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FUNGAL ENDOPHYTE INTERACTIONS AND MECHANISMS OF FUNGAL-MEDIATED PLANT DROUGHT TOLERANCE

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FUNGAL ENDOPHYTE INTERACTIONS AND MECHANISMS OF FUNGAL-MEDIATED PLANT DROUGHT TOLERANCE

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Dedication

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FUNGAL ENDOPHYTE INTERACTIONS AND MECHANISMS OF FUNGAL-MEDIATED PLANT DROUGHT TOLERANCE

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Plants are ubiquitously colonized by diverse communities of horizontally-transmitted fungal endophytes, that can drastically alter plant physiology. Though many endophytes are mutualist, effects are context-dependent and can shift from pathogenic to mutualistic depending on abiotic and biotic factors. However, our understanding of endophyte effects comes almost exclusively from test of individual fungi, which may miss important community level processes that can alter fungal effects. Using *Panicum* grasses, I examined mechanisms underlying fungal interactions on plant physiology. I studied interactions in the context of plant drought responses, as climate models predict and increase in the intensity and frequency of drought. Scaling up from pairwise endophyte-plant studies will allow us to develop fungal applications that are more generalizable in real-world agricultural settings.

Throughout my dissertation, I characterize the effects of altered precipitation and fungal interactions on plant physiology. To examine impacts of altered precipitation, I measured leaf-level and whole-plant carbon and water exchange in C4 grasses grown in extreme dry, extreme wet and mean levels of precipitation. Within this system, both extreme increases and decreases in precipitation inhibited plant gas fluxes, with all plants (*Andropogon gerardii, Panicum virgtaum,* and *Sorghastrum nutans*) responding

similarly. To understand how fungal interactions effect plant performance, I compared the physiology of *P. virgatum* grown with six fungal pairs, the corresponding 12 individual fungi, and a no-fungus inoculum in low and high soil moisture. In most cases, plants responses to fungal pairs were non-additive (greater or less than expected) relative to effects of corresponding individual fungi. Furthermore, similarity of fungal stress tolerance and metabolic profiles predicted effects of fungal pairs in high and low soil moisture, respectively. To further understand mechanisms behind fungal interactive effects, I grew *P. virgatum* with 10 fungal pairs, in which each fungus was paired with one another, the five corresponding fungi, and a no-fungus inoculum in low and high soil moisture. Two of the five species dominated effects on the plants, such that outcomes of interactions could be predicted by the presence of these fungi within a pair. Furthermore, overall fungal effects on plant physiology could be predicted in the plant metabolome.

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Introduction[1]

Plant productivity is primarily limited by water availability in many ecosystems (Knapp and Smith 2001), with water limitations likely to be exacerbated, as climate models predict an increase in the intensity and severity of extreme drought events in many regions (IPCC 2014). Grasslands may be particularly vulnerable to extreme changes in precipitation as mean annual precipitation is a primary driver of grassland productivity (Knapp and Smith 2001). Plants have developed many mechanisms in which to respond to drought, with strategies generally leading to plant escape, resistance, or tolerance of drought (Chaves et al. 2002, Chaves, Maroco and Pereira 2003, Juenger et al. 2005). Though often overlooked, fungal symbionts can also mediate plant stress responses and directly alter the above-mentioned strategies (Elmi and West 1955, Rodriguez et al. 2009, Worchel, Giauque and Kivlin 2013, Kivlin, Emery and Rudgers 2013).

Grasses predominantly associate with arbuscular mycorrhizal (AM) fungi belowground and endophytes aboveground (Leuchtmann 1992, Smith 1974). Endophytes are further divided into two broad classes based on host colonization, transmission, and function: vertically transmitted (VT) or horizontally transmitted (HT) endophytes (Rodriguez et al. 2009). The role of AM fungi and VT endophytes in mediating plant drought tolerance is fairly well characterized, but we know very little about mechanisms underlying symbioses among grasses and HT endophytes. Arbuscular mycorrhizal fungi

[1] Work presented in the introduction comes primarily from Worchel EW, Giauque HE and Kivlin SN. 2013. Fungal symbionts alter plant drought responses. Microbial Ecology 65: 671-678
Connor planned, executed and wrote up project. Giauque contributed to planning and executing. Kivlin supervised project

can promote drought avoidance by producing hyphae with access to small soil pores, expanding belowground water uptake surface area (Auge 2001). Endophytes can influence plant stomatal conductance and osmotic adjustment, with biochemical mechanisms generally underlying the effects of VT endophytes (Clay and Schardl 2002).

However, the direction of plant–fungal symbioses is often dependent on biotic and abiotic factors, with the same fungal taxa conferring different costs or benefits under different conditions. For instance, under drought conditions, the relative benefit from a fungal symbiont may be different depending on the photosynthetic pathway of the host plant. Plants with C4 physiology have greater water use efficiency and stomatal conductance than C3 species; therefore, they may rely less on fungal symbionts for water acquisition (Edwards et al. 2010). In addition to plant photosynthetic pathway, abiotic conditions can dictate the interaction between a plant host and its fungal symbionts. For example, a beneficial fungal symbiont can become parasitic when environments are not stressful (Johnson, Graham and Smith 1997). As such, both abiotic and biotic factors are likely to contribute significantly to the shift from mutualism to parasitism in regards to fungal effects on plant performance under drought.

For part of my graduate work, I quantified the relative strength and direction of plant biomass responses in grasses to both AM fungi and VT endophytes under a range of drought conditions using a meta-analysis of previously published studies (Worchel et al. 2013). Through this approach, I also examined the influence of environmental conditions and plant host characteristics contributing to fungal effects on grasses. However, only AM fungi and VT endophytes were manipulated often enough to be compared quantitatively, thus this approach could not be used for HT endophytes. To fill this

knowledge gap, I experimentally manipulated soil moisture and HT endophytes colonization in C4 grasses, as outlined in Chapters 1-3.

Specifically, using a dataset containing 86 comparisons from 51 different studies, I performed a factorial meta-analysis to determine how AM fungi and VT endophytic fungal symbionts affect growth of grasses under drought (Worchel et al. 2013). The meta-analysis also addressed (1) how the effect of fungal symbionts on plant growth was influenced by biotic (plant photosynthetic pathway) and abiotic (level of drought) factors, and (2) if there is a phylogenetic signal of fungal symbionts on grass growth under well-watered and drought conditions. Based on this dataset, only AM fungi categorically influenced the growth of grasses under drought, although VT endophytes had a similar trend. Furthermore, the outcome of grass–fungal interactions under drought was context dependent, with three important factors contributing to outcomes. First, fungal symbionts increased plant biomass more as water limitation increased. Second, plant photosynthetic pathway affected the AM fungal–plant symbiosis, with C3 grasses receiving a greater benefit from associating with AM fungi than C4 grasses under both well-watered and drought conditions. Finally, while VT endophytes had little effect on plant biomass overall, there was a significant phylogenetic signal for biomass in response to VT endophyte presence, with grasses in the *Elymus* genus receiving the most benefit from their VT endophyte symbionts. As individual plant biomass is a large determinant of plant survival during a drought (Tilman and El Haddi 1992), identifying key drivers of symbioses between grasses, AM fungi and VT endophytes will improve our ability to harness the plant microbiome for managing plant drought responses. However, due to the

lack of data available on HT endophytes, patterns are yet to be generalizable this ecologically important symbioses.

Thus, to address this knowledge gap, my dissertation work focuses exclusively on HT endophytes (hereafter 'endophytes'). Similar to VT endophytes, effects on host plants are context-dependent and can shift from pathogenic to mutualistic depending on abiotic and biotic factors (Saikkonen et al. 1998). However, these endophytes differ from VT endophytes in that they have broader host ranges, being isolated from all terrestrial plant species and biomes to date, and they are transmitted from plant-to-plant via the environment, allowing for both symbiotic and free-living lifestyles (Rodriguez et al. 2009). Furthermore, colonization patterns differ widely: in contrast to colonization by a single dominant VT endophyte (Wille, Aeschbacher and Boller 1999), plants harbor a diverse community of endophytes with up to 20 different endophytes species within one plant (Arnold et al. 2003).

However, our understanding of endophyte effects comes almost exclusively from test of individual fungi, which may miss important community level processes that can alter fungal effects. Specifically, competitive interactions among symbionts can result in antagonistic effects on host performance through limiting the productivity of competing species or reducing overall species diversity (Kennedy et al. 2007). Conversely, complementarity among symbionts can lead to synergistic effects when species have functionally distinct traits that provide complementary benefits (Thonar et al. 2014). As grasses are typically colonized by multiple symbionts, patterns observed in pairwise studies of endophyte colonization may not translate to the field.

Therefore, my chapters focus on the effects of altered precipitation and fungal interactions on plant physiology. In chapter 1, I identify the effects of extreme changes in precipitation on carbon and water fluxes in three native Texas, C4 grasses (*Andropogon gerardii, Panicum virgatum*, and *Sorghastrum nutans*). Currently, our understanding on the impact of altered precipitation comes primarily from studies that focus on persistent moderate changes in precipitation. These studies are likely to underestimate impacts on plant communities as moderate changes in precipitation may be too mild to affect population dynamics, or plants may have the opportunity to acclimatize (Watkinson et al. 2003, Chaves et al. 2003). Specifically, extreme climatic events can push species to biological thresholds, inhibiting plant functions and shifting community composition. To identify the impacts of extreme changes in precipitation, I measured leaf-level and whole-plant CO2 and H2O exchange in grasses grown in three precipitation treatments: extreme dry, mean, and extreme wet based on historical rainfall records.

In chapter 2, I examine the effects of fungal interactions on plant physiology in drought and well-watered conditions. I then develop a framework to predict outcomes of fungal interactions on plant responses. Plants are colonized by multiple endophyte species that can drastically effect plant stress responses (Redman et al. 2011, Rodriguez et al. 2008). Fungal interactions may result in additive effects on the host plant, which could be predicted simply based on individual fungal behavior. Alternatively, interactions among fungi may result in non-additive synergistic or antagonistic effects on plant performance that are more challenging to predict. These outcomes are likely driven by the degree of niche overlap among species, with competition increasing in parallel with ecological similarity (Loreau and Hector 2001). To examine if effects of fungal

interactions on the plant host could be predicted from their niche overlap, I compared the effects of six pairs of fungi, with a wide range of niche-overlaps, to the corresponding individual fungal species on *Panicum virgatum* in water-stressed and well-watered conditions.

Finally, in chapter 3, I identify fungal species that largely control outcomes of fungal interactions on plant responses, and link fungal effects to underlying changes in the plant metabolome. As mentioned above, effects of endophyte interactions on plant responses may be due to either facilitative or competitive processes among endophytes. Alternatively, an individual endophytic fungus may come to dominate overall function, such that effects are caused by a single species in the mixture. This can occur through selection effects in which competition leads to the dominance of the most productive species (Cardinale et al. 2006, Loreau and Hector 2001). To understand mechanisms underlying fungal interactive effects, I examined the effects of fungal interactions and drought on the performance and metabolomics profiles of *Panicum hallii*. Plants were grown with one of five individual fungi, a two-species fungal mixture, in which all individual fungi were paired with one another, or a fungus-free control inoculum in water-stressed and well-watered conditions.

Understanding the mechanisms driving endophyte effects on plant drought responses is particularly timely given the predictions for more frequent droughts in the future (IPCC 2014). However, to translate endophytes benefits into the field, endophytes must be studied within a community context. The experiments described here represent the first steps to understanding plant-endophyte symbioses beyond pairwise associations.

Chapter 1: Effects of extreme changes in precipitation on the physiology of C4 grasses[2]

INTRODUCTION

Climate models predict an increase in the intensity and severity of weather events, with warming temperatures and prolonged dry periods leading to more extreme drought and flooding in many regions (IPCC 2014). Extreme weather events are more intense relative to past historical climate and are expected to have large impacts on ecosystem function via their effects on plant growth, physiology and survival (Gutschick and BassiriRad 2003, Smith 2011). However, despite the significant impact of extreme events on natural systems (Jentsch, Kreyling and Beierkuhnlein 2007, Petterson et al. 2008, Smith 2011), most studies focus on persistent moderate changes in temperature or precipitation. When changes in climate are moderate, drought may be too mild to affect population dynamics, or plants can have the opportunity to acclimatize (Watkinson et al. 2003, Chaves et al. 2003), resulting in little apparent impact on ecosystem function over time (França et al. 2000, Grime et al. 2000, Suttle, Thomsen and Power 2007, Zavaleta et al. 2003).

Extreme weather events, in contrast, can push species to biological thresholds from which function cannot recover. For instance, severe water limitation can cause plant transpiration to exceed absorption, resulting in loss of cell turgor, relative water content, and cell volume to a point where cellular functions are irreversibly impaired (Lawlor and

[2]We also examined the effects of extreme precipitation on fungal endophyte communities within these grasses. A brief introduction, methods and results of this study are in Appendix 1 'Effects of extreme changes in precipitation on fungal endophyte communities'.

Cornic 2002). Extreme flooding can cause hypoxia in the rhizosphere, which leads to inhibition of ATP synthesis and nutrient deficiency (Steffens et al. 2005). Adverse effects of extreme events can have long-term impacts on community dynamics and can affect ecosystem responses such as net ecosystem exchange of carbon (Reichstein et al. 2013). However, extreme events are not always detrimental; some studies report stable productivity despite extreme rainfall (Grant et al. 2014). Yet our understanding of the impacts of extreme events comes primarily from observational studies, with extreme manipulation experiments being rare, and the majority of both observational and manipulation experiments focused on changes in aboveground net primary productivity (Knapp et al. 2016).

Grasslands may be particularly vulnerable to extreme drought and flooding because mean annual precipitation is a primary driver of grassland productivity (Knapp and Smith 2001). Despite the long-held assumption that the dominant grasses in tallgrass prairies of the United States are ecologically equivalent (Polley et al. 1992, Weaver 1931), plant physiological responses to water can be species-specific, making ecosystem responses to changes in precipitation difficult to predict (Fay et al. 2003). The concept of ecological equivalency is often rejected when climate variables are directly manipulated in prairie and grassland ecosystems (Knapp 1985, Connor, Sandy and Hawkes 2017, Silletti and Knapp 2002, Smith, Hoffman and Avolio 2016). For example, when exposed to increased rainfall variability, where intervals between rainfall was lengthened by 50%, the two most abundant and functionally similar grasses in a central Great Plains rainfall manipulation site exhibited contrasting responses to rainfall variability, with no change in productivity of *Andropogon gerardii* but reduced cover and flowering of *Sorghastrum*

nutans (Fay et al. 2003). Furthermore, differences in plant responses are complex, as plant species differ not only in function but also in the speed or lag-time in which they respond to stress.

We propose that ecosystem responses to extreme weather events will depend on the severity of the event and how different plant species respond to those conditions over time. To address this issue, we examined the effects of extreme changes in precipitation on leaf-level and whole-plant CO_2 and H_2O exchange of three native C4 bunchgrasses over three years. The grasses were *Andropogon gerardii, Panicum virgatum*, and *Sorghastrum nutans*, which were historically dominant in tallgrass prairies (Shantz 1923) and have potential for use as low-input, non-irrigated biofuel sources (Adler et al. 2009). The grasses were grown in three precipitation treatments: extreme dry, mean, and extreme wet based on historical rainfall records. We measured leaf-level CO_2 and H_2O exchange and plant growth in all treatments at three time points across three years (July 2012, July 2013 and July 2014) and whole-plant CO_2 and H_2O exchange across all precipitation treatments at one time point (July 2013).

We hypothesized that extreme dry conditions impair leaf-level physiology, resulting in declining leaf-level fluxes over time. In contrast, we expected extreme wet conditions to have a saturating-effect, where additional precipitation does not translate into changes in plant physiology, such that differences are unlikely to be observed among the mean and extreme high rainfall treatments. We further predicted that plant responses to external climatic factors would depend on precipitation treatment, with ambient temperature affecting plants experiencing drought stress in the extreme low treatment more compared to unstressed plants in the mean or extreme wet rain treatments. At the

plant-level, expected leaf-level effects should translate to a net carbon loss in the low treatments and a net carbon gain in the mean and extreme wet treatment. Finally, consistent with other studies, all physiological responses to the treatments were expected to be species-specific.

METHODS

Study site

The experiment was conducted in a rainout shelter facility located at the University of Texas Lady Bird Johnson Wildflower Center in Austin, Texas, USA. The rainout shelters were constructed from steel frames (18 m wide x 73 m long) that are 6.0 m tall at the center with 1.8 m open sides. Shelter roofs were 6-mm thick polyethylene film with 91% light transmission (IGC Greenhouse Megastore, Danville, IL, USA). Mean maximum temperature at the site is 35.8 °C (August), mean minimum temperature is and 3.7 °C (January) and in the year measurements were collected (2012, 2013 and 2014) the maximum temperature was 36.5, 36.8 and 36.5 °C (August, August and August) and the minimum temperature were 5.0, 3.1 and 1 °C (January, December and January). The site is located on Speck stony clay loam soils (pH = 7.9); rocks greater than 5-cm in diameter were sieved out when the plots were constructed.

Experimental design

We used a randomized complete block design with three rainfall treatments replicated across four blocks and six grass species grown in the plots as a split-plot factor. The extreme low, mean, and extreme high rainfall treatments were designed to mimic the local historical precipitation record and were 349, 885, and 1331 mm yr^{-1}. The extreme treatments are not symmetric around the mean because they are based on the respective

ten years surrounding the driest, mean, and highest rainfall in the 87-year historical record for Austin (National Centers for Environmental Information: https://www.ncei.noaa.gov/), with applications created using a stochastic weather generator, LAR-WG 5.5 (Semenov et al. 1998) calibrated to those ten years. Irrigation was applied using 90° sprinklers (Hunter HP2000, Hunter Industries Inc., San Marcos, CA, US) on 1-m risers placed in the four plot corners. All plots were instrumented to monitor soil moisture (ECH2O 10HS sensors, Decagon Devices, Pullman, WA, USA) and temperature (thermocouple wire, Omega Engineering Inc., Stamford, CT, USA) with data collected hourly (CR1000 datalogger, Campbell Scientific Inc., Logan, UT, USA). Note that the full design included two soil types; here we limited measurements to the native clay soils (Speck series; stony clay loam).

Six native perennial C4 grasses were grown in 2.5 m x 2.5 m plots: *Andropogon gerardii* Vitman, *Leptochloa dubia* (Kunth) Nees, *Panicum virgatum* L., *Schizachyrium scoparium* (Michx.) Nash, *Sporobolus compositus* (Poir.) Merr., and *Sorghastrum nutans* (L.) Nash. Seeds of the six species were purchased from Native American Seed (Junction, TX) or provided by the Ladybird Johnson Wildflower Center Seed Bank. The seeds were germinated and grown in seedling trays on field soils under ambient greenhouse conditions for 12 weeks before transplanting into field plots on August 19, 2010. In the field plots, plants were arranged in a grid with 0.5-m spacing and three individuals per species in stratified random locations (18 total plants per plot). Plants were watered 2-3 times per week at 1000 mm yr^{-1} to allow for establishment before rainfall treatments began on May 22, 2012. For all plant measurements, we focused on three species:

Andropogon, *Panicum*, and *Sorghastrum*. In each plot, a single individual of each plant species was randomly selected for measurement of physiology.

Plant size measurements

In July 2012, 2013, and 2014, we measured plant canopy height (H), maximum basal diameter (w1), and the diameter perpendicular to the maximum (w2). We estimated cylindrical plant volume as $V = \frac{\pi \times W_1 \times W_2 \times H}{4}$.

