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Precipitation increases the abundance of some groups of root-associated fungal endophytes in a semiarid grassland

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Abstract. Diverse communities of Root-Associated Fungal (RAF) species, including Dark Septate Endophytes (DSE), inhabit grasses in semiarid soils. These aridlands are often distinguished by large but variable precipitation events (monsoons). We sought to compare the composition and fungal load of the RAF community inhabiting the roots of *Sporobolus cryptandrus*, a dominant forage grass, exposed to variable precipitation events. We used a rainfall manipulation experiment at the Sevilleta National Wildlife Refuge (SNWR) to examine the abundance and composition of the RAF communities using molecular and microscopic techniques. Molecular data reveals that the RAF communities are dominated by Ascomycetes and few sequences were related to Arbuscular Mycorrhizal Fungi (AMF). The most dominant group (*Paraphaeosphaeria* spp.) clearly increased in abundance in response to water amendments and the addition of similar volumes of water appeared to increase similarity among the RAF communities, irrespective of size and distribution of precipitation events. Microscopic examinations of the roots also revealed an increased fungal load when exposed to elevated moisture, irrespective of rainfall frequency. We suggest that the increase in these cosmopolitan and abundant groups of Ascomycete fungi, in part, represents a common but complicated strategy of water translocation in a variety of soil types, similar to that proposed and shown in the arbuscular mycorrhizal fungi (AMF). Additional work should examine whether many (or most) members of the Ascomycete RAF also can facultatively broker water in the larger biological network in the soil.

Key words: dark septate endophytes; endophytic fungi; Sevilleta National Wildlife Refuge; *Sporobolus cryptandrus*.

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Introduction

Aridlands constitute approximately one-third of the earth's land surface and, by most accounts, continue to increase in area (Noy-Meir 1973, Schlesinger et al. 1990, UNESCO 1977). Grasses make up the predominant plant form in nearly all of these areas (Peters et al. 1997) which provide habitat and forage for a great diversity of organisms. Recent evidence suggests that many grass species within these areas form intimate and long-lasting symbiotic relationships with a large and diverse community of fungal endophytes, particularly within their roots (Jumpponen 2001, Kovacs and Szigetvari 2002, Lucero et al. 2006, Petrini 1996, Smith and Read 1997). This root-associated fungal (RAF) endophyte community often is dominated by a few taxonomically undescribed fungal clades (Khidir et al. 2010, Porras-Alfaro et al. 2008). For example, two or three fungal groups consistently inhabited the roots of nearly all *Bouteloua gracilis* plants (a common forage warm-season grass) within six sites across the latitudinal distribution in North

America (Herrera et al. 2010). Thus, grass-fungal interactions appear to be common and consistent over wide areas in aridland ecosystems.

Accumulating evidence supports the idea that many arid and semiarid ecosystems are maintained by nutrient dynamics made possible, in large part, by fungi catalyzing the cycling of nutritional resources (Allen 2007, Collins et al. 2008, Hasselquist et al. 2010). This "fungal loop" hypothesis, suggests that fungal hyphal networks link plants, soil and biological crusts to functionally integrate and exchange nutrients (including water) among these linked guilds. This process may be particularly important in many aridlands because precipitation events large enough to penetrate deep into soil (where the majority of the roots are located, Kurc and Small 2007) are rarely consistent and often only available in bursts known as "monsoons" (Kurc and Small 2007, Knapp et al. 2002, Loik et al. 2004). These monsoons vary by location, but are only common during a few weeks or months of the year. Consequently, because grasses in these areas usually only have shallow fibrous rather than tap root systems (Canadell et al. 1996, Gibbens and Lenz 2001), they are particularly dependent on these rare precipitation events to access free water. Alternatively, these plants may supplement their water needs by tapping into the hyphal network of a large and diverse community of RAF. As already demonstrated with Arbuscular Mycorrhizal Fungi (AMF, Marulanda et al. 2003), fungal hyphae can efficiently respond to and absorb soil water because of their large surface area-to-volume ratio made possible by an extensive mass of fungal mycelium extending from the roots. The response of these RAF communities, including non-AMF species, to variable precipitation events has not been fully characterized and examined in arid regions where monsoons typically occur. Yet, it is unclear whether (and how) this newly described consortium of non-AMF species changes under variable precipitation events during the monsoon season (the season with the highest net primary productivity for the grasses). Accordingly, the objectives of this study were to:

