variable and conserved regions to allow for robust alignments. Sequences were trimmed, quality checked, and clustered into operational taxonomic units (OTUs) based on 97% sequence similarity (Giauque & Hawkes 2016).

In a previous study, we characterized the effects of 20 fungi on plant responses to water availability (Giauque & Hawkes, 2013). For the current work, we screened an additional 15 fungal isolates using the same methods. Briefly, *P. virgatum* seedlings were grown with single fungal isolates or with no fungi (as a sterile control) and subjected to high (15%) or low (3%) soil moisture conditions. Each treatment combination was replicated five times for a total of 410 plants. To estimate evaporative water losses, a

further ten replicates without plants were included with five for each moisture treatment in each trial. There were a total of six trials to test all 35 taxa. We measured whole-plant water loss, number of wilt-free days, plant height, and number and status of tillers at three-day intervals (days 1, 4, 7, 10, 13, 16, 19). Whole-plant water loss was measured as the loss of water by weight from each planting box at each time point, adjusted for average water loss from plant-free controls during the same time period (Meurs & Stranghellini, 1992). The average rate of water loss was calculated over time (g water d^{-1}). Relative growth rate was calculated based on the change in plant height over time (cm d^{-1}). On day 19, shoots and roots were harvested, dried, and weighed. Whole-plant transpiration efficiency (TE) was calculated as total plant biomass divided by total water loss (mg ml^{-1}).

Plant sampling

From 2011 to 2014, we sampled fungal endophytes from five native, C4 bunchgrasses (*Panicum hallii, Panicum virgatum*, *Andropogon gerardii, Schizachyrium scoparium* and *Sorghastrum nutans*). The *Panicum* species were sampled from multiple sites across a steep precipitation gradient in Central Texas, while *Andropogon*, *Schizachyrium*, and *Sorghastrum* were sampled from a single site on the gradient. Sampling occurred as described in Giauque and Hawkes (2015).

Sampling was restricted to the Edwards Plateau to maintain consistent vegetation and soil types. Sampling was performed as detailed in Giauque and Hawkes (2015). At each site, spatial location, climate historical means and annual values for precipitation and temperature (Oregon State University, http//prism.oregonstate.edu, created 15 August 2014), and soil moisture and nutrients were recorded as described in Giauque and Hawkes (2015).

Tillers were sectioned into three 2-mm fragments, which were surface sterilized using 0.5% sodium hypochlorite and 70% ethanol (Arnold *et al*., 2000), placed on petri dishes containing 2% potato dextrose agar (PDA) and 50 ppm ampicillin, and incubated at room temperature. Plates were assessed daily for fungal growth and newly emerged hyphae were transferred to new PDA plates to obtain pure cultures. Once in pure culture, 1-cm x 2-cm diameter cores were subsampled for (1) use in plant-endophyte interaction assays (2) long-term storage in 2 ml of RNase/DNase-free water at room temperature for trait assays (Burdsall, 2003), and (3) immediate DNA extraction.

For DNA-based identification, we used both the ITS and LSU regions of rDNA, as in Giauque & Hawkes (2015), because these capture both variable and conserved regions to allow for robust alignments (Liu *et al*., 2012; Porter & Golding, 2012). The ITS region (~500-800 bp) was amplified using the primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al*., 1990; Gardes & Bruns, 1993). The D1/D2 region of the LSU (~650 bp) was amplified using the general fungal primers NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') AND NL4 (5' GGTCCGTGTTTCAAGACGG3 3') (O'Donnell, 1993). Each 25-µl PCR reaction contained approximately 10 ng of fungal DNA, 0.75 U *Taq* polymerase, 1x PCR buffer, 2 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} dNTPs, and 0.5 µmol L^{-1} each of primers. Thermal cycling reactions used the following conditions: 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; 1 cycle of 72°C for 5 min. Amplified products were sequenced on an ABI3730XL DNA Analyzer (Applied Biosystems, Carlsbad, California, USA) at the DNA Sequencing Facility at the University of Texas at Austin.

Sequences were trimmed and quality checked and clustered into operational taxonomic units (OTUs) based on 97% sequence similarity, as detailed in Giauque and Hawkes (2015). In total, 70 unique OTUs were identified. Thirty-five of these isolates were screened for function within the plant, to identify potential fungal strategies governing the outcome of the plant-fungal interaction.