Leaf-level CO_2 and H_2O exchange measurements

Photosynthesis (A_{max}) and stomatal conductance (g_s) were measured on leaves of non-flowering tillers annually in July from 2012 to 2014 using a LI-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA). All measurements were taken between 9 am and 12 pm using the LI-COR 6400 LED light source at ambient temperature, ambient humidity, and with CO_2 reference levels maintained at 400 umol mol^{-1}. Leaves were sealed in the LI-6400 chamber and measurements were recoded once gas exchange reached a steady state (~2 min). Air temperature was recorded by the LI-6400 and soil volumetric water content by 10HS sensors (Decagon Devices Inc., Pullman, WA, USA).

Plant-level CO_2 and H_2O exchange measurements

We measured whole-plant CO_2 exchange and H_2O vapor fluxes in July 2013 using a custom transparent flux chamber with an infrared gas analyzer (IRGA; LI-COR 7500, LI-COR Biosciences, Lincoln, NE, USA). The cylindrical chamber (0.45 m diameter x 0.9 m height) was constructed of 5-mm thick cast acrylic with 92% light transmission (Interstate Plastics, Sacramento, CA, USA). The IRGA and one mixing fan were attached to the chamber lid (3-mm thick cast acrylic); two additional fans were mounted on acrylic

down-poles attached to the lid. Chamber height could be doubled for taller plants by attaching a second chamber segment. The chamber was sealed to the soil with a clear vinyl skirt attached to the chamber bottom and overlain by a heavy steel chain (Huxman et al. 2004).

Fluxes were measured during 40-60s intervals to estimate whole-plant net ecosystem CO_2 exchange (NEE) and evapotranspiration (ET), following St. Claire et al. (2009). Ecosystem dark respiration (R_e) was measured by wrapping the chamber in reflective polyethylene insulation and repeating the measurement. Fluxes of NEE (mmol CO_2 m^{-2} s^{-1}), ET (mmol H_2O m^{-2} s^{-1}), and R_e (mmol CO_2 m^{-2} s^{-1}) were measured as $(V \times C)/(A \times t)$ using the HMR package in R, which fits the best linear or curvi-linear model of changes in concentration over time (dC/dt) for each flux measurement (Hutchinson and Mosier 1981, Pedersen, Petersen and Schelde 2010, Pedersen 2015). Approximating the chamber as a right cylinder, headspace volume (V; m^3) was calculated by subtracting plant volume from total chamber volume (Dossa et al. 2015); area (A; m^2) was calculated as the cross-sectional area of the chamber; concentration (C) of CO_2 and H_2O were measured as mmol m^{-2}; and time was measured in seconds.

STATISTICS

Plant volume, A_{max}, and g_s were analyzed using a split-split-plot ANOVA with precipitation (whole-plot treatment, fixed effect), grass species (subplot treatment, fixed effect), year (sub-subplot treatment, random effect), and block (random effect). Precipitation X block was used as the whole-plot error, precipitation X grass species X block was used as the subplot error, and the precipitation X grass species X year X block error was used as the sub-subplot error. Whole-plant gas fluxes (NEE, ET, R_e) were

analyzed using a split-plot ANOVA as above, but without year. Factors were considered significant at $P < 0.05$. When main effects were significant, Tukey post-hoc tests were used to examine differences among precipitation levels, species, or dates; when interactions were significant, each treatment was analyzed across levels of the other treatment using one-way ANOVA with Tukey post-hocs. Statistical analyses were performed in R using the STATS package (R Core Team 2016).

To examine how variation in leaf-level flux responses to precipitation treatment might be altered by environmental conditions at the point of sampling, we conducted stepwise multiple regressions. Separate regression models were run for A_{max} and g_s against the independent variables temperature, soil moisture, precipitation treatment, and the interaction of each variable with precipitation treatment. The interaction terms identify if different treatments were affected differentially by seasonal variations. Air temperature and soil moisture at time of measurement were used to capture seasonal climate variation. Independent variables did not display multicollinearity based on variance inflation factors (VIF < 2) using the vif function in the car R package (Fox and Weisberg 2001). The best explanatory model was selected based on Akaike Information Criteria (Akaike 1974).

RESULTS

Plant size

Plant size varied with the interaction of rain treatment and year, with plants in the low treatment shrinking over time, plants in the mean treatment getting larger over time, and no change for plants in the high treatment over time (Fig 1a). Plant volume also differed across species, with *Andropogon* generally being the smallest and *Panicum* the

largest of the three; *Sorghastrum* differed from both except in 2014 when it was the same as *Andropogon* (Table 1, Fig 1b). Only *Panicum* changed significantly across years overall, with larger plants in 2014 compared to earlier years (Fig 1b).

Leaf level gas exchange measurements

Both A_{max} and g_s were affected by precipitation treatment (Table 1), with ~55% less carbon fixation (A_{max}) and ~40% less water lost (g_s) in the extreme dry condition compared to the mean and extreme wet treatments, which did not differ from each other (Figure 2). Differences among plant species in A_{max} and g_s only occurred at the first date (July 2012), when *Sorghastrum* fixed 96% more carbon and lost 114% more water than both *Andropogon* and *Panicum* (Table 2; Figure 2b, 2d). Across years, A_{max} was significantly lower in 2012 compared to 2013 and 2014, which did not differ (Figure 2b), whereas g_s was significantly lower in 2013 compared to 2012 and 2014, which did not differ (Figure 2d). There were no interactions of species x precipitation or year x species x precipitation for either A_{max} or g_s.

Based on regression analysis, A_{max} was best predicted by precipitation treatment ($R^2 = 0.126$), with increasing rates as precipitation level increase. Both precipitation treatment and ambient temperature explained g_s ($R^2 = 0.095$) (Table 3), which increased with precipitation but decreased with increasing temperature (Figure 3). There were no interactions retained in the models.

Plant level gas exchange measurements

Only whole-plant ET was significantly affected by precipitation treatment, with more water loss in the mean compared to the extreme low and high treatments (Figure 4b). Both NEE and R_e exhibited the same patterns, but were not significantly different

(Figure 4a, c). There were no differences in whole-plant fluxes among the three plant species and no interaction of species by precipitation treatment.

DISCUSSION

We found that an extreme 2.5x decrease in precipitation was more important than an extreme 1.5x increase for leaf-level physiology of native Texas grasses, with reductions of 55% and 40% in A_{max} and g_s. At the plant-level, however, plants were more active in the mean rainfall treatment: water fluxes were 74% higher on average, and carbon fluxes (though not significant) were 68% (NEE) and 100% (Re) higher on average, suggesting that both extreme rainfall treatments constrained overall physiology. By 2014, plants were also 46%-88% larger in the mean treatment, relative to the extreme high and low treatments; in contrast, plant sizes in the extreme high and extreme low treatments were stable and declined over time, respectively. The differences between moderate and extreme rainfall challenge our expectations for the future as inhibition of whole-plant fluxes did not depend on whether precipitation was increased or decreased.

However, in contrast to our expectations, *Andropogon gerardii, Panicum virgatum*, and *Sorghastrum nutans* responded similarly to the precipitation treatments after the first year, despite consistent size differences among the three species. These findings differ from other studies, where *Sorghastrum* physiology was sensitive to both moderate increases and decreases in precipitation, but *Andropogon* was not (Fay et al. 2003, Silletti and Knapp 2001). Furthermore, *Andropogon* and *Sorghastrum* also differentially responded to moderate temperature and water stress at the molecular level, suggesting distinct genetic pathways for coping with stress (Smith et al. 2016). The variation in species equivalence among studies may be explained by different local

ecotypes of these grasses across the central US, which represents a north-south temperature gradient and east-west rainfall gradient. For example, *Panicum virgatum* found in the southern Great Plains is more drought-tolerant than northern populations (Aspinwall et al. 2013). Furthermore, species differences may break down as stress increases and biological thresholds are reached.

When ambient temperature and soil moisture were considered, precipitation treatment remained the primary controller of A_{max}. This is consistent with other studies, in which temperature showed no effect on A_{max} (meta-analysis across 85 studies: Wu et al. 2011) even when coupled with increased rainfall variability (Fay et al. 2011). For g_s, however, temperature was as important as precipitation treatment, suggesting that leaf-level carbon and water fluxes will be decoupled with warmer and drier climates. This could lead to less efficient plant carbon fixation. Furthermore, the lack of interaction between precipitation treatment and ambient climate conditions runs counter to our hypothesis that plant responses would be more sensitive to temperature when already stressed by low rainfall. Grasses in this system have C4 photosynthesis, which is adapted to high temperatures, such that temperature differences between years were likely too limited to be a testable factor.

The responses to extreme precipitation observed here differ from rainfall experiments that imposed only moderate shifts in precipitation, in that plant size, carbon, and water fluxes typically increase with increases in precipitation (Wu et al. 2011, Patrick et al. 2007). For instance, a meta-analysis of 85 studies by Wu et al. (2011) reported that moderate increases in precipitation resulted in a 56% increase in NEE, a 40% increase in photosynthesis and a 28% increase in aboveground production, whereas moderate

drought caused a 45% decrease in NEE, a 9% decrease in photosynthesis, and a 37% decrease in aboveground production. Extreme levels of precipitation, both increases and decreases, can have significant consequences on soil water and nutrient dynamics that are not captured when precipitation changes are moderate, thus underestimating plant responses to projected changes in climate (Knapp et al. 2008).

Plant responses to extreme precipitation may also be less predictable than studies of moderate shifts in rainfall, given the equivocal findings from studies that manipulate extreme precipitation (Heisler-White et al. 2009). For instance, extreme precipitation treatments are not always detrimental; many studies report stable ANPP despite heavy rainfall (Grant et al. 2014, Kreyling et al. 2008, Mirzaei et al. 2008), prolonged drought (Grant et al. 2014, Jones et al. 2016, Kreyling et al. 2008, Jentsch et al. 2011), or extreme variability in precipitation patterns (Jones et al. 2016, Suttle et al. 2007); and in one case a positive effect on ANPP was reported (Dreesen et al. 2012). In contrast, drastic reductions in ANPP (Hoover, Knapp and Smith 2014, Knapp et al. 2002) and plant CO_2 fluxes (Ciais et al. 2005, Hoover et al. 2014, Bloor and Bardgett 2012, Knapp et al. 2002, Jentsch et al. 2011) have also been reported in response to extreme decreases in precipitation. Furthermore, a few studies report rapid recovery of grassland functions, with carbon fluxes recovering within weeks of a single extreme dry event (Bloor and Bardgett 2012) and productivity recovering as early as one year after a prolonged extreme drought (Hoover et al. 2014, Grant et al. 2014, Bloor and Bardgett 2012), suggesting that resilience rather than resistance may be buffering community responses to extreme events.

Multiple mechanisms may be responsible for the contrasting sensitivities observed across studies to extreme precipitation patterns; including past climatic history (mesic vs. arid ecosystems), community composition, and traits of the most dominant species (Heisler-White et al. 2009, Polley et al. 2014). Both extreme increases and decreases in precipitation may have disproportionately large effects in arid climates, where soil water holding capacity is low, soil carbon and nutrient reserves are limited, and plant communities are often less diverse (Wilcox et al. 2017, Levesque et al. 2013, Knapp et al. 2015). Alternatively, ecosystems that appear to be insensitive to changing rainfall may be moderated by biotic factors, such as complementarity in species responses and species interactions. For instance, functional diversity can buffer sites against stress through asynchrony of drought responses and higher likelihood of containing drought tolerant species (Diaz and Cabido 2001, Bloor and Bardgett 2012). Species interactions can further stabilize community responses via facilitation or complementary responses in competition intensities (Grant et al. 2014). Although biotic stabilizing mechanisms do not appear to play a large role when considering the three grasses in this study, local field communities include a mixture of functionally more diverse species.

Our approach has limitations. First, the observed decoupling of leaf-level photosynthesis and whole-plant CO_2 fixation may be methodological, indicating the inability of single-leaf based measurements to capture the variability of fluxes within a plant. The lack of correlation has been shown in other systems (Poni et al. 2014), with variability attributed to differences in light diffusion and leaf age (Escalona et al. 2016). Furthermore, leaf-level fluxes often overestimate carbon assimilation especially in

stressful conditions where photosynthetic rates are low (Pons and Welschen 2002). Our measurements of whole-plant CO_2 and H_2O were taken at one time point, in year two year of the precipitation manipulations, and thus we do not know the long-term impacts of extreme climatic changes (Jones et al. 2016). Furthermore, in a similar system, plant responses were more sensitive to rainfall interval than to an overall decrease in precipitation (Knapp et al. 2002)which was not tested here. Finally, we only measured aboveground plant responses, and belowground responses may be more important particularly under extreme stress (Wilcox et al. 2017).

Overall, our findings differ from experiments that only impose moderate changes in precipitation in two ecologically important ways. First, we observed that whole-plant physiology of three C4 grasses was constrained by both extreme increases and decreases in precipitation, causing large reductions in carbon fixation and evapotranspiration, which are generally not observed with moderate precipitation change. Second, differences in leaf and whole-plant level fluxes among the three grass species were not observed after the first year of the experiment. If the lack of species-specific responses is more common in the southern Great Plains where stressful conditions are more common, carbon losses from these grasslands may be disproportionately large because functional differences will not stabilize flux responses. Thus, accurate predictions of carbon fluxes depend on understanding the influence of extreme changes in precipitation on plant growth and physiology, as extreme events are more likely to occur in the future.

Chapter 2: Stress-dependent non-additive symbiont interactions[3]

INTRODUCTION

Plants are colonized by diverse communities of horizontally transmitted fungal endophytes (Arnold and Lutzoni 2007) that actively affect the ability of the plant to tolerate environmental stressors, including salinity, heat, and drought (Rodriguez et al. 2008). However, studies of plant-endophyte stress responses have focused exclusively on individual fungi, and thus may have missed important community processes that can influence the symbiosis. The effects of multiple fungi on a plant host can be additive in the absence of fungal interactions, as might occur if fungi are spatially segregated in the plant. However, when fungi interact, the outcomes can be non-additive and therefore difficult to predict. This is particularly relevant for fungal endophytes, which have recently been targeted for development as management tools in agriculture (Mei and Flinn 2010).

Interspecific interactions among symbionts can affect the host plant via (1) facilitation or complementarity that synergistically increase symbiont benefits, and (2) competitive or antagonistic processes that limit species diversity and reduce symbiont benefits. Ecological theory predicts that these outcomes are driven by the degree of niche overlap among species, with competition increasing in parallel with ecological similarity (Loreau and Hector 2001) and positive interactions more likely when fungi have functionally distinct traits. For instance, endophyte taxa differ in their ability to affect plant drought responses (i.e., regulating stomatal conductance or cellular desiccation

[3]Connor EW, Sandy M and Hawkes CV 2017. Microbial tools in agriculture require and ecological context: stress-dependent non-additive symbiont interactions. Agron J. doi: 10.2134/agronj2016.10.0568 Worchel planned, executed and wrote up project. Hawkes and Sandy supervised project

(Elmi and West 1955), such that combining endophytes with these traits may improve overall plant performance. Interspecific interactions have not been studied in leaf endophytes, but both complementarity and competition have been observed among fungal root symbionts (Kennedy et al. 2007, Jansa, Smith and Smith 2008, Thonar et al. 2014). For example, competition prevented closely related and functionally similar arbuscular mycorrhizal fungi from co-occurring in roots of *Plantago lanceolate* (Maherali and Klironomos 2007) and niche differentiation allowed two root endophytes to coexist in *Phragmites australis* (Ernst et al. 2011).

If niche overlap determines the strength of competition, fungal trait similarity may provide a framework for predicting interactive fungal effects on plants. Trait-based approaches to niche partitioning assume taxa with similar traits have similar ecological requirements and will therefore compete more intensely (MacArthur and Levins 1967). This approach is ideally suited to microbial organisms that are difficult to study in nature, because it requires no prior assumptions regarding mechanisms that link fitness to environmental conditions. Instead, trait-based approaches aim to predict when interspecific interactions will be competitive or complementary, and can point to potential mechanisms. Furthermore, if mechanisms driving both endophyte effects on plants and endophyte interactions are similar, traits could be directly linked to interactive fungal effects on plants. Given the chemical nature of both (via the production of bioactive secondary metabolites), this link could be established with fungal metabolites produced in culture (Schulz and B.Boyle 2002); however this would require similar fungal interactions in culture and in symbiosis. Alternatively, fungal traits such as stress tolerance that integrate metabolite production with other mechanisms may be more

predictive of plant responses. Despite there being a direct test of endophyte traits, fungal traits have been used in other systems to predict niche processes; such as trade-offs between competitiveness and tolerance to moisture stress regulating interactions in wood-decay fungi (Crowther et al. 2014), as well as soil fungi (Lennon et al. 2012).

The environment may also affect competition and shift fungal effects on the host plant from positive to negative (or vice versa). The context-dependency of endophyte effects is well documented in multiple plant-endophyte symbioses (Donoso et al. 2008, Giauque and Hawkes 2013) and is often attributed to a change in the level of environmental stress (Saikkonen et al. 1998). As the environment becomes more stressful, the benefits of harboring an endophyte are expected to increase relative to the costs. When scaling up to multiple fungal species, the environment may also influence their competitive interactions. The paradigm in plant communities, for example, is that facilitative interactions are more common with increasing stress across environmental gradients (Bertness and Callaway 1994). For endophytes, similar facilitative interactions can occur when species have different mechanisms of stress tolerance, use different approaches to detoxifying an environment, or mobilize different nutrients (Saunders, Glenn and Kohn 2010). Alternatively, stressful environments may simply limit species abundances and thus reduce the likelihood of competitive interactions (Chesson 2000). Thus, target fungal communities would ideally be beneficial under all conditions or beneficial under stress and otherwise neutral.

Our objective was to understand how endophyte interactions affect plant stress responses and whether those outcomes could be predicted by similarity of fungal traits in culture. To this end, we grew the C4 grass, *Panicum virgatum*, with 12 individual

endophytic fungi, six mixtures of two of the individual fungi, and in fungus-free controls under low and high soil moisture. We examined the role of fungal niche overlap in plant responses based on a wide range of fungal traits in culture that are putatively related to behavior in symbiosis, including fungal stress tolerance, enzyme production, and substrate use, and metabolite production. We broadly hypothesized that the effects of fungal pairs on plant performance would be both non-additive and dependent on stress level, reflecting shifting niche overlap. Specifically, we expected (1) more super-additive effects when fungi were more dissimilar and under stress - conditions where competitive interactions should be weakened, (2) more sub-additive negative effects on the plant when fungal traits were more similar and in unstressed – conditions under which competition should be heightened, and (3) additive effects when fungi where dissimilar and unstressed – conditions where fungi might not interact. We further expected that fungal traits could qualitatively predict outcomes as positive, negative, or neutral for the plant, focusing on metabolites that might ultimately prove useful in application development. Finally, we hypothesized that the degree to which non-additive effects differed from expected additive effects could be predicted quantitatively, with greater deviations under the stress of low soil moisture, and deviations increasing linearly with increasing dissimilarity among fungal traits.

METHODS

Selecting endophyte pairs

Endophyte pairs were selected from a pool of 33 taxa isolated from *Panicum* grasses in central Texas (Giauque and Hawkes 2013, Giauque 2016). To select fungal pairs that would create a wide range of plant responses independent of phylogenetic

history, we selected taxa across a range of similarity in their effects on plants, while limiting their phylogenetic distance.