1. Molecularly characterize and compare the RAF community of a dominant grass species (*Sporobolus cryptandrus*) exposed to variable and additional precipitation events.
2. Microscopically assess and compare the relative fungal load within the roots of *S. cryptandrus* exposed to variable and additional precipitation events.

Methods

Study site

Most of the study was conducted at the Monsoon Rainfall Manipulation Experiment (MRME; GPS coordinates: 34°20.641 N and 106°43.615 W) at the Sevilleta National Wildlife Refuge (SNWR; Socorro County, NM), a Long Term Ecological Research Site (LTER) in central New Mexico, USA. The MRME was established in 2006 to study the effects of altered precipitation regimes on the viability, productivity and sustainability of the semiarid ecosystems (Thomey et al. 2011). The MRME is located on the eastern side of SNWR on sandy loam soil (Buxbaum and Vanderbilt 2007). The site is divided into a total of 13 plots, each 8x13m and randomly assigned to one of three watering regimes: 0 (receive only ambient precipitation; 3 plots), 5 mm (5 plots) and 20 mm (5 plots). The 5 mm plots receive ambient rainfall in addition to four, 5 mm artificial watering events each month (during the monsoon season, July through September). The 20 mm plots receive ambient rainfall and an additional 20 mm artificial watering event each month (July through September). Thus, the 5 and 20 mm plots receive equal volumes of additional water, but distributed variably over the season. Ambient precipitation data was assessed using recordings from SNWR-LTER meteorological station #49, which is closest to the MRME (approximately 1 km south). Additional meteorological datasets for SNWR are available within the LTER website ⟨http://sev.lternet.edu⟩.

Several plant species, including black grama (*Bouteloua eriopoda*), sand dropseed (*S. cryptandrus* Torr. A. Gray), and a few species of forbs dominate these plots. Since our laboratory has a sizeable database describing the baseline RAF community of *S. cryptandrus*, we opted to focus our efforts on assessing the RAF community of this plant.

Sampling of roots

Twenty six *S. cryptandrus* plants were randomly selected (two plants/plot; least mean interdistance between plants = 6.4 m, SD = 2.7) with the mean height of the tallest tiller = 0.86 m (SD = 0.11; N = 26). A few roots of these same plants were carefully harvested during two different collection bouts (July and again in August, 2009) using U.S. Fish and Wildlife Service permit 22522. Roots were first sampled on July 14th after the 20 mm plots were watered once (with 20 mm on July 13th) and the 5 mm plots twice (each instance with 5 mm, July 6th and 13th). The roots from the same plants were sampled again on 29 August (after the 20 mm plots had been watered twice and 5 mm plots eight times). Six to eight roots (>6 cm long) from each plant were carefully sifted out by hand and harvested with sharp scissors. The roots were collected only if they were connected to green leaf tissue, had root hairs, and appeared asymptomatic. Following the collection of the roots, any disturbed soil surrounding the plant was replaced carefully. Cut roots were placed in sterile resealable bags and placed in a portable cooler (5°C) for transport to the laboratory for processing.

Processing of roots

To remove soil and debris, roots from each plant were washed separately (and within 24 h) utilizing high pressure tap water and occasional sprays of a 1% Triton X solution. Then, the roots were surface sterilized for one min with a 5% Clorox and 1% Triton X solution before being rinsed twice in sterile distilled water (as described elsewhere, Herrera et al. 2010). Clean and surface-sterilized roots from each plant were cut into two approximately equally-sized sections and placed into 2 separate 1.5 ml microcentrifuge tubes and frozen at −30°C until the roots were assessed (within seven months of collection). Root sections from the first tube were used for molecular assessment and root sections from the second tube were used for microscopic assessment.