Plant trials

Seeds were surface sterilized in 0.5% sodium hypochlorite and 70% ethanol and seedlings showing no fungal outgrowth were transplanted into plant culture boxes (Magenta GA-7; Magenta Corporation, Chicago, IL) containing sterile sand. Nutrients were provided as 5 ml of filtered 1/4 strength Hoagland's solution (Hoagland & Arnon, 1950). Plants were kept in an isolated greenhouse at ambient light and temperatures ranging from 27-35°C (day vs. night). Plants were allowed to grow for 3-4 weeks at 15% soil moisture prior to fungal inoculation, when seedlings reached at least 4 cm height.

To inoculate the plants with target endophytes, the fungi were cultured in liquid PDA and diluted in water as needed to obtain 10^5 spores ml^{-1}. Each plant received 1 ml of inoculum by pipetting directly onto the shoot (Rodriguez *et al*., 2008). Control plants were mock-inoculated with the same volume of sterile water. After inoculation, plants were grown for 1 week to allow the fungi sufficient time to colonize the plant (Rodriguez *et al*., 2008) before the drought treatment was imposed. To ensure the inoculation was successful and that non-target fungi had not contaminated plants, leaf tissue was taken from a subset of replicate plants, surface sterilized, and re-cultured on PDA to compare to expected morphotypes.

When the low moisture treatment was imposed, soils were allowed to dry to 3% soil moisture (7-10 days), which created extreme drought stress for *P. virgatum* (Barney *et al*., 2009; Barney & DiTomaso, 2010). This level of soil moisture occurs throughout

the gradient during drought years, but is particularly common at the western end of the Edwards Plateau precipitation gradient. Moisture levels were checked every three days and adjusted to maintain the treatments based on whole-pot weight.

Fungal osmotic stress tolerance and growth assays

To assess fungal growth rate and response to osmotic stress, we grew fungal isolates in liquid 1x M9 media (Miller, 1972) supplemented with 200 ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 0.1 ml of 1 M $CaCl_2$ per liter of solution. Osmotic stress was created by adding varying sodium chloride concentrations, which were chosen to mimic osmotic stress levels the fungal isolates were likely to have experienced in nature. Treatments included the addition of 0 g/L, 40 g/L, 80 g/L, 120 g/L and 160 g/L of sodium chloride, which represent -30 kPa, roughly field capacity, -300 kPa, -600 kPa,1000 kPa and -1200 kPa, close to the permanent wilting point of soil (Tolk, 2003). Liquid media was inoculated by taking plugs ($1mm^2$) from fungal monocultures grown on 2% potato dextrose agar plates. Fungi were grown for 21 days, until the majority of isolates reached stationary growth, at 30°C in 8 ml tubes, shaken at 100 rpm. To harvest each isolate, cultures were centrifuged at 15,000 rpm for 10 minutes and rinsed in 4 ml autoclaved water twice. Cultures were re-suspended in 8 ml of autoclaved water and filtered through pre-weighed grade 1 filter paper. Filter paper was dried at 100°C for 24 hours and then placed in a dessicator for at least 1 hour prior to weighing. Dry fungal weight was ascertained by subtracting filter paper weight from total dry weight. There were 4 replicates per treatment with 5 different stress levels.

While most studies in plant induce osmotic stress using polyethylene glycol (PEG), NaCl induces greater levels of osmotic stress at lower concentrations, allowing us to screen a wider array of stress levels. Additionally, we screened a subset of fungi for osmotic stress tolerance using both NaCl and PEG (with osmotic potentials reaching

~100 kPa) and growth rates, thresholds, and osmotic sensitivity were highly correlated between NaCl and PEG treatments (data not shown). A study focused on *Fusarium graminearum* similarly found that fungal responses to PEG and NaCl were similarly correlated (Ramirez *et al*., 2004) .