Endophytes were previously screened by Giauque (2016) for their individual effects on five plant responses in *Panicum virgatum* under drought and well-watered conditions: plant biomass, relative growth rate, transpiration efficiency, days to first wilt, and survival. These data were used to calculate similarity of fungal effects on plants for every possible two-species endophyte combination using the Euclidean distance method (Griffith, Rayner and Wildman 1994, Walker, Kinzig and Langridge 1999) with the *stats* package in R (R Core Team 2016). To control for phylogenetic distance, we eliminated any combinations that had a cophentic distance of less than 1 and confirmed that there was no relationship between cophenetic distances and Euclidean distances with a linear regression analysis. The remaining two-species fungal combinations were grouped into two pools representing pairs with higher than average or lower than average similarity of effects on plants, with the average being 1.86. We then selected three pairs of endophyte taxa without replacement from each pool to generate a total of six pairs (Table 2.1; Figure 2.1, additional details in Appendix 1 'Calculation of endophyte phylogenetic distances'). Similarity of fungal effects on plants was only used to select endophyte pairs and not used as an indicator of niche overlap because (1) data was obtained from an unrelated study and as such we did not expect values to completely align with our data, and (2) our primary goal was to identify links between fungal traits that can be easily studied in culture and possible mechanisms regulating fungal interactions in symbiosis.

Characterizing endophyte culture traits

We screened the 12 selected fungi for traits in culture that putatively relate to behavior in symbiosis: osmotic stress tolerance, enzyme activities, resource use, and metabolite profiles. All traits were measured on individual fungi; in addition, metabolites were measured on co-cultures of the six target fungal pairs. We used standard methods: (1) osmotic stress assays used fungal growth at five levels of NaCl (Bell and Gonzalez 2009) to measure osmotic sensitivity and tolerance; (2) enzyme assays fluorometrically measured activity of three carbon-degrading enzymes, α-1,4-glucosidase, β-1,4-glucosidase, and cellobiohydrolase (Saiya-Cork, Sinsabaugh and Zak 2002); (3) resource use profiles were based on utilization of 95-substrates in Biolog FF microplates (Biolog, Hayward, California, USA) with average substrate use calculated for carbohydrates, amino acids, amines, polymers, carboxylic acids; and (4) metabolic profiles of fungal extracts were generated from reverse-phase liquid-chromatography coupled with ultraviolet spectroscopy and electrospray ionization mass spectrometry (LC-ESIMS) analysis. Metabolite compounds were identified by retention time and UV spectra, and compound abundance was calculated from absolute peak area. (A more detailed description of culture trait assays can be found in the appendix 1 'Fungal trait assays).

Culture trait dissimilarity between paired fungi was calculated for each of the four trait groups. Trait values were scaled to a mean of 0 and SD of 1 (Lamanna et al. 2014) and dissimilarity was calculated using the Euclidean distance method in the *stats* package in R (R Core Team 2016). As such, dissimilarity among traits is represented as continuous values increasing from 0, with 0 representing two fungi that share the same traits and increasing values representing greater dissimilarity. We used the classical method of measuring trait dissimilarity in preference to other common measurements

(Petchey and Gaston 2002, Podani and Schmera 2006, Villéger, Mason and Mouillot 2008) because our data included few fungal species and as such dendogram path-lengths are likely to underrepresent dissimilarity (Griffin et al. 2009). In addition we had no missing data, and all trait data was continuous (no mixed data types) thus negating limitations of Euclidean based measurements (Podani and Schmera 2006).

Experimental test of plant responses to two-species endophyte mixtures

To assess how interactions between endophytes affected plant performance, we grew *P. virgatum* plants inoculated with the six different pairs of endophyte species, the corresponding 12 individual endophytes, or without fungi. Plants were grown under either high (15%) or low (5%) gravimetric soil moisture, which we know from prior work represent unstressed and stressed conditions (Giauque and Hawkes 2013). Each treatment combination was replicated four times for a total of 152 plants.

Panicum virgatum seeds (Native American Seed, Junction, TX) were surface sterilized in 0.5% sodium hypochlorite (4 min) followed by 70% ethanol (90 sec), rinsed in sterile water, and germinated on dampened sterile filter paper in petri dishes. After one week, germinated seeds showing no evidence of contamination were planted into sterile planting boxes consisting of a clear top chamber (8 x 8 x 45 cm) constructed of Propafilm (LI-COR Biosciences, Lincoln, NE) wrapped on a metal frame and attached to a 5-L pot (7 x 7 x 45 cm; Stuewe and Sons, Tangent, Oregon USA). Pot drainage holes were lined with 20-μm nylon mesh to reduce the probability of fungal contamination. Planting boxes were filled with autoclaved sand and nutrients were provided as 5 mL of filtered ¼ strength Hoagland's solution (Hoagland and Arnon 1950). Pre-treatment plants were grown for two months in an isolated greenhouse at ambient light and temperature with

15% soil moisture. Immediately prior to fungal inoculation, plants were tested for fungal contamination by culturing leaf segments into 2% potato dextrose (PD) agar and 50 ppm ampicillin plates, and any plants showing fungal growth were discarded.

One week before plant-endophyte trials commenced, the 12 fungal isolates were cultured into PD broth in 50 mL conical tubes at room temperature with constant shaking (110 rpm). To create consistent inoculations, fungal hyphae were concentrated via centrifugation (10 min at 14000 rpm), washed and re-suspended in sterile water to obtain 10^5 hyphal fragments ml^{-1}. Plants inoculated with single isolates received 2 mL of hyphal suspension directly pipetted onto plant shoots (Rodriguez et al. 2008), plants with an endophyte pair received a 2-mL mixture of 1 mL of hyphal suspension from each fungal isolate, and control plants were given a mock inoculum of 2 mL sterile water. One week after inoculation, the soil moisture treatment was imposed; use of one week was chosen based on previous work demonstrating that fungi become established during this time (Giauque and Hawkes 2013). Moisture treatments were maintained by weight, with all pots checked and adjusted every 4 days. Plant locations in the greenhouse were also randomized every 4 days to avoid spatial effects.

To assess the effects of endophyte interactions and soil moisture on plant performance, we measured plant height, days to first wilt, and total number of tillers. Wilt was assessed daily while plant height and tiller number were measured every 7 days. Plant relative growth rate was calculated as the change in plant height over time (cm day^{-1}). Measurements were made in a laminar flow hood that was UV-sterilized between plants to prevent cross-contamination. On day 21, final plant measurements were made and target fungal inoculum was confirmed by placing 2-mm^2 samples of surface-

sterilized leaf tissue from each plant on petri dishes as above. All plants used for analyses were colonized by the appropriate individual or pair of fungi and had no contamination.

Quantifying effects of fungal interactions

We examined two metrics to identify the effects of fungal interactions on plant responses: (1) expected-additive effects of plant responses to fungal pairs compared to individual fungi, and (2) the deviation of the observed effect of fungal pairs on plant responses from the expected-additive effect. Each metric was calculated for each plant responses under low and high soil moisture. If effects of fungal pairs are significantly different from additive, then interactions among fungi are important in shaping the outcomes of plant-endophyte symbioses. Following Wardle et al. (1997), we used the classic approach to testing additive vs. non-additive effects and compared fungal pair effects to the expected-additive effect of the component monocultures. Expected-additive effects ($\bar{Y}_E$) were calculated as the average of the mean effect of each component endophyte in monoculture. To quantify the magnitude of non-additive effects, we followed (Loreau and Hector 2001) and calculated deviations from expected-additive. Deviations were calculated as the difference of the mean observed effect of fungal pairs to the expected-additive effect.

STATISTICS

Plant treatment responses

We first examined the overall treatment effects on plant responses using ANOVA. Specifically, we tested for the main effects of endophyte treatment (including all monocultures, mixtures and the control), soil moisture level (low and high), and their interaction. We tested the effect of fungal treatment, rather than the effect of fungal

richness, because we had only monocultures or pairs (Jansa et al. 2008); we also did not test composition because we did not use a complete replacement design (Schmid and B.Hector 2002). When significant interactions were found ($P < 0.05$), each treatment was analyzed across levels of the other treatment using one-way ANOVAs; in cases where fungal treatment was significant, we compared single and paired fungal treatments, under low and high soil moisture separately, to the respective fungus-free controls with Dunnett's multiple comparison posthoc tests (Dunnett 1955). Fungi that were not significantly different from the fungus-free control were considered commensal and those that were significantly greater or less than the control were considered beneficial or detrimental, respectively. Post hocs were Bonferroni-corrected for multiple comparisons to maintain family-wide confidence limits at 90%; in our model with 38 individual contrasts the cutoff was $P < 0.003$.

Additive and non-additive effects of fungal endophyte mixtures

Observed mixture effects were considered significantly different from additive if the 95% CI did not overlap expected-additive effects ($\bar{Y}_E$). We interpreted super-additive effects as those significantly greater than $\bar{Y}_E$ and sub-additive effects as those significantly lower than $\bar{Y}_E$. Note that unlike in studies of plant additivity, we were unable to partition sampling effects or species complementarity effects because plant responses to single endophyte species within mixtures could not be separated (Loreau and Hector 2001).

Qualitative indicators of additive vs. non-additive fungal pairings

To identify potentially useful indicators of qualitative fungal interaction effects, we focused on fungal metabolites. We examined whether metabolites produced by fungal pairs could indicate a pairing as super-additive, sub-additive or additive using the

Dufrene-Legendre indicator analysis (Dufrene and Legendre 1997) using the *labdvs* package in R (Roberts 2015). This analysis calculates an indicator value for each metabolic compound as the product of its relative frequency (presence-absence) and relative average abundance (peak area) in each group (super-additive, sub-additve or additive) and uses a random permutation test (n = 999) to calculate a significance value.

Plant response deviations from additive

For analyses of deviations, we focused only on plant responses that differed significantly from the expected-additive model (plant growth rate and days to first wilt). We used ANOVA to examine the deviation from additive as a function of endophyte-pair treatment (mixtures only), soil moisture level (low and high), and their interaction. When significant interactions existed ($P < 0.05$), each fungal treatment was analyzed across levels of soil moisture using one-way ANOVAs and we used the Bonferroni correction to maintain family-wide confidence intervals of 90%; with 12 contrasts the cutoff was $P < 0.008$

Quantitative predictors of plant response deviations from expected-additive effects

To examine how the deviation of plant responses from additive depended on fungal trait dissimilarity, we conducted stepwise multiple regressions. Separate regression models were run for the deviations of plant growth rate and days to first wilt. In both models, the independent variables were soil moisture and the trait dissimilarities of fungal pairs in osmotic stress tolerance, enzyme activity, resource use, and metabolic profiles, as well as the interactions of moisture and each trait. Soil moisture treatment was coded as a dummy variable to capture the potential environmental dependence of these relationships. Trait groups did not display multicollinearity based on variance inflation

factors (VIF ≤ 4) using the vif function in the car R package (Fox and Weisberg 2001). The best explanatory model was selected based on Akaike Information Criteria (Akaike 1974). When significant interactions existed, we used linear regressions to analyze the effect of dissimilarity between fungal pairs under low and high moisture separately.

RESULTS

Plant treatment responses

Overall, plants grew faster under high vs. low soil moisture (Table 2.2), and growth rate and days to first wilt varied across fungal treatments (Table 2.2). However, both plant growth rate and days to first wilt were significantly affected by the interaction of fungal treatment and soil moisture (Table 2.2), reflecting shifting effects of fungal treatments between low and high moisture. The vast majority of fungal-plant combinations did not differ from fungus-free control plants and were thus considered commensal (Table 2.3). Only 13% of cases were beneficial compared to fungus-free controls and these occurred only in the low moisture treatment (equally distributed between individual fungi and mixtures); 5% of fungal inocula had negative effects relative to fungus-free controls and were found only for individual fungi in the high moisture treatment (Table 2.3). Plant tiller number was not affected by fungus, soil moisture, or their interaction; all plants had 1 to 3 tillers.

Additive and non-additive effects of fungal endophyte mixtures

For plants inoculated with mixtures of two fungal species, tiller number followed the expected-additive model based on plants inoculated with the corresponding individual fungi (Figure 2.2). However, there were non-additive growth and wilting responses, in which plants performed greater than or less than expected compared to an additive model

(Figure 2.2). Among non-additive responses 79% were super-additive and 11% were sub-additive, but the specific responses depended on fungal pair and moisture treatment. In some cases, non-additive responses shifted the outcome of plant-endophyte symbioses along the parasite to mutualist continuum (Table 2.3). For instance, in low soil moisture the individual fungi comprising pair 4 were both beneficial for growth rate on their own, but commensal in mixture. Conversely pair 5 individual effects on plant growth were detrimental and commensal, but were beneficial in mixture. Furthermore, inoculation with some pairs of fungi reduced the moisture dependency of individual fungal effects on plants. Specifically, for pairs 1, 3, and 5 the moisture-dependent effects of individual fungi on plant growth rate and days to first wilt disappeared when the two fungi were grown in mixture (Table 2.3).

Qualitative indicators of additive vs. non-additive fungal pairings

On average, fungal pairs produced 8 metabolites (SD = 3), of which there were large variations in repression of compounds (between 4 and 10; SD = 2) and small variations in the production of new compounds (between 1 and 4; SD = 1) compared to the corresponding fungi in monoculture. There were six indicator metabolites for super-additive responses and three for sub-additive responses, but these indicator metabolites were only identified in low soil moisture. In contrast, the eight metabolites that were associated with additive effects occurred in both high and low moisture (Table 2.4; Figure 2.3). Some metabolites were unique to a single outcome whereas others were indicative of more than one condition. For example, two of the four metabolites associated with super-additive effects on days to first wilt under low moisture were also associated with additive effects on wilt under high moisture.

Plant response deviations from additive

Fungal pairs affected the deviation of plant growth rate and days to first wilt from an expected-additive model, but the effect was dependent on soil moisture (Table 2.5, Figure 2.4). Plant growth deviated from additive more under low than under high soil moisture conditions for fungal pairs 3, 4, and 5 by +113%, -327%, and +238%, respectively. For fungal pairs 4 and 5, plant wilt also deviated more from additive in low than high moisture, by -905% and +331%. In contrast, for plant wilt with fungal pair 2, the deviation from additive was +306% greater in high compared to low moisture.

Quantitative predictors of plant response deviations from expected-additive effects

Based on regression models, moisture and fungal trait dissimilarity explained 51-92% of the variation in how plant responses deviated from the expected additive model (Table 2.6, Figure 2.5). Deviations of plant growth rate and days to first wilt were best explained by dissimilarity of fungal metabolic profiles and dissimilarity of fungal stress tolerance, but these effects varied between high and low moisture (Figure 2.5). In low soil moisture conditions, super-additive effects on plant growth rate and days to first wilt occurred with increasing dissimilarity in fungal metabolic profiles (Figure 2.5a and 2.5c). Under high soil moisture, super-additive effects on plant growth rate and days to first wilt were more likely when fungi were more dissimilar in stress tolerance traits (Figure 2.5b and 2.5d).

DISCUSSION

Pairwise interactions between leaf endophytes resulted in both additive and non-additive effects on plants. In cases where interactions were additive, prediction of fungal interaction effects on plants will be a simple function of the individual fungal effects. For

non-additive interactions, we found individual metabolites that were indicative of the direction of the effect, and the size of the deviation was largely predicted from fungal niche overlap, as defined by dissimilarity among fungal traits in culture. Consistent with our expectations, endophytes that were less similar in stress and metabolic traits increased plant growth rate and days to first wilt. These results provide support for niche complementarity as a driver of fungal interactions and demonstrate that fungal niche-based processes at the leaf-level can scale up to the plant. However, the environmental dependence of some fungal combinations means that development of fungal consortia for real-world applications will need to consider the dynamics of these interactions.

Facilitation and complementarity among dissimilar fungi may allow for the development of leaf endophyte communities with unique benefits, much like microbial root consortia have been employed in pathogen biocontrol (Dunne et al. 1998, Raupach and Kloepper 1998, Whipps 2001). We made inroads into methods for identifying appropriate mixtures of taxa via fungal secondary metabolites indicative of interaction outcomes. Across taxa, there were two metabolites uniquely associated with super-additive increases of plant growth rate and two associated with super-additive increases in days to first wilt. We also found metabolites that indicated multiple conditions: suggesting effects may be quantitative; such dose-dependent effects have been observed for many microbial-derived metabolites that act as inhibitors (Duke et al. 2000). Ultimately, key positive metabolites may serve as direct pathways to mitigate plant stress in agricultural systems. Although substantial work is needed to translate initial patterns into useful tools, robust processes already exist for developing fungal metabolites into pesticides and fungicides (Demain and Fang 2000). Moreover, recent studies have linked

fungal metabolites to gene clusters, laying the foundation for large scale screening of fungal genomes for target compounds and for metabolic engineering for increased compound production (Pickens, Tang and Chooi 2011, Ballester et al. 2015).

In addition to qualitative outcome prediction, fungal metabolic and stress trait dissimilarity predicted the magnitude of deviations from expected additive interactions for plant growth and wilting. Increasing super-additive effects between fungi with more dissimilar traits further supports a niche-based complementarity mechanism and suggests that fungal traits in culture can be used to identify benefits in the host. In some cases, metabolite production might also affect how endophytes cope with stress; for instance, endophytes can produce osmolytes that reduce cellular desiccation and phenolic antioxidants that can scavenge harmful superoxides and hydroxyl free radicals (Schulz and B.Boyle 2002, Malinowski and Dariusz P.Belesky 2006, Yuan, Chu-long and Fu-cheng 2010). However, here these traits were not significantly correlated ($R2 = 0.13$, $P = 0.247$), perhaps because our approach to metabolite characterization did not capture osmolytes or because stress tolerance mechanisms in fungi were not chemical in nature. Similarly, other traits we thought might reflect interaction mechanisms were poor predictors, such as those related to fungal growth and enzyme production. It is likely that many fungal traits in culture are not reflective of fungal behavior in symbiosis. In addition, predictive traits could change with conditions such as plant life stage or plant physiology and resulting carbon availability to symbionts.

Fungal interactions, their effects on plants, and their predictive traits depended on whether plants were grown in water-stressed or well-watered conditions, which could limit applicability. Using different traits to predict outcomes in different environments

might be feasible in some circumstances, but ideally fungal consortia would have consistently beneficial effects and predictors. We only observed consistent commensal behavior for pairs 1, 2, and 4 in mixture. For stress management, fungi that are beneficial under stress and otherwise neutral could also be effective; we found pairs 5 and 6 in mixture shifted from commensal to beneficial for plants only under low soil moisture. Furthermore, several of the fungi used in our experiment are known to be plant pathogens in other systems, such as A. niger in maize (Zea mays) and peanuts (Arachis hypogaea) (Palencia, Hinton and Bacon 2010), and C. lunatus in sorghum (Sorghum bicolor) (Katilé et al. 2010). Because endophytes are known to shift along the mutualism-pathogen continuum under different environments and plant hosts (Saikkonen et al. 1998), exploiting the chemical mechanisms behind fungal-mediated benefits may be a more effective strategy in agriculture.