Molecular assessment

For samples collected in July and August, 2009, at least three root sections (maximum of 6, depending on size) from each plant were pooled and ground utilizing a separate sterile mortar and pestle, sand and liquid nitrogen. Qiagen's DNeasy Plant Mini Kit was used to extract the DNA per manufacturer's instructions. Fungal specific primers, ITS1F and ITS4, were used to amplify the ITS region (ITS1, ITS2 and 5.8S of fungal rDNA) following Gardes and Bruns (1993) and as described by Khidir et al. (2010). After being amplified, ITS rDNA from each plant was sent to the Genome Center at Washington University (St. Louis, MO) for cloning and sequencing. Clean sequences from the clone libraries were edited with SEQUENCHER (Gene Codes, Inc., Ann Arbor, MI). Individual Operational Taxonomic Units (OTUs) were defined as sequences sharing 97% base-pair similarity with at least 40 basepair overlap, as commonly done in other ecological studies (Khidir et al. 2010, O'Brien et al. 2005). Chimeric sequences were deleted from the dataset as suggested by O'Brien et al. (2005). Randomly selected sequences for each OTU were submitted to GenBank under accession numbers, HQ38937-HQ389539. Because taxonomic information is often lacking or incorrect (Nilsson et al. 2006), we opted to assign only the taxonomic order to each of the sequences in our submissions. Any provisional taxonomic identity of our OTUs (including the order) were still vetted by checking the support and publication status of similar sequences in GenBank and other more refereed sites (UNITE, Kessy et al. 2010, EMERENCIA, Nilsson et al. 2005). These provisional taxonomic identifications and abundances for each OTU were complied in an excel spreadsheet (Supplement).

Similarities among the RAF communities in plants exposed to different watering regimes were compared using Statistica's (Statsoft Inc., Tulsa, OK) Multidimensional Scaling (MDS, Kruskal 1964) module and utilizing a matrix of Morisita-Horn (Magurran 1988) similarity indices based on SEQUENCHER OTUs (97% similarity and 40% overlap). Moreover, because previous work in the same study area (Khidir et al. 2010) suggested only a few groups (related to *Paraphaeosphaeria* spp.; *Fusarium* spp.; *Moniliophthora* spp.; *Monosporascus* spp.; and Sordariales spp.) dominated the RAF community of *S. cryptandrus*, we focused our effort on assessing how variable precipitation events affected the prevalence of these core fungal groups.

Microscopic assessment

Root sections were thawed and prepared for microscopic assessment using a modification of Vierheilig and Piché (1998) method. Each 1.5 ml tube containing roots from one plant was filled with a 1% HCl solution containing lactophenol cotton blue and allowed to stain at room temperature for 30 min. The roots were then stored into different 1.5 ml tubes containing acidified glycerol (1% HCl) until the roots were imaged (July and August collections imaged within one and six months, respectively) to assess their fungal load.

A total of three digital images were taken at 200X (under a compound microscope using either a Sony 3-chip RGB camera or a DFC420C high resolution Leica camera) at haphazardly selected locations from at least two (July samples) or three (August samples) randomly selected roots (a total of six or nine images/plant, respectively). The relative fungal load within each root section was estimated utilizing the line-intercept method (McGonigle et al. 1990) for both dark septate endophyte (DSE) and hyaline hyphae. The proportion of intercepts hitting DSE or hyaline hyphae was calculated for each image and arcsine transformed (arcsine of the square-root of each proportion) to normalize their distribution (Zar 1999). These transformed values were used to calculate a relative fungal load for each plant collected in July and August (by collapsing the average proportion of each image within each root within each plant).

Three separate one-way ANOVAs were used to determine if plants within different plots (watering regimes) in July harbored different fungal loads. The proportion of intercepts hitting fungi (either DSE, hyaline, or both) were used as the dependant variable and watering amendments (0, 5 mm, 20 mm) used as the independent variable. This analysis was repeated for roots collected in August. A post-hoc paired sample t-test was conducted with all 26 plants to determine if fungal load was higher in roots collected in August (and exposed to more precipitation events) compared to those obtained in July.