Resource use

To assess fungal resource use, we grew each fungal isolate on Biolog (Biolog, Hayward, California, USA) plates. To inoculate the plates, we grew each isolate on 2% potato dextrose agar plates and scraped fungal conidia into a 2 ml microcentrifuge tube. We added 1 ml of FF Inoculating Fluid (Biolog, Hayward, California, USA) and ground up suspended conidia using a sterilized micropestle. We transferred the inoculum to a 50 ml falcon tube and added 40 ml of FF Inoculating Fluid and standardized each inoculum to equal ~1.6 x 10^8 cells/ml (or an absorbance of 0.2 nm when read at 600nm), to ensure even inoculation. Biolog plates were inoculated with 100 μl/well, with 3 replicates per isolate, and were stored at 26°C. The plates were read on a SpectraMax M3 Microplate Reader (Molecular Devices, Sunnyvale, California, USA) for determination of absorbance at 490 nm and 750. Absorbances were read every other day, starting on the third day following inoculation, for 25 days. Metabolic activity of the isolates was based on absorbance values 9 days after inoculation, given that most isolates reached stationary growth within 1-2 days following that time point (Hobbie *et al*., 2003). For Biolog assays, substrates were categorized as carbohydrates, amino acids, amines, polymers, or carboxylic acids. We used scores from the first PCA axis for each substrate category, which explained 47-61% of the variation in substrate use among endophytes.

Cellulose-degrading enzymes and ACC deaminase production

Fungi are capable of producing a wide range of extracellular enzymes, depending on resource requirements. We screened all 36 isolates for production of cellulose-

degrading enzymes (α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), and cellulobiohydrolase (CBH) and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity. To screen for cellulase production, we grew each isolate on 1x M9 media (Miller, 1972) supplemented with 200 ml 20% cellulose, 1 ml of 1 M $MgSO_4$, and 0.1 ml of 1 M $CaCl_2$ and 30 g agar per liter of solution. Enzyme assays were performed fluorimetrically as detailed in Saiya-Cork, *et al*. (2002). Five 1 m^2 plugs were taken from each cellulase plate and ground into a suspension in 125 ml of 50 mM, pH 5.0, acetate buffer and homogenized for 1 minute using an immersion blender. The resulting suspensions were stirred continuously while 200 µl aliquots were dispensed into 96 well-microplates, with 16 replicate wells per sample per assay. Two hundred microliters of acetate buffer and 50 µl of 200 µM substrate solution (AG = 4-MUB-α-D-glucoside, BG = 4-MUB-β-glucoside, and CBH = 4-MUB-β-D-cellobioside) were added to each sample well. Quench standard wells received 50 µl of standard (10 µM methylumbelliferone) and 200 µl of sample suspension. Reference standard wells received 50 µl of standard plus 200 µl acetate buffer. There were eight replicate wells for each blank, negative control, and quench standard. The microplates were incubated in the dark at 20°C for 1 hour. To stop the reaction, 10 µl of 1 M NaOH was added to each well. Fluorescence was measured using a microplate fluorometer with 365 nm and 450 nm emission filters. After correcting for negative controls and quenching, enzyme activities of each isolate were expressed in $nmol^{-1}$ mm^{-2} (Saiya-Cork *et al*., 2002).

ACC deaminase activity was screened colorimetrically, based on the protocol developed by Li *et al*. (2011). We grew each isolate in potato dextrose media for 14 days, shaking at 100 rpm at 30°C. Cultures were centrifuged at 12,000 rpm for 10 minutes and the pellet was rinsed with Dworkin Foster (DF) media (Penrose & Glick, 2003) twice. The pellet was resuspended in DF media, supplemented with 0.5 M ACC

(final concentration = 0.3 mM ACC) and standardized to equal concentrations of ~1.6 x 10^8 cells/ml (based on OD at 600nm). We transferred the suspension to a new 8 ml tube and added 6 ml of DFF+ACC media. The cultures were incubated at 26°C for 14 days, shaking at 100 rpm. After incubation, we transferred 1.5 ml of culture to a new microcentrifuge tube, centrifuged at 8,000 g for 5 minutes, and removed 1,000 µl of supernatant to a new tube. We added 120 µl of ninhydrin reagent (Li *et al*., 2011) and 60 µl of sample and standards to each well of a chimney top PCR plate. The samples were run with 8 replicates and each standard was run in duplicate. Standards consisted of DF+ACC media with varying concentrations of ACC (0.5M, 0.25M, 0.1M, 0.05M, 0.3 mM, 0.25 mM, 0.1 mM, and 0.05 mM). We incubated the plate at 100°C for 30 minutes and transferred the samples to a clear, flat-bottomed 96-well plate. To determine the amount of color change, we measured absorbance at 570 nm and calculated current concentration of DF-ACC media in sample wells. If concentration was significantly lower than the 0.3mM standard, we classified that isolate as a producer of ACC deaminase.

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