Our approach has limitations. We tested only six pairs of fungi, which demonstrates the importance of interactions and identifies predictive traits, but does not allow for development of a broad predictive framework for the outcome of interactions. Focusing on fungal pairs is also unrealistic, since C4 grasses and crops are typically colonized by dozens of fungal taxa and these could change observed pairwise interactions (Kennedy et al. 2007). Furthermore, our experiment was short-term and focused on juvenile Panicum; although this is a critical life stage, juveniles invest more in tiller height and roots than in tiller production (Lowry et al. 2015) and thus limit our ability to detect other effects such as on reproduction. We also did not measure the effect of endophyte treatment on plant biomass because all plant tissue was used to confirm fungal inoculation success. In addition, metabolite data were based on ethyl acetate extractions,

which will target predominantly hydrophobic metabolites (Blanks and Prausnitz 1964) and thus will not extract water-soluble sugars that may be important osmolytes. Extractions were also taken from fungal cultures and do not necessarily represent activity in the leaf. Finally, at this point further structural data is needed (e.g. high resolution mass spectrometry and nuclear magnetic resonance spectroscopy) to accurately identify specific metabolites; however, this provides a starting point for future work which will isolate and identify metabolites with bioactivity towards plant stress responses. A more comprehensive approach that captures the full metabolite spectrum will be employed to identify the best initial targets, which can then be isolated, characterized, and tested directly on plants for drought stress amelioration Finally, extractions were run on a low-resolution mass spectrometer meaning that we cannot accurately identify specific metabolites; however, this provides a starting point in which to isolate and identify metabolites with bioactivity towards plant stress responses. A more comprehensive approach that captures the full metabolite spectrum will be needed to identify the best initial targets, which can then be isolated, characterized, and tested directly on plants for drought stress amelioration.

Overall, our study highlights key challenges to predicting fungal effects on plants and provides a future direction to employing endophytes for agricultural plant management. First, interactions among fungal symbionts can affect the outcome of plant-fungal symbioses across a range of mutualistic to antagonistic. Second, effects of fungal interactions can shift due to changes in soil moisture. As such, identifying combinations of beneficial fungi will also require identifying communities that have either consistent effects as environment fluctuate or only for which the degree of shift is acceptable, i.e.,

super-additive/synergistic when needed under drought and otherwise commensal. Third, we identified traits in culture that can be used to predict when endophyte interactions will be beneficial or harmful to the plant host. This knowledge can be used to create more complex combinations of beneficial endophytes. Taken together, these results support the need to move beyond single-inoculum studies of fungal effect on plants and instead study fungi in a community context.

Chapter 3: Chemical mechanisms underlying plant-fungal interactions

INTRODUCTION

Symbiotic associations between plants and endophytic fungi are ubiquitous, with mutualistic to antagonistic interactions that directly affect plant fitness. Despite their ecological importance, the basic mechanisms governing plant-endophyte symbioses remain poorly understood, with the exception of vertically transmitted endophytes in cool-season grasses (Clay and Schardl 2002). Much less known is about horizontally transmitted fungal endophytes, which reside within tissue of nearly all studied plants (Rodriguez et al. 2009). Horizontally transmitted endophytes (hereafter "endophytes") are typically defined as asymptomatic fungi living inside plant tissues (Rodriguez et al. 2009), but can have a broad range of non-pathogenic effects on the host plant (Carroll 1988). Here, we propose that individual endophyte effects on the host plant are altered by both the biotic and abiotic context, and we examine chemical mechanisms underlying those effects.

Endophytes are well known for affecting plant stress responses (Redman et al. 2011, Rodriguez et al. 2008, Giauque and Hawkes 2013, Arnold et al. 2003); however, the evidence for endophyte stress effects comes almost exclusively from tests of individual fungi. Yet most plants are colonized by a community of endophytic fungi that have the potential to interact, which can change how the symbiosis impacts the plant. For instance, both facilitative and competitive interactions among root-fungal symbionts can generate non-additive effects on plant hosts (Jansa et al. 2008, Thonar et al. 2014, Kennedy et al. 2007, Guske, Schulz and Boyle 2004, Connor et al. 2017). In an earlier study on *Panicum* plants, we found that fungal interactive effects on plant drought

responses were primarily non-additive, with effects dependent on the degree of trait similarity between the interacting fungi (Connor et al. 2017).

Alternatively, community effects can be caused by a single species in the mixture, as is common in diversity experiments (Cardinale et al. 2006, Loreau and Hector 2001). Individual endophytic fungi may come to dominate overall function via selection effects in which competition leads to the dominance of the most productive species. Such species effects are only occur expected to occur if function and species abundance are tightly linked; however, recent studies have shown negative selection effects, where less abundant species can dominate function when function is not measured in terms of biomass or yield (Jiang 2007, Bruno et al. 2005). This may hold true for endophyte communities, where mutualisms are maintained by controlled fungal growth within plants (Christensen, Bennett and Schmid 2002) and many effects are driven by fungal produced compounds, such that endophytes with better chemical factories per unit hyphal length may exert greater control on the symbioses.

Effects of both individual and mixtures of endophytes are often plastic in their response to the environment (Saikkonen et al. 1998, Giauque and Hawkes 2013, Schulz and Boyle 2005, Connor et al. 2017). As such, plant-endophyte symbioses can switch from mutualistic to pathogenic as the environment changes (Giauque and Hawkes 2013, Ren and Clayy 2009). For individual endophytes, there is no strong link between the environment and direction of fungal effects, with both pathogenicity and mutualisms occurring when plants are stressed (Ren and Clayy 2009, Schulz and Boyle 2005, Andrews, Hecht and Bashirian 1982, Kannadan and Rudgers 2008, Donoso et al. 2008, Giauque and Hawkes 2013). However, when scaling up to multiple fungal species, the

influence of the environment on competitive processes may negate these individual-level processes. For instance, within plant communities, facilitative interactions are often more common with increasing stress across environmental gradients (Bertness and Callaway 1994). Alternatively, in an earlier study with *Panicum* plants, we saw that inoculations with pairs of endophytes reduced the moisture dependency of individual fungal effects on plants (Connor et al. 2017).

Understanding the underlying mechanisms governing fungal effects on plants would better explain the complex and often unpredictable outcomes of plant-endophyte interactions. In general, endophyte effects are expected to be driven by chemical mechanisms, with the fungi producing compounds that have bioactivity against plant stressors (Strobel and Daisy 2003, Hardoim et al. 2015, Waqas, Khan and Lee 2014). The most well-known example is the production of toxic alkaloids to reduce herbivory in the tightly co-evolved symbiosis between vertically transmitted *Epichloe* endophytes and C3 grasses (Clay and Schardl 2002). Horizontally transmitted fungi also produce a plethora of bioactive secondary metabolites; although to date the functions in planta of only a few of these compounds have been characterized. In our own studies, we have linked fungal metabolites in culture to fungal interactive effects on plant moisture responses (Connor et al. 2017); however, these mechanisms must be tested *in planta* to be generalizable to the field.

To understand how biotic and abiotic context control the outcomes of plant-endophyte symbioses, we examined the effects of fungal interactions and drought on the performance and metabolomics profiles of *Panicum hallii* Vasey (Hall's Panicgrass). *Panicum hallii* is a short-lived, native, warm-season grass related to the biofuel species

Pancium virgatum. We tested the hypotheses that (1) plant responses to moisture depend on fungal treatment, (2) in some cases, effects of fungal treatment are controlled by a specific fungal species rather than the pair per se (species effects), (3) metabolomics profiles explain the effects of fungal treatment on plant responses to moisture.

METHODS

Experimental test of plant responses to two-species endophyte mixtures

To assess how endophytes and moisture affect plant performance, we grew *P. hallii* plants inoculated singly with five different endophyte species and with every pairwise endophyte combination (10 pairs), or with mock inoculum to create fungus-free controls. Plants were grown under either high (15%) or low (5%) gravimetric soil moisture on sterile sand soils amended with X% Hoagland solution. We know from prior work on *Panicum virgatum* in the same soils that 5% moisture limits growth and survival, whereas 15% moisture supports high growth rates (Connor et al. 2017, Giauque and Hawkes 2013). Each treatment combination was replicated five times for a total of 160 plants.

Panicum halii seeds were collected from wild populations at the Lady Bird Johnson Wildflower Center in Austin, Texas in May 2016. Seeds were surface sterilized in 0.5% sodium hypochlorite (4 min) followed by 70% ethanol (90 sec), rinsed in sterile water, and germinated on dampened sterile filter paper in petri dishes. After one week, germinated seeds showing no evidence of contamination were planted into sterile Magenta boxes (Magenta GA-7; Magenta Corporation, Chicago, IL) modified with 20-μm nylon mesh windows to allow water loss while preventing cross contamination. Plant

and fungal pre-treatment growth, and fungal inoculations followed Connor et al 20017 (Chapter 2).

Briefly, plants were grown for 8 weeks in an isolated greenhouse at ambient light and temperature with 15% soil moisture; plants were kept sterile until they reached the adult stage. Immediately prior to fungal inoculation, plants were tested for fungal contamination by culturing leaf segments into 2% potato dextrose (PD) agar plates with 50 ppm ampicillin, and any plants showing fungal growth were discarded. One week before plant-endophyte trials commenced, the five fungal isolates were cultured into PD broth in 50 mL conical tubes at room temperature with constant shaking (110 rpm). Fungal hyphae were concentrated via centrifugation (10 min at 14000 rpm), washed and re-suspended in sterile water to obtain 10^5 hyphal fragments mL^{-1}. Plants inoculated with single isolates received 2 mL of hyphal suspension directly pipetted onto plant shoots, plants with an endophyte pair received a 2-mL mixture containing 1 mL of hyphal suspension from each fungal isolate, and control plants were mock inoculated with 2 mL sterile water.

Plants were grown with fungal and moisture treatments in the greenhouse for 4 weeks, until just before flowering. High and low water treatments were maintained weekly by weight. We measured plant growth rate as the change in height over time, plant size as fresh shoot biomass (rather than dry biomass because the tissue was needed for metabolomic extractions), whole-plant water loss by weight compared to plant-free controls, and survival as the percentage of tillers that did not wilt (tiller wilt). Plant height and water loss were assessed every three days, whereas tiller wilt and biomass were assessed at harvest. All measurements were made in a laminar flow hood that was UV-

sterilized in between plants. The logit transformation were applied to tiller wilt because values were proportions (Warton and Hui 2011). Plants were harvested; aboveground plant material was clipped, weighed, immediately frozen in liquid nitrogen, and stored at -80 °C until metabolite extractions.

Identifying effects of fungal inoculum on plant metabolomics profiles

Plant metabolomics profiles were obtained from whole plant crude extracts using methanol:chloroform:water extractions on frozen leaf tissues with three replicates per treatment (Theodoridis et al. 2012, Maia et al. 2016). Briefly, frozen samples were ground in liquid N_2 and approximately 40 mg of ground sample was immediately added to an ice cold methanol:chloroform:water solution (600:300:300 μL). Samples were vortexed for 15 s and placed in ice on a shaker for 30 min at 130 rpm, with samples vortexed every 5 min. To separate the organic and aqueous phases, samples were centrifuged (10,000g, 10 min, 4 °C) and the layers were transferred by syringe into glass vials. The organic phase was then stored at -80 °C until vacuum evaporation, after which the pellet was reconstituted in 500 μL of methanol. LC-MS chromatograms were obtained from liquid chromatography coupled to time of flight mass spectrometry (LC/TOF/MS) (Q-TOF Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS) using high resolution electrospray ionization in positive mode at the Department of Chemistry Mass Spectrometry facility at the University of Texas at Austin.

Plant metabolomics profiles were created from chromatographically separated and single-spectra mass spectral data using the *XCMS* package in R (Smith et al. 2006, Tautenhahn, Bottcher and Neumann 2008, Benton, Want and Ebbels 2010). Data was preprocessed using the following steps: (1) peak identification and matching (full width

at half maximum (FWHM) of 30 s; extracted ion base peak chromatograms at 0.1 m/z), (2) retention time correction (least-squares method), (3) peak matching (FWHM of 10 s) and (4) filling in missing peaks. A matrix was then created for all detected compounds (identified by m/z and retention time) based on peak intensities. To obtain a matrix containing compounds associated with fungal colonization in plants, the average intensity of compounds from fungus-free control plants (separated by soil moisture) was subtracted from each compound and intensities were scaled to have a zero mean and a unit variance (Worley and Powers 2013).

STATISTICS

Effect of fungal treatment and soil moisture on plant performance

We used MANOVA to analyze how plant physiological responses (growth rate, shoot biomass, water loss, and tiller wilt) varied with moisture treatment, fungal treatment, and their interaction. Significant factors were compared to the respective fungus-free controls with Dunnett's multiple comparison post-hoc tests (Dunnett 1955). For significant interactions, fungal treatment was also analyzed across moisture levels using one-way ANOVA with Bonferroni-corrections to maintain family-wide confidence limits at 90% (in our model with 45 individual contrasts the cutoff was $P < 0.002$). We also tested for fungal species effects on plant performance by analyzing individual plant responses as a function of the presence of each fungal species, moisture treatment, and their interactions (Jacob, Hertel and Leuschner 2013). This analysis allows us to explicitly test species identity effects separate from fungal treatment effects, as each fungus is present in five different fungal treatments. We limited the species analyses to cases where there was a significant main effect of fungal treatment or fungal treatment x

moisture treatment interactions. Models were Bonferroni-corrected for multiple comparisons, such that significant effects occurred at $P < 0.017$.

Chemical mechanisms underlying fungal effects

We analyzed how the plant metabolome varied with moisture and fungal treatments, using PERMANOVA with Euclidean distances using adonis in the vegan package in R, with each term entered last in the model to obtain Type III sums of squares (Oksanen et al. 2017, Anderson and Walsh 2013). Because there were significant effects of fungus and fungus x moisture, we examined the role of individual fungal species in shaping the plant metabolome by running PERMANOVA with the presence of each fungus, moisture treatment, and their interaction.

Linking metabolomics profiles to plant responses

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Linking metabolomics profiles to plant responses

To examine how plant physiological responses were related to their metabolomics profiles, we first reduced each multivariate dataset using nonmetric-multidimensional scaling (NMS) with Bray-Curtis distances in PC-ORD (McCune and Mefford 2011). All data were standardized to unit variance and only response variables from plants with a paired metabolomics profile (n=3) were included in the NMS. Thus, plant physiological responses to moisture and fungal treatments for each replicate plant, as well as their associated metabolomics profiles, were represented by single points in an ordination space where increasing distance in ordination space represents greater dissimilarity among samples (McCune and Mefford 2011). For the plant response NMS, we identified the combinations of plant responses represented by each NMS axis using multiple linear regressions with the *stats* package in R (R Core Team 2016).

We used multiple linear regression to examine how overall plant physiological responses to fungal treatments depended on plant metabolomics profiles, including their respective NMS axis 1 and 2 scores. Regressions were run separately for NMS scores in low and high soil moisture based on the significant interaction of moisture and fungal treatment on plant responses (above mentioned MANOVA). Metabolome patterns associated with fungal treatments, fungal species, and moisture were visualized with dendograms and clustered image maps using the complete linkage method in the *mixOmics* package in R (Gonzalez et al. 2012, Cao et al. 2016). For significant factors, we further identified the compounds associated with those effects via bipartite networks. Because bipartite networks are inferred from pair-wise similarity matrices obtained from corresponding ordination analyses, we used partial least squares-discriminate analyses (PLS-DA) to reduce the overall model (Gonzalez et al. 2012, Cao et al. 2016). Specifically, to ensure PLS-DA models were not over-fit, we validated models using a 'leave-one-out' cross validation scheme (Westerhuis et al. 2008). The optimal number of components and variables for the model was then determined based on the minimum error rate (Wopereis et al. 2009). Correlations among treatments (presence of a specific fungus or moisture level), were calculated from the components retained from the PLS-DA and relevant compounds were obtained by setting the threshold to 0.60, indicating biological significance (Cao et al. 2016). Compounds were then identified using the Metlin database (Smith et al. 2005).

RESULTS

Effect of fungal treatment and soil moisture on plant performance

Plant responses to moisture depended heavily on fungal treatment, with significant interactions affecting growth rate, shoot biomass, and water loss (Table 3.2; Figure 3.1). Low moisture only minimally reduced plant growth rate (23%), shoot biomass (33%), and water loss (32%) compared to high moisture, whereas fungal treatment altered these same responses by 86%, 67% and 200% on average relative to fungus-free controls. Individual fungal treatments were also variable, ranging from 1.4 to 47 fold differences between the treatments with the largest and smallest effects across plant responses (Figure 3.1). The interaction of fungal treatment and moisture also affected shoot biomass, water loss and tiller wilt; but significant differences of fungal treatment across moisture levels only occurred at Bonferroni corrected cutoff of $P < 0.002$ for shoot biomass and tiller wilt (Table 3.2; Table 3.3).

Fungal species effects occurred for *Aspergillus* and *Nigrospora*, which mattered more than the fungal pair identity for plant growth rate, shoot biomass, and water loss (Table 3.4). The presence of *Aspergillus* reduced plant growth rates by 124% regardless of moisture (Figure 3.2). Other *Aspergillus* effects depended on moisture treatment: in high soil moisture shoot biomass decreased by 47% decrease and water loss increased by 148%, whereas in low soil moisture water loss decreased by 200% and shoot biomass was unaffected. Plants inoculated with a treatment containing *Nigrospora* had a 29% decrease in growth rate and 36% decrease in shoot biomass relative to treatments without *Nigrospora*, and these effects were consisted across moisture treatments.

Effect of fungal treatment and soil moisture on plant metabolomes

Fungal treatment and the interaction of fungal and moisture treatments explained 46% and 23% of the variation in plant metabolomics profiles, respectively (Table 3.5).

Fungal treatments partly reflected species effects of *Aspergillus* (R^2 = 7.7%) and *Nigrospora* (R^2 = 2.1%) on the plant metabolome, which were also moisture dependent (Table 3.7, Figure 3.3). In contrast, moisture on its own only explained 4% of plant metabolome variation.

The presence of *Aspergillus* was linked to 33 and 38 different compounds in low and high soil moisture. The presence of *Nigrospora* was similarly correlated with 19 different compounds in high soil moisture (Table 3.9); no compounds were associated with *Nigrospora* in low soil moisture. A large percentage of the compounds were unknown (*Aspergillus*: 64% and *Nigropsora*: 34%), meaning that they were not contained in the Metlin database. Of the identified compounds, only one is known to be exclusively produced by fungi (*Aspergillus:* Cytochalasin E); all others have been previously identified from either plants or both plants and fungi. Approximately half of the identified compounds associated with *Aspergillus* are involved in the oxylipin pathway, which regulates plant and microbial defense and development. In contrast, 63% of the identified compounds associated with the presence of *Nigrospora* were anthocyanin glycosides, which protect plants against a wide range of abiotic (e.g., UV damage, drought) and biotic (e.g., pathogen) stress. All of the compounds positively associated with *Aspergillus* or *Nigrospora* were negatively associated with plants colonized by other fungal treatments.

Linking metabolomics profiles to plant responses

Nonmetric-multidimensional scaling axes 1 and 2 cumulatively captured 94% of the variation in plant physiological profiles in both low and high soil moisture (Figure 3.3). For plant physiology in low soil moisture, NMS axis 1 was inversely related to plant

growth rate (R^2= 0.696, $P < 0.001$) and shoot biomass (R^2= 0.781, $P < 0.001$; Table 3.9; Figure 3.3b). For plant physiology in high soil moisture, NMS axis 1 was positively correlated with plant growth rates (R^2= 0.863, $P < 0.001$) and shoot biomass, (R^2= 0.736, $P < 0.001$), but negatively correlated with water loss (R^2= 0.678, $P < 0.001$; Table 3.9; Figure 3.3b). The NMS of plant metabolomics profiles described 74% and 83% of the variation in metabolomes in low and high soil moisture, respectively. When the plant metabolomics NMS axes 1 and 2 were used as independent variables to explain plant physiological NMS axes 1 and 2 in multiple regressions, the physiology NMS axis 1 was best predicted by the metabolomics NMS axis 1 under both low (R^2 = 47%) and high (R^2 = 66%) soil moisture ($P < 0.001$; Table 3.8; Figure 3.3c, 3f).