Because preliminary evidence indicated that fungal loads varied widely within and between plants (and to confirm results obtained in 2009), we also assessed fungal loads within the roots of 5 additional *S. cryptandrus* plants during the dry season in May of 2010 before and after two simulated rainfall events. The focal plants were selected along a 50 m transect in an area 6km northeast of the MRME plots (but within the same basin; GPS coordinates: 34°24.110 N and 106°40.611 W), with each plant being approximately 10 m away from the nearest focal plant. A 0.5 m^2 area surrounding each of the plants was showered with 20 mm of tap water utilizing a Dramm watering can for two successive days (a total of 40 mm). Using the same methods described above, root samples from all five plants were taken prior to watering (22 May, 2010) and then 48 h after the second (and last) watering event (25 May). A subset of these roots was assessed microscopically for fungi as described previously. Six roots from each plant (3 images/root) were assessed. This time, however, to decrease any possible observer bias, the measurements of all images were conducted by two naïve undergraduate students trained to identify DSE and hyaline hyphae on screen but otherwise not informed of the hypotheses being tested. Values from both students were not statistically significantly different for each plant and so were averaged and used for subsequent analyses. No molecular analyses were performed on these root samples.

Because roots were obtained from the same five plants, a one-tailed paired t-test was used to estimate the effect of watering on fungal load (proportional data transformed as described previously), with mean number of transformed transects in contact with fungal hyphae (surrogate for fungal load) for each plant (18 images/plant, three images from each of six roots) as the dependant variable and watering status as the independent variable for five paired samples.

Results

Rainfall patterns

Grasses have few and evanescent opportunities to access free water at SNWR. Based on data obtained from meteorological station #49 (SNWR database), most monsoonal events (>5 mm) occur primarily during July–September (Fig. 1), although some occasional pre-monsoonal rains occur in the spring of some years (Kurc and Small 2007). Furthermore, preliminary pilot

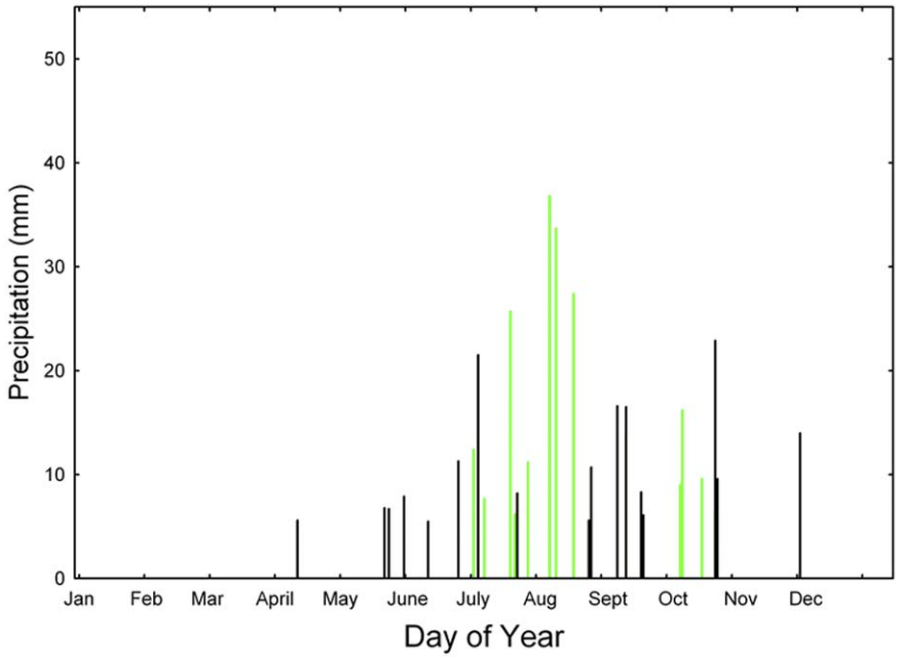

Fig. 1. Monsoonal (>5 mm) events during 2008 (green bars) and 2009 (black bars) at Sevilleta National Wildlife Refuge meteorological station #49 near MRME plots.

studies examining water infiltration into the soil adjacent to the MRME plots suggested that watering (with a Dramm watering can) with 5 mm and 20 mm of tap water only allowed water to penetrate less than 3cm and 10 cm into the soil, respectively. Most monsoonal events also typically occur during the hottest part of the year when the evaporation and transpiration rates are highest (Kurc and Small 2007).