DISCUSSION

The plant mycobiome is a critical component of plant drought responses and may matter more than drought itself. We found that fungal treatment affected plant physiology 4-23X fold more than soil moisture, which was associated with underlying changes in the plant metabolome. Species effects further emphasize the role of biotic context in fungal effects on plants. Overall, these results are consistent with a strong role for biotic context, where outcomes of fungal interactions are dependent on the identities of the fungi within a community. Although soil microbial communities can also affect plant drought responses (Lau and Lennon 2012), this is the first demonstration that foliar endophytic fungal symbionts outweigh abiotic context in determining plant performance.

In some cases, the effect of fungal treatment could be directly linked to the presence of specific fungal species. In the presence of *Aspergillus* or *Nigrospora*, plants grew slower (47%, 29%) and were smaller (124%, 38%); plants with *Aspergillus* also lost

more water (148%) in high soil moisture and less water (200%) in low soil moisture. Both of these species have previously been reported to have negative effects on plant growth, including antagonistic interactions with wheat, ryegrass, potato, wisteria and switchgrass (Dewan and Sivasithamparam 1988, Giauque and Hawkes 2013, Louis et al. 2014, Soylu, Dervis and Soylu 2011, Wright et al. 2008).The dominance of the two most antagonistic fungi in our system on plant responses suggest that antagonisms may have stronger species effects that mutualist, however our study is limited by a small number of fungi.

Aspergillus and *Nigrospora* effects on plant physiology were paralleled in their metabolomes, with compounds that were indicative of the presence of each of these species in the plant. The presence of *Aspergillus* had the strongest effect on the plant metabolome, with metabolomes dominated by only a few highly abundant compounds. Many of these compounds are involved in the oxylipin pathway; oxylipins are produced by both plants and fungi, and are important in regulating plant-fungal cross-talk (Christensen and Kolomiets 2011). Fungal oxylipins are important in fungal growth, sporulation, production of mycotoxins, and production of degradative enzymes (Brodhagen et al. 2008 2004, Endogenous lipogenic regulators of spore balance in Aspergillus nidulans, Fischer and Keller 2016). However, plant oxylipins, which accumulate in plants during stress as reactive oxygen byproducts, can interfere with these processes in fungi presumably by signal mimicry, and thus act to regulate fungal development and reduce mycotoxin production (Gao et al. 2009 2014, Lipids in Aspergillus flavus-maize interaction)}. However, the role of plant oxylipins in mediating fungal pathogenicity appears to be species-specific, with *Fusarium* and *Cochliobolus*

fungal species becoming more virulent in plants where the oxylipin enzyme LOX was knocked out (Gao et al. 2007). Nevertheless, the majority of metabolites linked to the presence of *Aspergillus* in this study were unknown, and will require structural characterization (via nuclear magnetic resonance spectroscopy) and *in planta* assays to identify and understand their potential roles in plant-endophyte interactions.

The presence of *Nigrospora* was associated with an accumulation of anthocyanins in plant leaves. Anthocyanins are flavonoids that are primarily known for protecting plants against UV-damage and abiotic stressors, such as drought (Bolouri Moghaddam and Van den Ende 2012, Buer, Imin and Djordjevic 2010).Anthocyanins can also play a role in inducing plant immunity to pathogen infection (Tauzin and Giardina 2014, Kunz et al. 2008, Gutha et al. 2010, Abdel-Farid et al. 2009), but it is generally believed that plants elicit immune responses to pathogen infection at the expense of abiotic stress-induced flavonoid accumulation (Serrano et al. 2012). For instance, microbial triggered immunity has been to shown to repress anthocyanin accumulation even in the presence of environmental stress in both *Sorghastrum* and *Arabidopsis* (Serrano et al. 2012, Lo and Nicholson 1998). However, because the presence of *Nigrospora* was generally antagonistic and anthocyanins were only upregulated in high water, our findings are better aligned with a role for anthocyanins in plant defense to microbial infection (Treutter 2006).

Other fungal treatments generally had more positive effects on plant growth and biomass, and reduced water loss compared to *Aspergillus* and *Nigrospora*. These fungal treatments also had strong effects on the plant metabolome, but only in certain conditions. For example, in high moisture, *Fusarium* increased plant biomass by 111%

and generated unique metabolomic patterns relative to all other fungal treatments. *Cochliobolus kusanoi* and *Cochliobolus kusanoi + Nigrospora* also produced novel metabolomics patterns, but without corresponding unique effects on plant physiology, suggesting that many effects of the endophytes were likely missed by the measurements made in this study. Based on these results, there are multiple interaction pathways in the plant-endophyte symbioses.

The distinct chemical pathways that we identified for *P. hallii* colonized by different fungal endophytes support a growing body of work on chemical mechanisms of interaction. For instance, pairing *Oryza sativa* with either a mutualistic or pathogenic fungus resulted in contrasting metabolomics profiles , including 11 unique compounds with the mutualist (Xu et al. 2015). Similarly, three ecologically similar bacteria caused an order of magnitude difference in the production of primary plant metabolites, such as sugars, in *Arabidopsis* (Ryffel et al. 2016). This diversity of chemical mediated pathways is reflected in fungal metabolic profiles, which genomic analyses have revealed can vary widely even among closely related species (Keller, Turner and Bennett 2005). Ultimately, more research will be needed to identify whether there are broadly shared pathways among fungi based on interaction types, mutualist vs. antagonist.

Though plants may respond differently to infection by different fungal species, we have shown that single species can control the pathways in which plants respond to multiple fungi.

Our approach has limitations. We tested interactions only for pairs of endophyte species, which demonstrates both the importance of interactions and the potential for a single species to exert a greater control on effects, but does not consider more realistic

multi-species interactions within plants. Furthermore, we identified dual plant and fungal metabolomics profiles, such that origin of metabolite could not be assigned. Fungal metabolite libraries are also far less complete than plant metabolite libraries, which might also explain why a majority of compounds were unknown. Furthermore, metabolite data were based only on chloroform:methanol:water extractions, which target predominantly hydrophobic metabolites (Blanks and Prausnitz 1964) and thus will not extract water-soluble sugars that may be important osmolytes in drought conditions.

Overall, our study reveals key mechanisms regulating plant-endophyte interactions. First, the strong species effects on both plant physiology and metabolomics indicates that a single species could be more important than a consortium. Species effects in mixtures could have profound implications for the development of endophytes as treatments in agriculture, if unintended consequences due to intraspecific interactions can be avoided. Furthermore, the link between plant physiology and metabolites provides a starting point for using fungal metabolites to regulate plant responses. Finally, the strong effect of fungal treatment relative to moisture supports the growing body of research suggesting plant responses to changes in climate must be studied in the context of symbioses.

Tables

		Plant Volume			A_{max}			g_s		
Factor	df	MS	F	P	MS	F	P	MS	F	P
Plot										
Block	3	0.012			12.840			0.001		
Precip	2	0.038	3.455	0.100	317.936	14.890	**0.005**	0.008	6.245	**0.033**
Residual	6	0.011			21.352			0.001		
Split-plot										
Spp	2	0.154	12.833	**<0.001**	73.858	3.081	0.071	0.010	5.7854	**0.012**
Spp X Precip	4	0.019	1.583	0.222	27.029	1.127	0.375	0.001	0.444	0.775
Residual	18	0.012			23.973			0.002		
Sub-plot										
Year	2	0.013	3.250	**0.046**	306.752	18.069	**<0.001**	0.026	18.039	**<0.001**
Year X Precip	4	0.025	6.250	**0.000**	17.537	1.0330	0.399	0.001	0.718	0.584
Year X Spp	4	0.015	3.750	**0.009**	105.627	6.222	**<0.001**	0.005	3.171	**0.021**
Year X Precip X Spp	8	0.004	1.000	0.447	20.003	1.178	0.329	0.003	1.892	0.080
Residual	54	0.004			16.976			0.001		

Table 1.1: Split-split-plot ANOVA for plant volume and leaf-level fluxes. Fluxes are photosynthesis (A_{max}) and conductance (g_s). Analyzed as a function of precipitation treatment ("Precip") and plant species ("Spp") with year and block as random factors. Bold type indicates significant effects at $P < 0.05$.

	A_{max}					g_s				
	Est	SE	*t*	*P*	R^2	Est.	SE	*t*	*P*	R^2
Full										
Temp						$-3.1e^{-3}$	$1.4e^{-3}$	-2.173	**0.032**	0.050
Precip	0.00	0.001	3.910	**< 0.001**	0.126	$2.6e^{-5}$	$1.1e^{-5}$	2.291	**0.024**	0.054
Full model				**< 0.001**	0.126				**0.005**	0.095

Table 1.2: Multiple regression for leaf-level photosynthesis (A_{max}) and conductance (g_s). Analyzed as a function of seasonal variations in climate ("Temp" = air temperature, "Moist" = soil moisture), and precipitation treatment ("Precip"). R2 values are only reported for significant factors in each model.

			NEE			**ET**			**R_e**		
	Factor	Df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Plot	Block	3	0.018			336.218			0.007		
	Precip	2	0.070	2.595	0.154	6445.177	7.279	**0.025**	0.162	2.667	0.148
	Resid	6	0.027			885.432			0.061		
Subplot	Spp	2	0.016	0.608	0.555	1789.154	2.208	0.138	0.052	2.268	0.132
	Precip X Spp	4	0.019	0.746	0.573	219.776	0.273	0.892	0.041	1.791	0.175
	Resid	18	0.026			806.192			0.023		

Table 1.3: Split-split-plot ANOVA for plant-level $C0_2$ and H_20 exchange. Analyzed as a function of precipitation treatment ("Precip") and plant species ("Spp") with block as a random factor.

Pair	Best BLAST match (Accession num.)	Best BLAST match (Accession num.)	Pair Dissimilarity	Known Life History Strategies	Accession nos.
1	Cladosporium (AY251074)	Pestalotiopsis (GU183121)	5.02	E, S P	KC582568 KC582587
2	Chaetomium (HM365261)	Cochliobolus (HE792897)	2.69	E, S E, P, S	KC582567 KP401907
3	Nigrosopra (GQ221860)	Penicillium (DQ339568)	2.65	E, P, S P	KC582580 KC582586
4	Alternaria (KJ541482)	Penicillium (JN642222)	1.61	P P, S	KC582561 KC582590
5	Acremonium (KJ194115)	Cercospora (GU214657)	0.37	E, P P, S	KP401945 KP401903
6	Aspergillus (FJ867942)	Cochliobolus (KC311473)	0.12	P, S E, P, S	KC582564 KC582570

Table 2.1: Fungal pairs (1-6) selected for plant trials. Best BLAST match of the fungi comprising the fungal pairs, dissimilarities in their fungal effects on plants, prior reports of life history, and Genbank Accession numbers reported. Previously known life history strategies are based on published literature regardless of host plant (Giauque 2016); E= endophyte, P= pathogen and S = saprophyte. The top and bottom accession numbers refer to the LSU sequences deposited in NCBI Genbank for the first fungus and second fungus in the pairs, respectively.

	Df	MS	F	*P*
Growth Rate				
Fungus	18	0.065	9.600	< 0.001
Moist	1	0.154	22.888	< 0.001
Fungus X Moist	18	0.071	10.502	< 0.001
Residual	114	0.007		
Tiller Number				
Fungus	18	0.393	1.002	0.463
Moist	1	0.164	0.419	0.519
Fungus X Moist	18	0.456	1.162	0.304
Residual	114	0.393		
Days to First Wilt				
Fungus	18	117.699	4.287	< 0.001
Moist	1	44.237	1.611	0.207
Fungus X Moist	18	182.181	6.635	< 0.001
Residual	114	27.456		

Table 2.2: ANOVA for plant responses. Analyzed as a function of fungal treatment, soil moisture and their interaction. Dependent variables are plant growth rate, tiller number, and days to first wilt. Abbreviations are fungal treatment = Fungus, soil moisture treatment = Moist.

		Growth Rate			Days to First Wilt		
	Moisture	Fungus A	Fungus B	Pair	Fungus A	Fungus B	Pair
Pair 1	High	0	0	0	0	0	0
	Low	0	0	0	0	0	0
Pair 2	High	-	0	0	0	0	0
	Low	0	0	0	0	0	0
Pair 3	High	0	-	0	0	0	0
	Low	0	0	0	0	0	+
Pair 4	High	0	-	0	0	0	0
	Low	+	+	0	+	+	0
Pair 5	High	0	0	0	0	0	0
	Low	-	0	+	0	0	+
Pair 6	High	0	-	0	0	0	0
	Low	+	0	+	+	0	+

Table 2.3: Fungal treatment effects on plant reponses. Fungi were considered beneficial (+), neutral/commensal (0) or detrimental (-) when plant responses were significantly greater than, equal to, or less than fungus-free control plants, respectively.

	Super-additive				Sub-additive				Additive			
	Num	RT	IV	*P*	Num	RT	IV	*P*	Num	RT	IV	*P*
Growth rate												
Low Moisture	1	5:42	0.75	0.001					10	5:13	1.00	0.001
	2	6:95	0.58	0.035					5	2:97	0.69	0.003
High Moisture									11	4:51	0.67	0.007
Days to first wilt												
Low Moisture	3	5:78	0.75	0.007	7	3:08	0.57	0.021	12	4:65	0.70	0.010
	4	6:29	0.75	0.005	8	4:27	0.57	0.018	10	5:13	0.60	0.009
	5	2:87	0.69	0.001	9	4:78	0.57	0.018	13	6:63	0.40	0.048
	6	2:71	0.65	0.003								
High Moisture									5	2:87	0.84	0.001
									6	2:71	0.63	0.005
									14	6:79	0.38	0.044

Table 2.4: Metabolites indicative of a fungal pair's effects. Num = number indicating unique metabolites; RT is the retention time (i.e., time of compound elution) on C18 100A HPLC column.; IV is the indicator value ranging from 0 to 1, with a value of 0 indicating that a compound is never observed within a group and a value of 1 indicating that a compound is only present in one particular grouping. *P*-values were obtained from a random permutation test (n = 999).

	df	MS	F	P
Growth Rate Deviation				
Pair	5	0.073	10.741	< 0.001
Moist	1	0.008	1.172	0.286
Pair X Moist	5	0.120	17.741	< 0.001
Residual	36	0.007		
Days to First Wilt Deviation				
Pair	5	130.343	3.383	0.013
Moist	1	78.797	2.045	0.161
Pair X Moist	5	482.359	12.518	< 0.001
Residual	36	38.535		

Table 2.5: ANOVA for plant deviations from additive. Analyzed as a function of fungal pairs, soil moisture and their interaction. Dependent variables are deviations of plant growth rate and days to first wilt from the expected additive response. Abbreviations are fungal pairs = Pair, soil moisture level = Moist.

	Deviation of Plant Growth Rate					Deviation of Days to First Wilt				
	Est.	SE	t	P	R2	Est.	SE	t	P	R2
High + Low										
Moist	0.305	0.138	2.210	0.069	0.008	16.959	7.981	2.125	0.077	0.025
Stress	-0.066	0.040	-1.667	0.146	0.079	-4.468	2.294	-1.948	0.099	0.089
Metab	0.085	0.014	5.944	0.001	0.319	4.181	0.831	5.028	0.002	0.143
Moist X Stress	0.164	0.056	2.921	0.026	0.117	12.761	3.245	3.933	0.007	0.220
Moist X Metab	-0.101	0.020	-4.957	0.003	0.691	-6.038	1.176	-5.135	0.002	0.509
Full model				0.011	0.875				0.121	0.871
High Moisture										
Stress	0.098	0.025	3.932	0.029	0.682	8.293	2.612	3.175	0.050	0.505
Metab	-0.015	0.009	-1.703	0.187	0.005	-1.857	0.947	-1.962	0.145	0.055
Full model				0.065	0.838				0.101	0.783
Low Moisture										
Stress	-0.066	0.050	-1.315	0.280	0.007	-4.468	1.925	-2.322	0.103	0.001
Metab	0.085	0.018	4.688	0.018	0.812	4.1805	0.698	5.994	0.009	0.785
Full model				0.041	0.881				0.021	0.923

Table 2.6: Estimated regression for plant response deviations. Analyzed as a function of moisture and fungal trait dissimilarity. Abbreviations: Stress = stress tolerance trait dissimilarity, Metab = metabolite profile trait dissimilarity.

Fungal taxa	Code	Sister Accession	LSU Accession	ITS Accession	Known Life History Strategies
Cochliobolus kusanoi	Cok	KC311473	KC582570	KP401875	E, P, S
Cochliobolus lunatus	Col	JN943410	KC582571	KP401884	E, P
Fusarium oxysporum	Fus	AY188919	KC582575	KP401863	E, S
Nigrospora sphaerica	Nig	GQ328855	KP401947	KP401946	E, P, S
Aspergillus terreus	Asp	FJ867934	KC582565	KP401873	E, P

Table 3.1: Fungi selected for plant trials. Fungal identities are based on phylogenetic analysis in Giauque and Hawkes (2016). We further indicate the code used in figures, accession of the closest ("sister") taxon based on best BLAST match, Genbank Accession numbers for LSU and ITS rRNA sequences, and life history strategies reported in the published literature; E = endophyte, P = pathogen and S = saprophyte.

		Growth Rate			Shoot Biomass			Water Loss			Tiller Wilt		
	df	MS	F	P	MS	F	P	MS	F	P	MS	F	P
Moist	1	0.041	16.237	<0.001	3.8e3	26.025	<0.001	30.538	63.829	<0.001	11.386	3.515	0.064
Fungus	15	0.029	11.418	<0.001	7.6e2	4.965	<0.001	2.490	5.110	<0.001	4.426	4.174	0.177
Moist X Fungus	15	0.004	1.499	0.124	4.5e2	3.120	<0.001	3.542	7.670	<0.001	5.970	1.966	0.027
Residuals	107	0.003			1.5e2			0.478			3.239		

Table 3.2: MANOVA on plant growth rate, shoot biomass, water loss and tiller wilt. Analyzed as a function of soil moisture ('moist'), fungal treatment ('Fungus') and their interaction.

		Growth Rate			Shoot Biomass			Water Loss		
	df	MS	F	P	MS	F	P	MS	F	P
Asp										
Moist	1	6.72	0.068	0.800	19.600	41.263	<0.001	0.015	0.004	0.951
Residual	8	98.442			0.475			3.815		
CoK										
Moist	1	1.3e3	6.098	0.034	0.118	0.086	0.776	2.112	0.461	0.516
Residual	8	219.857			0.136			4.578		
CoL										
Moist	1	736.939	5.883	0.046	0.275	4.476	0.067	3.614	1.571	0.245
Residual	8	125.265			0.061			2.300		
Fus										
Moist	1	5.3e3	9.895	0.026	0.051	0.322	0.586	0.000	0.000	1.000
Residual	8	538.052			0.158			5.555		
Nig										
Moist	1	0.1664	0.002	0.963	0.676	3.615	0.0948	8.446	1.786	0.218
Residual	8	73.787			0.187			4.728		
Asp + CoK										
Moist	1	8.446	1.786	0.218	90.313	1.504	0.260	2.112	0.382	0.554
Residual	8	4.728			60.029			5.530		
Asp + CoL										
Moist	1	87.120	0.819	0.400	20.306	14.668	0.005	0.015	0.005	0.948
Residual	8	106.310			1.384			3.350		
Asp + Fus										
Moist	1	24.090	0.182	0.683	29.756	43.880	<0.001	4.992	1.026	0.341
Residual	8	132.433			0.678			4.866		

Table 3.3: Post comparisons for significant moisture x fungal treatment interactions. Post hocs were corrected for multiple comparisons to maintain family-wide confidence limits at 90% using Bonferroni corrections; in our model with 45 individual contrasts the cutoff was $P < 0.002$.