During 2009, precipitation fell within the 10-year running average for the site (Fig. 2). Plants in 0 mm plots received an additional 39.8 mm of rainfall between the two sampling bouts in July and August. About one-third (16.3/39.8 mm) of this precipitation fell within a 24 h period on 23–24 August. Moreover, of the total precipitation that fell in 2008 and 2009 (272.0 and 244.9 mm, respectively), 79.5% (216.3/272) and 75.2% (184.2/244.9) occurred in monsoonal events during 13 and 17 d, respectively. During 2010, only 10.8 (on 22 April) and 1.4 mm (on 3 May) of precipitation fell at the site in the two months prior to our collection of roots. Consequently, the five plants assessed on the 22 May had access to little to no precipitation prior to being watered.

Molecular assessment

The 13 plants (one/plot) assessed in July and again in August generated a total of 26 clone libraries that were pooled into six groups (three watering regimes: 0, 5, and 20 mm × assessed twice: July and August). These clone libraries yielded a total of 1858 clean sequences, though 45 of these sequences were considered chimeric and removed. The remaining 1813 sequences (a mean of 139.5 sequences/plant; $n = 13$ plants; $SD = 25.0$) were used in the study (Table 1).

Although an uncontrolled wildfire from dry lightning strikes burned through the entire site on 4 August, 2009, we did not observe an effect on the below-ground RAF community composition between the two sampling periods (though the abundance of sequences of a few groups changed). For example, with the possible exception of roots collected from 20 mm plots in August, rarefaction, richness and diversity of RAF were not obviously different among the three watering regimes (Supplement).

As with previous studies (Herrera et al. 2010, Khidir et al. 2010, Porras-Alfaro et al. 2008) AMF were notable by their near absence (only 3 sequences); instead, the RAF communities again