Asp + Nig										
Moist	1	20.851	0.275	0.614	15.500	8.035	0.022	59.497	790.877	<0.001
Residual	8	4.866			1.929			0.0752		
CoK + CoL										
Moist	1	15.956	0.043	0.844	0.247	1.701	0.233	1.109	0.119	0.740
Residual	8	373.610			0.145			9.319		
CoK + Fus										
Moist	1	270.529	1.558	0.252	0.077	0.842	0.389	1.468	0.736	0.419
Residual	8	173.684			0.091			1.995		
CoK + Nig										
Moist	1	32.913	0.472	0.514	0.084	0.822	0.391	0.050	0.009	0.927
Residual	8	69.792			0.102			5.555		
CoL + Fus										
Moist	1	782.239	3.705	0.103	7.396	0.816	0.393	0.311	0.043	0.841
Residual	8	211.104			9.067			7.278		
CoL + Nig										
Moist	1	975.274	11.310	0.016	8.333	0.883	0.375	2.813	0.285	0.608
Residual	8	86.228			9.437			9.869		
Fus + Nig										
Moist	1	311.813	4.3113	0.077	0.000	0.002	0.970	0.610	0.113	0.745
Residual	8	72.324			0.215			5.404		

Table 3.3: Continued post comparisons

		Growth rate			Shoot biomass			Water loss		
Source	df	MS	F	P	MS	F	P	MS	F	P
Moist	1	0.011	3.330	0.070	2.8e3	15.390	<0.001	0.826	0.523	0.471
Asp	1	0.177	55.001	<0.001	4.0 e3	21.725	<0.001	69.112	43.768	<0.001
Cok	1	0.004	0.124	0.725	0.502	0.003	0.958	1.015	0.643	0.424
Col	1	0.001	0.241	0.624	10.264	0.056	0.814	0.664	0.420	0.518
Fus	1	0.000	0.056	0.814	826.274	4.499	0.036	0.781	0.495	0.483
Nig	1	0.019	5.986	0.016	2.5 e3	13.678	<0.001	0.003	0.002	0.967
Moist* Asp	1	0.007	2.310	0.131	2.6 e3	14.032	<0.001	44.516	28.191	<0.001
Moist* Cok	1	0.002	0.545	0.462	415.838	2.264	0.135	0.566	0.358	0.550
Moist* Col	1	0.000	0.071	0.790	372.262	2.027	0.157	3.629	2.298	0.132
Moist* Fus	1	0.000	0.020	0.889	40.747	0.222	0.638	0.281	0.178	0.674
Moist* Nig	1	0.000	0.019	0.891	627.976	3.419	0.067	1.455	0.922	0.339
Residuals	142	0.003			174.645			1.579		

Table 3.4: ANOVA of plant responses. Analyzed as a function of soil moisture treatment, presence of each fungal species, and their interactions. For fungal species abbreviations see Table 3.1.

Source	df	MS	F	R2	P
Moist	1	2317.443	4.184	0.040	0.001
Fungus	14	1240.928	2.240	0.301	0.001
Moist X Fungus	14	962.076	1.737	0.233	0.001
Residual	44	553.899		0.422	
Total	73			1.000	

Table 3.5: PERMANOVA for plant metabolomics profiles. Analyzed as a function of moisture treatment (Moist), fungal treatment (Fungus), and their interaction.

Source	df	MS	F	R2	P
Moist	1	2441.671	3.818	0.042	0.001
Asp	1	4471.251	6.992	0.077	0.001
Cok	1	743.123	1.162	0.013	0.252
Col	1	582.609	0.911	0.010	0.531
Fus	1	729.309	1.140	0.013	0.299
Nig	1	1237.823	1.936	0.021	0.023
Moist*Asp	1	1705.864	2.667	0.030	0.006
Moist*Cok	1	892.331	1.395	0.015	0.142
Moist*Col	1	1013.998	1.586	0.018	0.075
Moist*Fus	1	887.949	1.388	0.015	0.134
Moist*Nig	1	2093.940	3.274	0.036	0.002
Residuals	62	639.522			
Total	73				

Table 3.6: PERMANOVA for species effect on the plant metabolome. Analyzed as a function of soil moisture treatment, presence of each fungal species, and their interactions on the plant metabolome. For fungal species abbreviations see Table 3.1.

Source	Est.	SE	*T*	*P*	R^2	Est.	SE	*T*	*P*	R^2
	Plant responses Low Moisture (NMS 1)					**Plant responses Low Moisture (NMS 2)**				
Low Moist										
Metab NMS 1	-0.823	0.141	-5.839	**<0.001**	0.469	0.175	0.126	1.390	0.173	
Metab NMS 2	-0.127	0.169	-0.754	0.456		0.240	0.150	1.596	0.119	
Full model				**<0.001**	0.462				0.121	
	Plant responses High Moisture (NMS 1)					**Plant responses High Moisture (NMS 2)**				
High Moist										
Metab NMS 1	-0.845	0.097	-8.687	**<0.001**	0.660	-0.009	0.012	0.083	0.934	
Metab NMS 2	-0.357	0.144	-2.488	0.018		0.007	0.166	0.040	0.968	
Full model				**<0.001**	0.707				0.996	

Table 3.7: Linear regression of each plant response NMS axis as a function of metabolomics NMS axes. Estimated regression parameters, standard errors, t-values, and *P*-values are indicated; factors were considered significant at the Bonferroni-corrected cutoff $P < 0.013$.

Source	NMS 1				NMS 2			
	Est.	**SE**	**T**	**R**	**Est.**	**SE**	**T**	**R**
Low Moist								
Growth rate	-5.134	0.766	-6.703	0.696	2.121	0.614	3.454	0.034
Shoot Biomass	-0.037	0.005	-8.060	0.781	-0.011	0.004	-3.146	0.013
Tiller wilt					-2.262	0.157	-14.375	0.823
Water loss								
Full model				0.911				0.874
High Moist								
Growth rate	3.476	0.239	14.564	0.863	1.405	0.185	7.593	0.002
Shoot Biomass	0.015	0.001	18.830	0.736				
Tiller wilt	-0.228	0.054	-4.210	0.012	2.365	0.042	56.371	0.960
Water loss	-0.204	0.010	-19.449	0.678	0.032	0.008	3.917	0.029
Full model				0.994				0.991

Table 3.8: Linear regression of plant responses to plant NMS axes in low and high soil moisture. Only factors that met the Bonferroni-corrected cutoff of $P < 0.013$ are shown

	mz	Retention time (s)	Moist	Identity	Origin	Function
Presence *Aspergillus*						
	173.866	1316	Both	Unknown		
	184.885	1304	Both	Unknown		
	219.174	667	High	Sesquiterpene	B	Signaling
	282.279	695	Both	Sphingolipids	B	Guard cell regulator; Plant immune response
	322.272	695	Both	Eicosadiynoic acid	B	Guard cell regulator; Plant immune response
	325.236	722	Both	Unknown		
	343.339	816	Both	Oxylipin	B	Plant-fungal 'cross-talk'
	355.246	612	Both	Eicosanoids	B	Guard cell regulator; Plant immune response
	356.25	612	Both	Unknown		
	371.211	612	Both	Amine alkaloid	P	Plant defense
	376.26	549	Both	Sphingolipids	B	Guard cell regulator; Plant immune response
	377.263	549	Both	Unknown		
	381.298	779	Both	Unknown		
	382.301	780	Low	Eicosanoids	B	Guard cell regulator; Plant immune response

Table 3.9: Metabolites that are associated with species-effects. Only the 20 compounds with the strongest correlations ($R^2 > 0.60$) are listed by their M/z and retention time from LC-MS. "Moist" refers to the soil moisture treatment in which the compound was identified; "Identity" is the assignment from the Metlin database; "Origin" and "Function" are based on literature reports that indicate whether the compound is known to be generated by plants ('P'), fungi ('F') or both ('B') and known functions.

398.242	550	Both	Unknown		
399.245	549	Both	Unknown		
400.248	549	Both	Unknown		
414.214	549	Both	Unknown		
431.179	810	Both	Alkaloid	B	Plant defense
432.182	810	Both	Unknown		
460.212	549	Both	Cytochalasin E	F	Growth inhibitor
773.493	549	Both	Eicosanoids	B	Guard cell regulator; Plant immune response
885.366	790	High	Isoflavone		Plant immune response
969.526	832	Both	Saponin	B	Plant immune response
Presence *Nigrospora*					
758.2	901	High	Anthocyanins	P	Antioxidant
759.2	901	High	Anthocyanins	P	Antioxidant
760.2	901	High	Phytosulfokine b	P	plant peptide growth factors
763.2	901	High	Anthocyanins	P	Antioxidant
764.2	901	High	Unknown		
833.2	938	High	Anthocyanins	P	Antioxidant
834.2	938	High		P	Antioxidant
837.2	938	High	Flavonoid	P	
838.2	938	High	Unknown		
839.2	938	High	Unknown		
908.3	974	High	Glycoside	P	Anti-fungal

Table 3.9: Continued metabolites that are associated with species-effects

909.3	974	High	Unknown		
911.2	974	High	Anthocyanins	P	Antioxidant
912.2	974	High	Unknown		
913.2	974	High	Unknown		
980.3	1020	High	Anthocyanins	P	Antioxidant
981.3	1020	High	Saponin	B	
982.3	1020	High	Anthocyanins	P	Antioxidant
987.2	1020	High	Unknown		

Table 3.9: Continued metabolites that are associated with species-effects

Figures

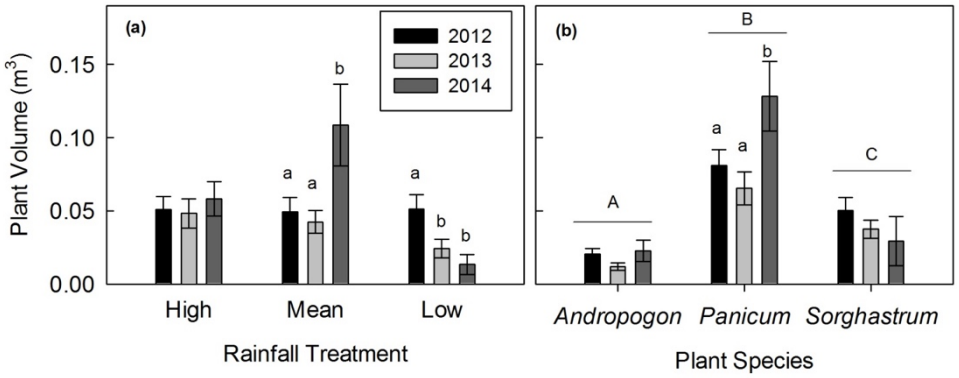

Figure 1.1: Plant size (volume) over time as a function of (a) rain treatment and (b) plant species. Uppercase letters indicate significant differences among species in posthoc comparisons; lowercase letters indicate significant differences in posthoc tests across years either (a) within a rain treatment or (b) within a species.

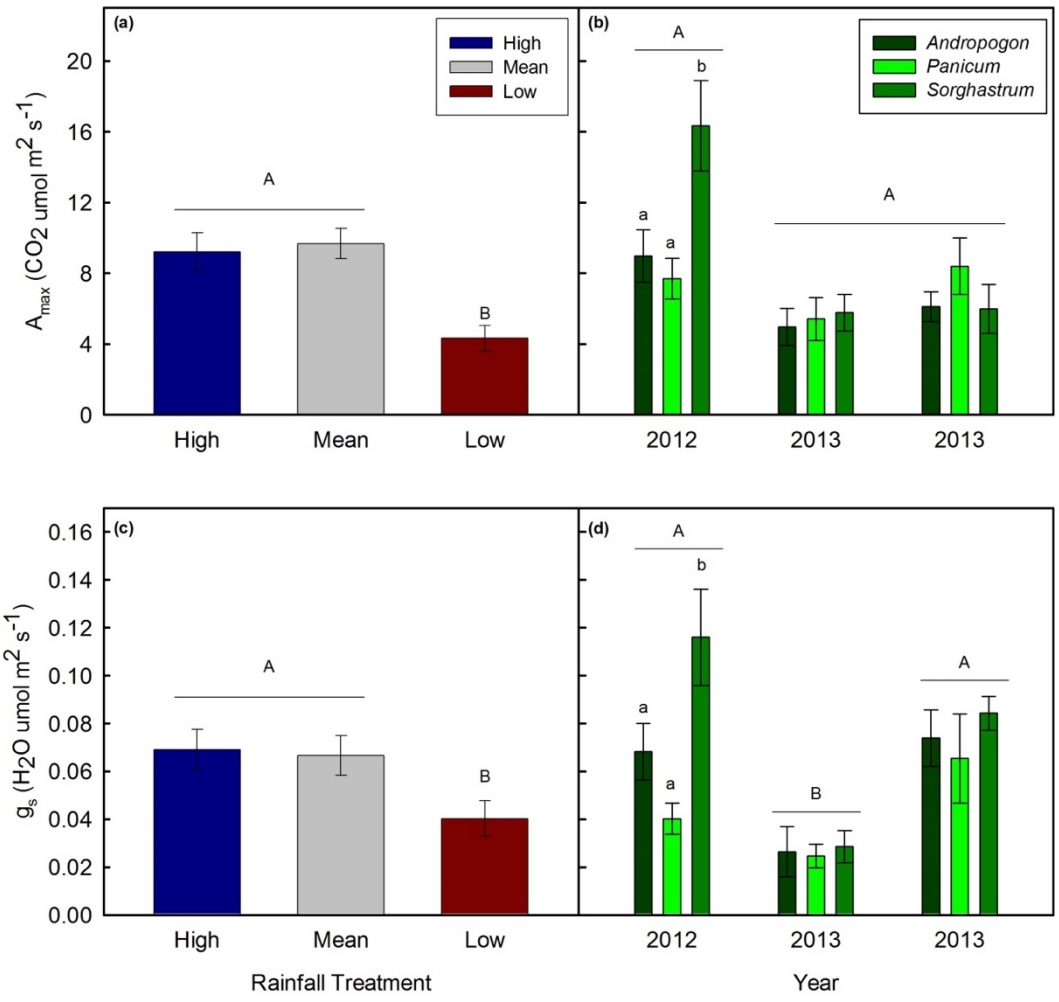

Figure 1.2: Differences in photosynthesis (A_{max}) (a) among precipitation treatments and (b) across years among plant species and differences in conductance (g_s) (c) among precipitation treatments and (d) across years among plant species. Upper case letters indicate significant difference across variables ($P < 0.05$) and lower case letters indicate significant differences within variables based on Tukey post hoc tests. Bars are means ± 1 SE (n= 36; n= 12; n= 36; n= 12).

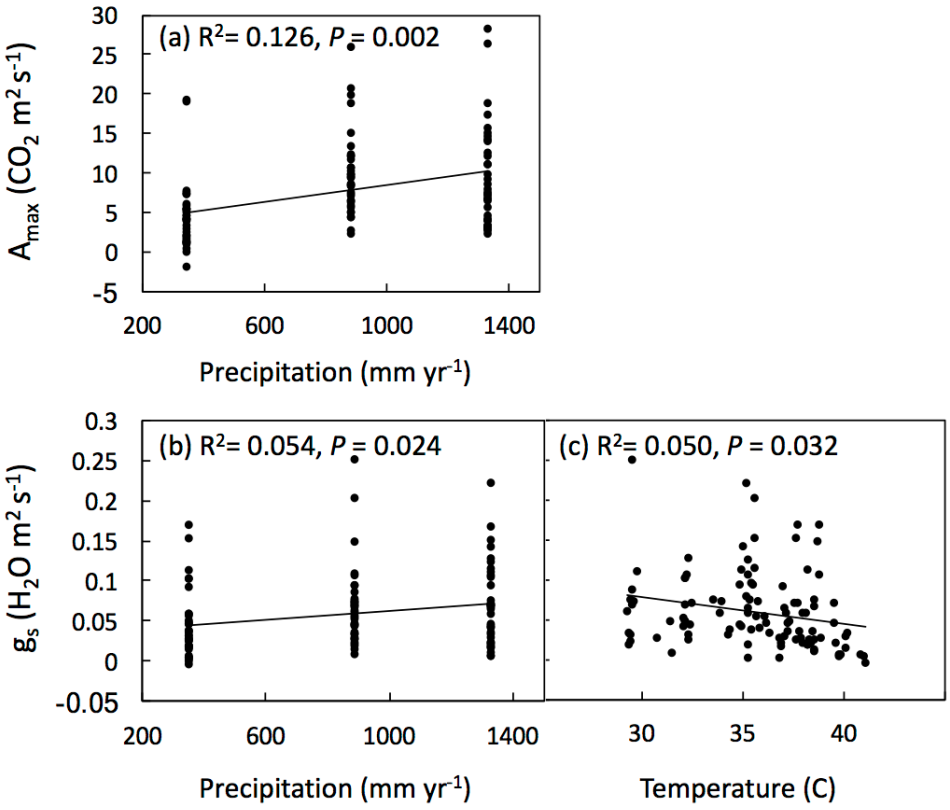

Figure 1.3: Relationship of plant photosynthesis (A_{max}) and (a) precipitation treatment, and relationship of conductance (g_s) and (b) precipitation treatment and (c) temperature. Significance are based in a linear regression.

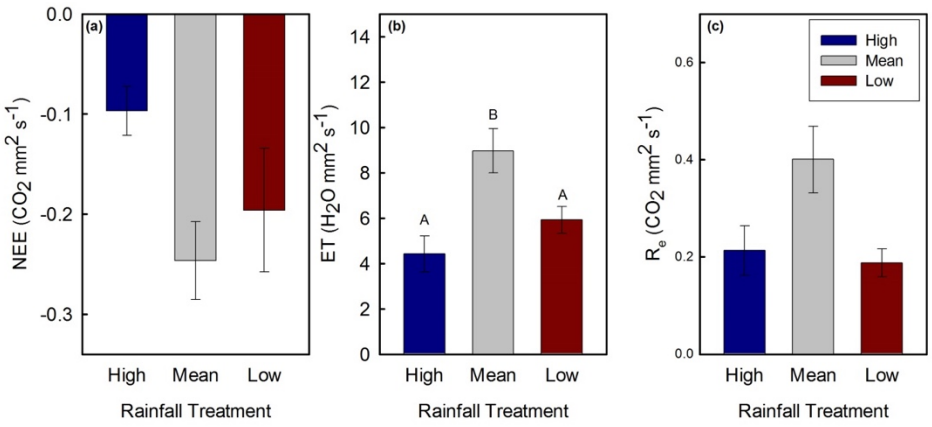

Figure 1.4: Differences in whole plant (a) CO_2 fluxes (NEE), (b) H_2O fluxes (ET) and (c) dark respiration (Re) among precipitation treatments. Letters indicate significant differences ($P < 0.05$) based on Tukey post hoc tests. Error bars are ± 1 SE (n = 12).

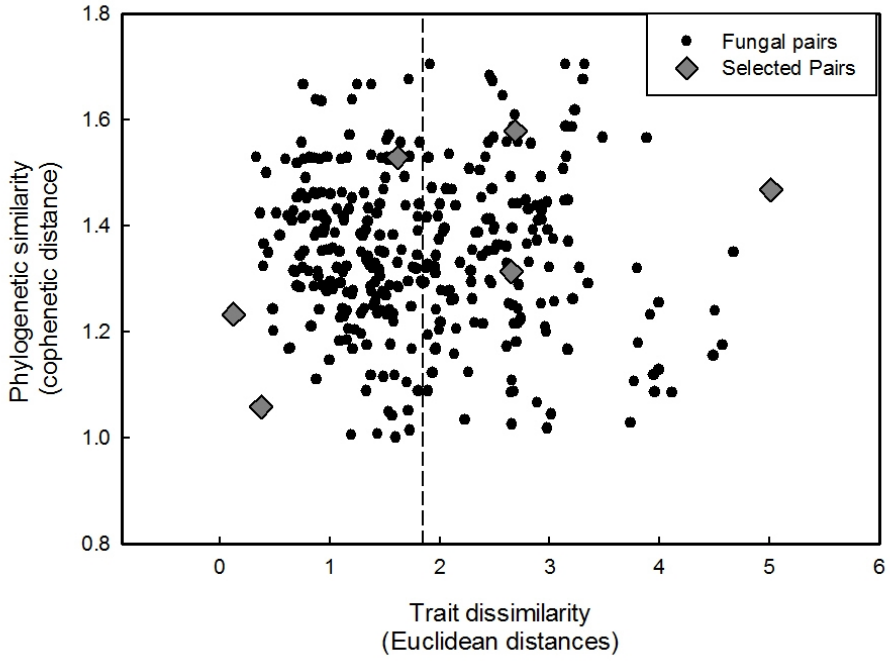

Figure 2.1: Relationship between pairwise dissimilarity of fungal effects on plant drought responses and phylogenetic distance. A total of 341 fungal pairs were considered in the pool (black circles); six pairs were chosen for the experiment (grey diamonds). The dotted line represents average trait similarity (1.85).

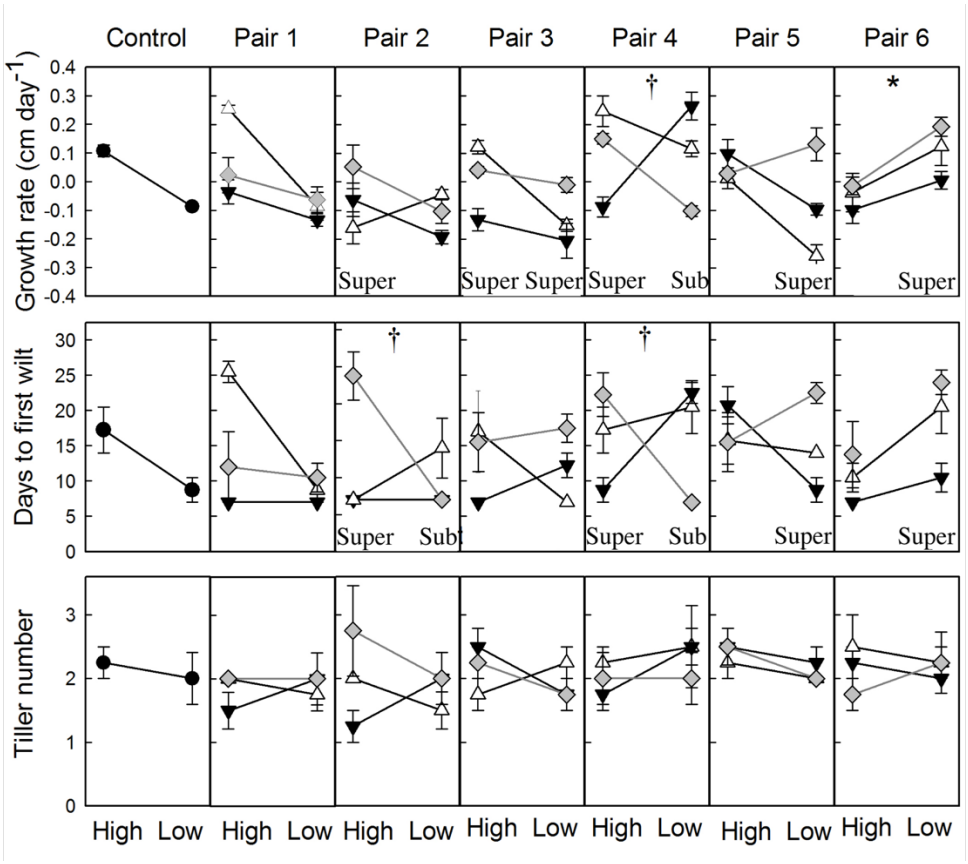

Figure 2.2: Effect of fungal endophyte treatments. For pairs, triangles represent plants inoculated with single endophyte species (white triangle up and black triangle down are the first and second fungus in each pair as indicated in Table 1) and gray diamonds represent plants inoculated with both endophytes. Non-additive fungal mixtures are indicated by 'Super' or 'Sub', for super-additive or sub-additive effects; all others were additive. Asterisks indicate fungal mixtures that differed between low and high soil moisture in posthoc comparisons with † indicating significance after Bonferroni corrections ($P < 0.003$) and * indicating a trend ($P < 0.05$). Error bars are ± 1 SE (n=4).

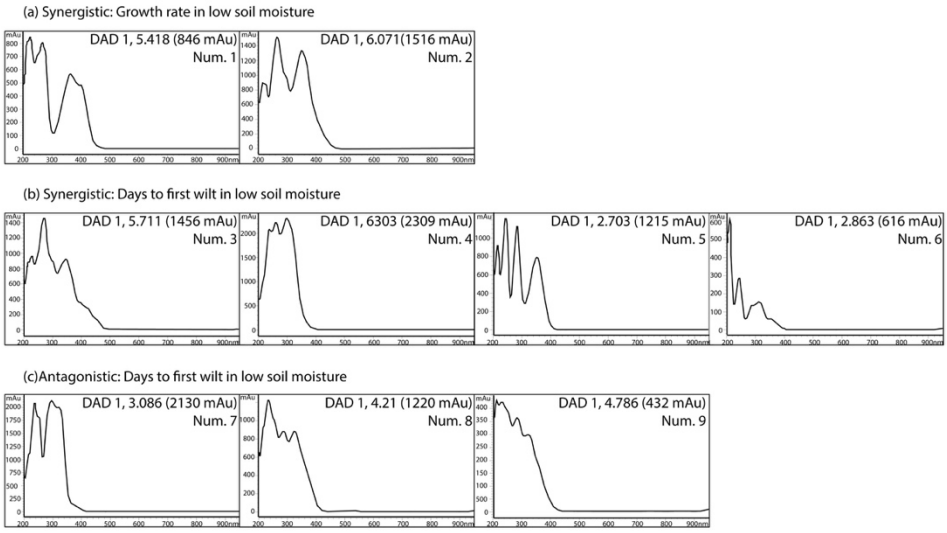

Figure 2.3: UV spectra of metabolites from indicative of non-additive effects in low moisture. (a) Metabolites 1 and 2 had synergistic effects on plant growth rate in low soil moisture, (b) Metabolites 3-6 had synergistic effects on days to tiller wilt in low soil moisture, and (c) Metabolites 7-9 had antagonistic effect on days to tiller wilt in low soil moisture. Retention times, maximum mAU and compound number corresponding to Table 2.6 are indicated for each spectrum. UV spectra were identified via DAD, a diode-array detector, at 254 nm.

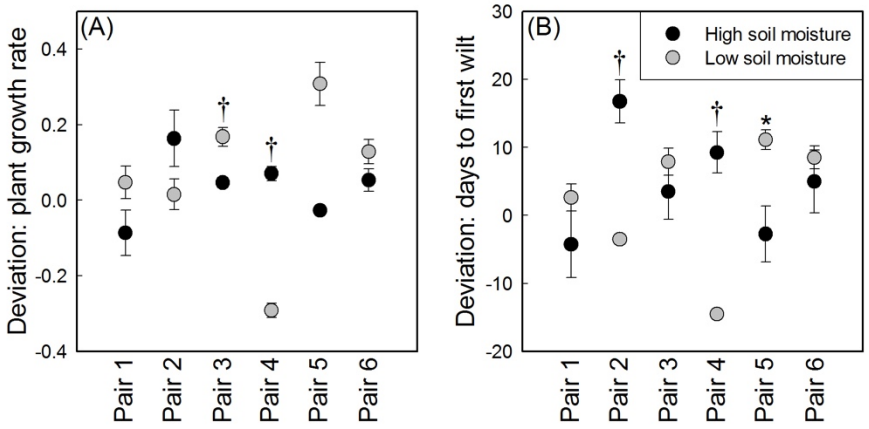

Figure 2.4: Deviations of plant growth rate and days to first tiller wilt. Deviations are the differences in the observed effects of plant responses inoculated with fungal pairs from the expected-additive effects of plant responses to the corresponding individual fungi. Asterisks indicate deviations that differed between low and high soil moisture in posthoc comparisons with † indicating significance after Bonferroni corrections ($P < 0.008$) and * indicating a trend ($P < 0.05$), as reported in supplemental table 5. Error bars are ± 1SE; n = 4.

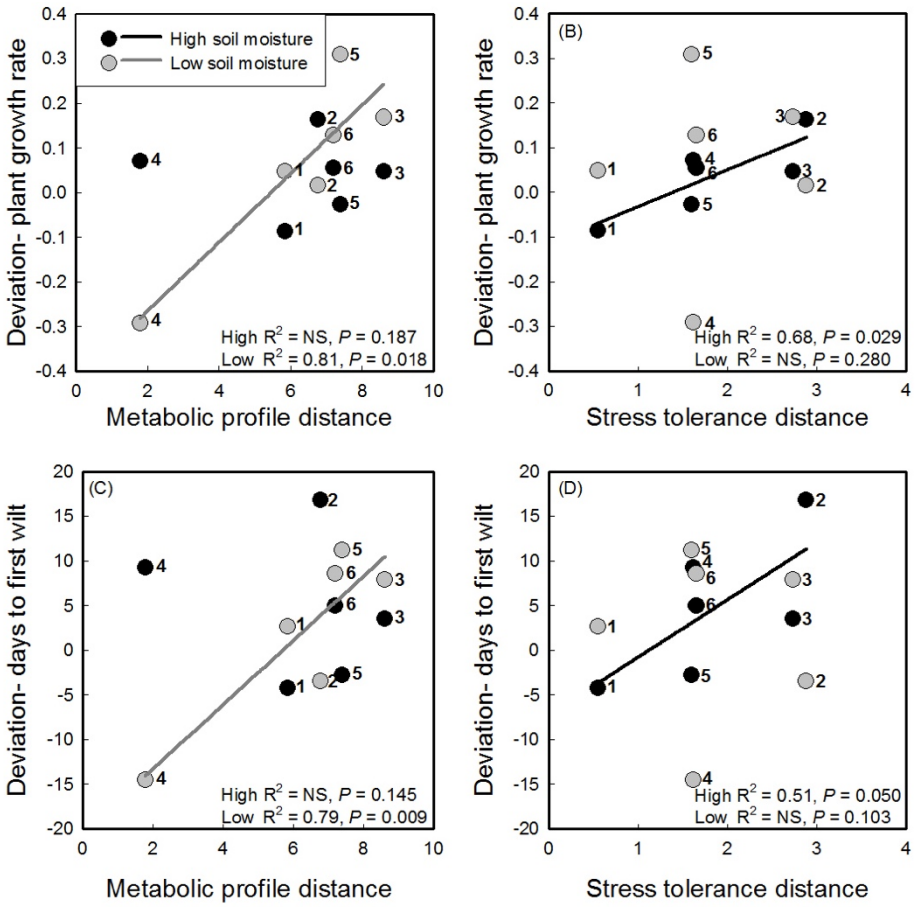

Figure 2.5: Relationships between the deviation of plant responses to fungal trait dissimilarity. Based on linear regression results, the deviations from an expected-additive model in plant growth and days to first wilt are plotted as a function of (a, c) dissimilarity in fungal metabolic profile and (b, d) dissimilarity in fungal stress response, with only significant regressions shown.

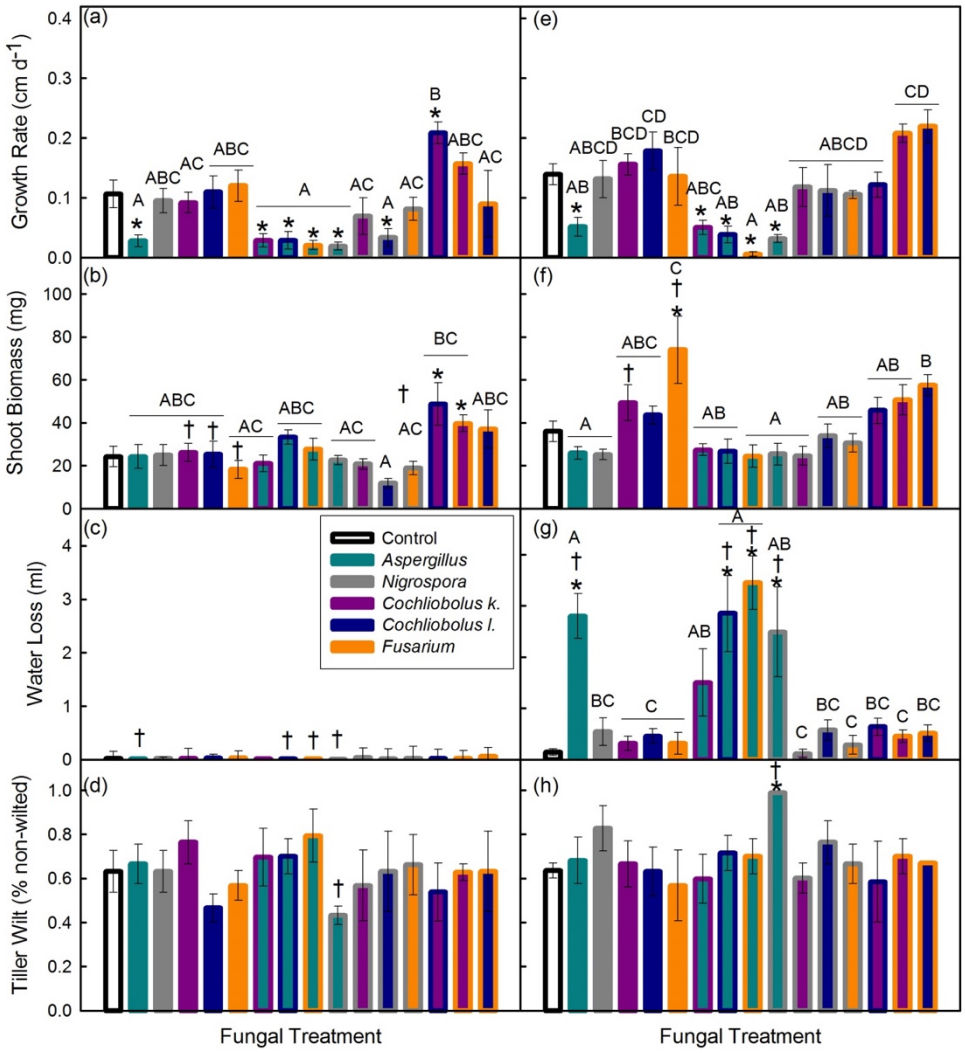

Figure 3.1: Effect of fungal endophyte treatments. Effects on plant growth rate, shoot biomass, water loss and tiller wilt under high (a-d) and low (e-h) soil moisture. For pairs, dual line and bar colors correspond to the two fungi within the pair. Asterisks indicate significant difference from control (fungus-free) plants; crosses indicate fungal treatments that significantly differed in low vs. high soil moisture; letters indicate significant differences among fungal treatments. Error bars are ± 1 SE (n=5).

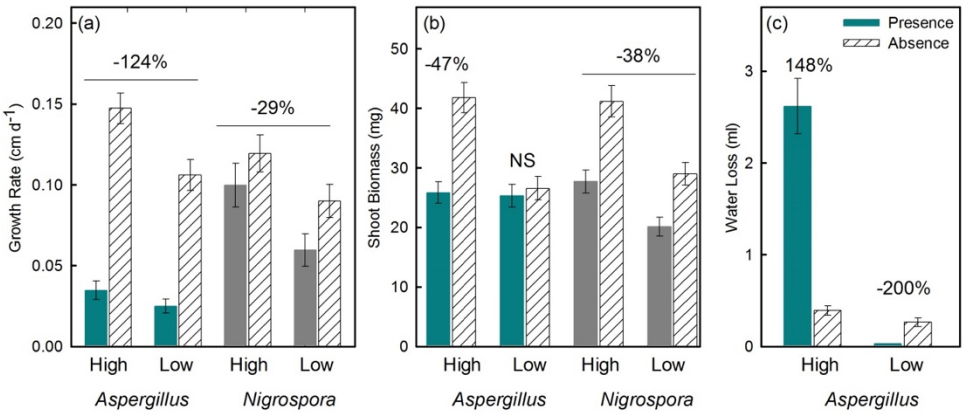

Figure 3.2: Species-effects on plant responses. Results shown for the presence of *Aspergillus* within treatment effected (a) plant growth rate (overall), (b) shoot biomass in high soil moisture and (d) water loss in high and low soil moisture; and the presence of *Nigrospora* within a treatment effected (a) plant growth rate (overall) and (b) shoot biomass (overall). Asterisks indicate significance at Bonferroni corrected levels of $P < 0.013$,

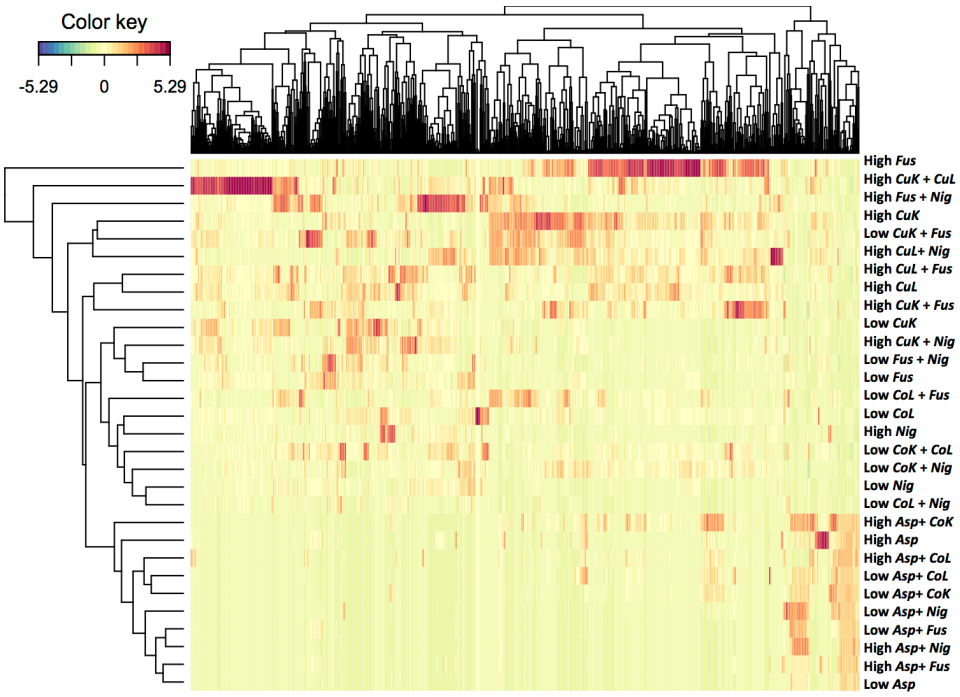

Figure 3.3: Dendograms and heatmap for plant metabolomics profiles. Rows are fungal treatments in high or low moisture and columns are metabolites.

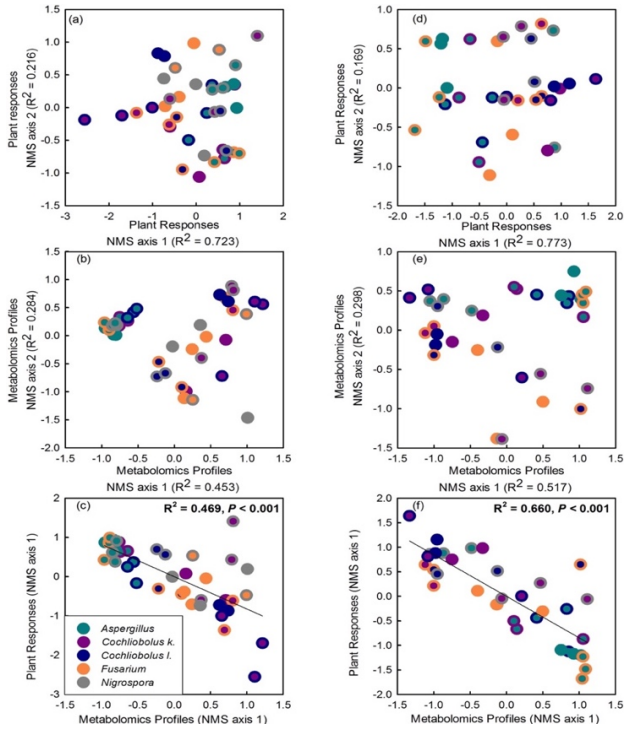

Figure 3.4: Nonmetric-multidimensional scaling of plant responses and metabolomics profiles. Results shown for plant responses in (a) low soil moisture (axis 1 R^2= 0.453, axis 2 R^2= 0.284; stress = 11.028) and (b) high soil moisture (axis 1 R^2= 0.517, axis 2 R^2= 0.298; stress = 9.790); plant metabolomics profiles in (c) low (axis 1 R^2 = 0.723, axis 2 R^2= 0.216; stress = 8.633) and (d) high soil moisture (axis 1 R^2 = 0.773, axis 2 R^2= 0.169; stress 11.578); and linear regressions between plant response NMS 1 and metabolomics profiles NMS 1 in (e) low and (f) high soil moisture.

Appendix

Effects of extreme changes in precipitation on fungal endophyte communities

Introduction: Extreme events can directly affect plant associated microbes, though imposing an environmental filter, or indirectly, though changes in their plant host. Here, we focus on horizontally-transmitted, Class 3, foliar fungal endophytes (hereafter, 'endophytes'). These endophytic associations are vital to plant health and function, especially in stressful conditions, with endophytes enhancing plant stress tolerance to salinity, heat and water stress (Redman et al. 2011). Despite their ecological significance, little is known on factors shaping their distributions, with most evidence from observational studies in natural environments.

For instance, endophyte distributions can vary across latitudinal gradients, with climatic variables often attributed to observed difference within communities (Giauque and Hawkes 2013, Arnold and Lutzoni 2007). In general, historical climatic variables are believed to be more important than short-term annual conditions in shaping endophyte distributions because endophytes have free-living and spore phases which allow them to temporarily escape harsh conditions. Furthermore, local fungal communities are usually impervious to colonization of new species, either through preempting resources and space or through niche modification (Werner and Kiers 2015). Despite this general notion, there are few studies which directly manipulate climatic variables, which would identify the effects of short-term annual conditions on endophyte community dynamics.

We propose that endophyte community responses to extreme weather events will depend on the severity of the event and how different plant species respond to those conditions over time. To address this issue, we examined the effects of extreme changes in precipitation on the community composition of fungal endophytes associated with three native C4 bunchgrasses over three years. The grasses were Andropogon gerardii, Panicum virgatum, and Sorghastrum nutans. The grasses were grown in three precipitation treatments: extreme dry, mean, and extreme wet based on historical rainfall records. We measured leaf-level CO2 and H2O exchange, plant growth and endophyte community composition in all treatments at three time points across three years (July 2012, July 2013 and July 2014). We further measured endophyte community composition of plants grown in an ambient treatment, representing the local species pool. At the fungal level, we hypothesized that precipitation will modify endophyte communities both directly and indirectly through changes in host plant physiology, such that changes in fungal community composition would be predicted by precipitation and host factors, including leaf-level fluxes. However, changes in fungal community composition would not change instantaneously, such that community differences would be greatest as time progressed.

Methods (Fungal culturing and identification): Three tillers were randomly selected from each plant. Following Arnold et al. (2000), tillers were surface sterilized in 95% ethanol (15 sec), 0.5% sodium hypochlorite (2 min), 70% ethanol (2 min), and sterile water (30 sec). Surface sterilized tillers were then sectioned into three 2-mm fragments and placed in petri dishes containing 2% potato dextrose agar (PDA) and 50 ppm ampicillin. Plates were incubated at room temperature and assessed daily for fungal

growth. Once hyphae emerged from a leaf fragment, the fungus was transferred to a new PDA plate to obtain pure cultures. Pure cultures were then used for subsequent DNA extractions.

Isolates were initially assigned to morphotypes based on morphological characteristics. Morphotype identity was then confirmed by sequencing at least three representatives from each morphotype group. Standard phenol- chloroform-isoamyl procedure was used for DNA extraction (Griffiths et al. 2000). Each 25-µl PCR reaction contained approximately 10 ng of fungal DNA, 0.75 U Taq polymerase, 1x PCR buffer, 2 mmol L-1 MgCl2, 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. We then sequenced the D1/D2 region of LSU of rDNA using the general fungal primers NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') AND NL4 (5' GGTCCGTGTTTCAAGACGG3 3') at the DNA Sequencing Facility at the University of Texas at Austin (O'Donnell 1993). Once trimmed and quality checked, sequences were aligned with SATé 2.24 and clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using UCLUST (Edgar 2010). Representative sequences of each OTU were then selected and re-aligned to an internal guide tree (More details of fungal identification in Appendix).

Once trimmed and quality checked (Arnold and Lutzoni 2007), sequences were aligned using SATé v2.2.7 (Liu et al., 2011) and clustered into initial operational taxonomic units (OTUs) based on 97% sequence similarity using UCLUST (Edgar, 2010). Representative sequences of each OTU were then aligned to an internal guide tree,

which was composed of 896 reference sequence of known Ascomycota fungi obtained from the SILVA database. All sequences were then aligned with SATé 2.24 using MAFFT for the initial alignment, MUSCLE for the merger, and FASTREE with a GTR+G20 model for tree estimation. Operational taxonomic units (OTUs) were again identified based on 97% sequence similarity using UCLUST.

Statistics: To ensure sufficient sampling of endophyte communities, we estimated species richness based on sampling effort for every precipitation treatment, plant species and year using the Chao1 estimator in the vegan package in R (Oksanen et al. 2017). To examine variations in endophyte community composition among precipitation treatment, plant species, year and their interaction, we used a blocked PERMANOVA on Bray-Curtis dissimilarities using the vegan package in R (Oksanen et al. 2017). Differences were visualized with nonmetric multidimensional scaling (NMDS) on Bray-Cutis distances. When community composition differed among effects, we examined the relationship among community composition, host factors and climatic variables using a partial redundancy analysis and linear regressions, with NMS scores as the dependent variables and plant physiology (Amax and gs), temperature and soil moisture as the independent variables. To further examine differences in endophyte communities, we identified endophyte species that were indicators for communities that differed significantly using the Dufrene-Legendre indicator analysis (Dufrene and Legendre 1997) with the labdvs package in R (Roberts 2015). This analysis calculates an indicator value for each species as the product of its relative frequency (presence-absence) and relative average abundance in each community and uses a random permutation test (n = 999) to calculate a significance value.

Results: Across all years and treatments, 23 fungal species were identified, representing a diverse set of species from the Dothideomycete, Lecanoromycete and Sordariomycte clades. Our sampling effort was robust across plant species, capturing between 89%-92% of the estimated species, and years, capturing between 90%-99% of the estimated species (Table A1.1). However, sampling effort was moderate within the different precipitation treatments, capturing between 73%-87% estimated species (Table A1.1). Fungal community composition differed among plant species and year but was not affected by precipitation treatment (Table A1.2). Among plant species, Andropogon and Sorghastrum had similar fungal communities, which were different than those within Panicum (Figure A.1a). However, there was only one species, Alternaria sp., that had a higher probability of colonizing Panicum (Table A1.3). Among years, fungal communities were similar in 2012 and 2013, which were different than those in 2014 (Figure A1.1b). Again, there was only one species, Dothideomycetes sp., that had a higher probability of colonizing plants in 2014 (Table A1.3). Though communities differed among plant species and year, plant host factors and climatic variables only explained 9% of the variation in fungal community composition based on a partial redundancy analysis. Specifically, plant host factors (species, Amax and gs) explained 4.1% of the variation and climatic variables (temperature and soil moisture) and year explained 5.0% of the variation. This weak relationship was confirmed with no significant relationship among community composition (NMS points) and plant host factors or climatic variables (Table A1.4).

Calculation of endophyte phylogenetic distances

We generated a large subunit reference dataset of Ascomycota fungi from the LSURef alignment download from the SILVA database. We extracted all Ascomycota sequences that contained the D1/D2 region corresponding to the sequenced region of the 32-tested endophyte species, resulting in 896 reference sequences. All sequences were aligned with SATé 2.24 using MAFFT for the initial alignment, MUSCLE for the merger, and FASTREE with a GTR+G20 model for tree estimation. Using the resulting tree, cophenetic genetic distances were calculated using the Picante package in R.

Fungal trait assays

Fungal osmotic stress tolerance and growth: Fungi were screened for osmotic sensitivity, osmotic threshold and growth. Fungal isolates were grown in liquid cultures of 1x M9 media supplemented with glucose (20%), 1 M MgSO4 (1 ml L-1), and 1 M CaCl2 (0.1 ml L-1). 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-1, 40 g L-1, 80 g L-1, 120 g L-1 and 160 g L-1 of sodium chloride, which represent -30 kPa, -300 kPa, -600 kPa,1000 kPa and -1200 kPa.

Prior to inoculation, fungal monocultures were grown on 2% potato dextrose agar plates. A 1-mm2 fungal plug was then inoculated into 10 ml tubes containing 8 ml of modified 1X M9 media. Fungi were grown at 30°C and shaken at 130 rpm. Isolates were harvested after 21 days, at which time the majority of isolates reached stationary growth. Isolates were harvested by centrifuging cultures at 15,000 rpm for 10 minutes, removing supernatant and rinsing plugs in 4 ml autoclaved water. This procedure was repeated once more to ensure all NaCl was removed from cultures and thus not included in fungal dry

weight measurements. Cultures were then 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 prior to weighing. In total, there were 4 replicates per treatment at 5 different stress levels. Fungal osmotic sensitivity was measured as the slope of fungal growth vs. stress; fungal osmotic tolerance was measured as the level of NaCl at which growth stopped; and fungal growth was measured as fungal dry weight at 0 g L-1 NaCl.

Fungal enzyme activities: Fungi were screened for the production of cellulose-degrading enzymes (α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), and cellulobiohydrolase (CBH). Prior to inoculation, fungal monocultures were grown on 1x M9 media supplemented with glucose (20%), 1 M MgSO4 (1 ml L-1), 1 M CaCl2 (0.1 ml L-1) and agar (30 g L-1). Five 1-mm2 fungal plug were placed into 125 ml of 50 mM acetate buffer (pH 5.0). Fungal plugs were ground up and solution was homogenized for 1 minute using an immersion blender. The resulting suspensions were stirred continuously while 200 µl aliquots were dispensed into 96 well-microplates. 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 16 replicate wells per sample per assay and eight replicate wells for each blank, negative control, and quench standard. Microplates were incubated in the dark at 20°C for 1 hour, after which time reactions were stopped by adding10 µl of 1 M NaOH 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.

Fungal resource use: Fungi were screened for substrate use of 95 substrates using Biolog FF microplates (Biolog, Hayward, California, USA). Prior to inoculation in mircoplates, fungal isolates were grown on 2% potato dextrose agar plates. Fungal mycelium was then scraped into a 2 ml microcentrifuge tube containing 1 ml of FF Inoculating Fluid (Biolog, Hayward, California, USA). Mycelium was ground up using a sterilized micropestle and the resulting suspensions were transferred to a 50 ml falcon tube containing 30 ml FF Inoculating Fluid. Suspensions were standardized to equal ~1.6 x 108 cells/ml (or an absorbance of 0.2 nm when read at 600nm) by the addition of more FF Inoculating Fluid. Microplates were inoculated with 100 μl/well, with 3 replicates per isolate, and 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. Location in incubator was randomized daily and absorbances were read every other day, starting on the third day following inoculation, for 25 days. Substrate use was based on absorbance values 9 days after inoculation, given that most isolates reached stationary growth within 1-2 days following that time point.

Fungal metabolic profiles: Co-cultures and pure cultures of endophytes were grown on PDA plates and incubated at room temperature with three replicates. All plates were inoculated with two 1-mm2 plugs of fungi spaced 5 cm apart; in co-cultures each plug was a different fungal species and in pure cultures the plugs were from the same isolate. Whole plates at stationary phase were extracted three times with ethyl acetate and

extracts were concentrated by vacuum. The dried extracts were re-dissolved in 500 μL methanol. Extracts were analyzed by liquid-chromatography mass spectrometry using an Agilent Series1200 LC with a diode-array detector coupled to an Agilent Technologies 6130 single quadrupolemass spectrometer. A Phenomenex Gemini 5u C18 100A column (50 x 2 mm) with a linear gradient of 5-95% CH3CN (vol/vol) over 12 min in H2O with 0.1% (vol/vol) formic acid at a flow rate of 0.5 mL/min was used for analysis. The elution was monitored by UV at 254 nm.

Fungal metabolite profiles were created from LC-ESIMS chromatograms using the following steps to detect and quality filter unique metabolites: (1) the data were quality filtered so that only peaks accounting for at least 5% of total peak area were retained; (2) chromatogram peaks were aligned and binned by retention time (± .02 sec); (3) absorbance spectra of all peaks within a bin were then manually checked to ensure bins represented same compounds; and (4) absorbance spectra of peaks in bins with similar retention times (≤ 0.05 sec) were checked to ensure that bins truly represented separate compounds. For missing values, we manually checked chromatograms to determine if compounds were present below the 5% cut-off. Compounds found below the cut-off were binned by retention times and absorbance spectrums were checked to ensure same compounds. The remaining missing values were determined to be absent and assigned a zero. For profile analysis, a matrix was created for all peaks based on absolute peak area at each retention time for each replicate. Peak areas were then normalized by the amount of fungal tissue extracted (fungal area cover from the plate used for extraction) and log-transformation to account for the skewed distribution of metabolic

data. Note that peak area is proportional to compound concentration, where a change in peak area indicates a change in compound concentration.

Appendix tables and figures

Table A1.1: Fungal richness within treatments and sampling effort based on Chao1 estimator.

	Richness	Sampling effort
Precip		
Low	16	73%
Mean	17	69%
High	22	79%
Ambient	18	87%
Species		
Andropogon	19	89%
Panicum	21	89%
Sorghastrum	18	92%
Year		
2012	21	99%
2013	17	98%
2014	13	90%

Table A1.2: Results of a blocked PERMANOVA for effects of precipitation ('Precip'), plant species ('Spp') and year on fungal community composition. Significance and contribution to explained variation in fungal community composition was determined by adding sequentially adding terms in the model.

		Fungal community composition			
Factor	df	MS	F	P	R
Precip	3	0.162	0.558	0.927	
Spp	2	0.793	2.731	0.004	0.040
Year	2	2.007	6.907	0.001	0.102
Residual	115	0.291			
Total	122	0.324			

Table A1.3: Indicator species analysis for fungal species that are indicative of being within different plant species and within different years. Genbank Accession numbers are for LSU region. IV is the indicator value ranging from 0 to 1, with a value of 0 indicating that a compound is never observed within a group and a value of 1 indicating that a compound is only present in one particular grouping. P-values were obtained from a random permutation test (n = 999).

Community	Fungal Species (Best BLAST Match)	Accession nos.	IV	P
Plant species				
Andropogon	Nigrospora	KP401947	0.301	0.019
Panicum	Alternaria	KC582560	0.246	0.004
Sorghastrum				
Year				
2012	Cadosporium	KC582572	0.360	0.002
	Cochliobolus	KP401907	0.297	0.001
	Preussia	KP401905	0.176	0.01
2013	Sordariomycetes sp1	KP401939	0.288	0.001
	Alternaria	KC582560	0.246	0.006
	Sordariomycetes sp2	KP401937	0.191	0.002
	Sordariomycetes sp3	KP401941	0.105	0.039
2014	Dothideomycetes		0.365	0.001

Table A1.4 Results of multiple regression for fungal community composition as a function of plant host factors (Amax and gs), seasonal variations in climate ("Temp" = air temperature, "Moist" = soil moisture), and precipitation treatment ("Precip"). R2 values are only reported for significant factors in each model.

	Community composition (NMS)				
	Est.	SE	t	P	R2
Full					
Amax	0.042	0.021	2.000	0.048	
gs	-3.576	1.779	-2.010	0.047	
Temp	0.030	0.017	1.764	0.080	
Full model				0.074	

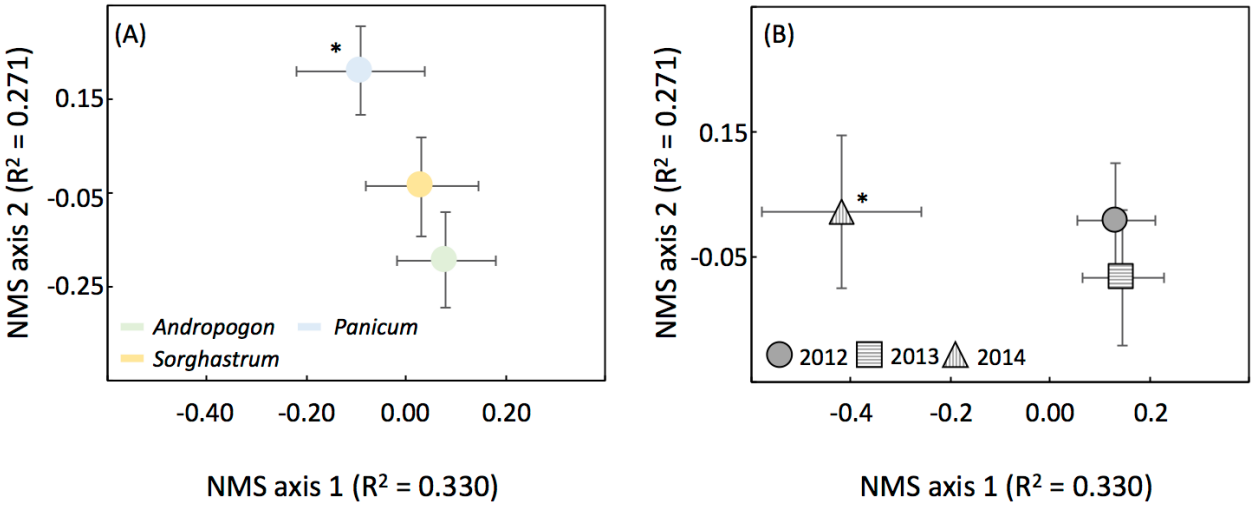

Figure A1.1 Nonmetric multidimensional scaling of endophyte communities by (A) plant species and (B) year using Bray-Curtis dissimilarities. Asterisks indicates a fungal community is significantly different. Bars are ± 1 SE.

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