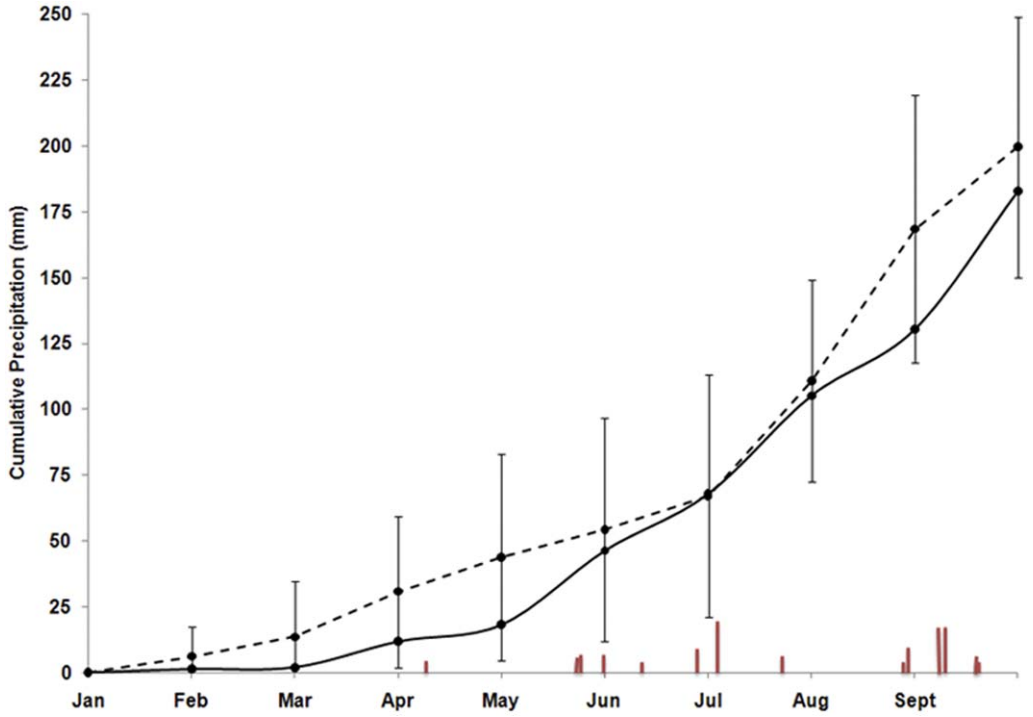

Fig. 2. Mean (±SD) cumulative precipitation during 1999–2009 recorded from meteorological station #49 at Sevilleta National Wildlife Refuge (dashed line). Precipitation during 2009 shown in dark line for comparison with monsoonal events shown as small bars on the X-axis.

were dominated by five core groups: four of them Ascomycetes (*Paraphaeosphaeria* spp., *Fusarium* spp., *Monosporascus* spp., and Sordariales spp.) and one of them a Basidiomycete (Agaricales: *Moniliophthora* spp.; Fig. 3). Although all plants harbored *Paraphaeosphaeria* spp., sequences of this group were most numerous in plants exposed to additional inputs of water. For example, the most frequent and pervasive OTU (OTU1, *Paraphaeosphaeria* sp.1) was more prevalent in 20 mm plots in July and August (Fig. 3).

Addition of water also appeared to increase similarity among the RAF communities. The MDS analysis indicated that, in general, communities receiving comparable amounts of water were more similar (Fig. 4). For example, the RAF community composition and the abundances of the number of sequences from OTUs obtained from the 0 mm plots in August most resembled those obtained from the 20 mm plots in July. Both plots received approximately 40 mm of water prior to sampling, albeit by different means.

Microscopic assessment

Seventy eight roots from 26 plants (3 roots/plant) were assessed microscopically from samples collected in July and August (156 roots overall). As with our previous study (Herrera et al. 2010), the within and between-root fungal abundance varied considerably (Fig. 5). Despite the variability, all plants and nearly all roots collected from the 5 and 20 mm plots harbored fungi. Most observations showed fungal hyphae throughout the root cortical tissue and extending out from the epidermis. Only 17 (21.8%) images of roots exhibited no fungi, and of these, seven (9.0%) belonged to plants in the 0 mm plots. Images from all roots and plants collected in August exhibited fungi. Only two images (both in July samples) exhibited what could be considered AMF vesicles.

In July, a mean proportion of 0.31 (SD = 0.28), 0.57 (SD = 0.28), and 0.52 (SD = 0.30) of intercepts crossed fungi in images obtained from roots in 0, 5, and 20 mm plots, respectively. These values were nearly statistically different (ANOVA on

Table 1. Description of root-associated fungal clone libraries obtained from *Sporobolus cryptandrus* plants collected during July and August 2009 from the Monsoon Rainfall Manipulation Experiment (MRME) plots at the Sevilleta National Wildlife Refuge (SNWR).

Collection date	Plots		Total sequences	No. sequences per plant		No. OTUs‡	No. singletons	Diversity values		Identity of most numerous sequences
	Added precip.	n†		Mean	SD			Shannon	Fisher's α	
July	0 mm–control	3	220	73.3	5.51	12	14	2.14	7.66	*Chaetomium* sp. *Moniliophthora* sp. *Monosporascus* sp.
July	5 mm	5	356	71.2	15.1	16	10	2.24	6.45	*Fusarium* sp. *Cercophora* sp. *Hymenochaete* sp.
July	20 mm	5	390	78.0	9.44	15	13	2.31	6.91	*Paraphaeosphaeria* sp. *Conocybe* sp. *Moniliophthora* sp.
July	Overall	13	966	74.3	11.0	34	29	2.98	15.1	*Paraphaeosphaeria* sp. *Fusarium* sp. *Pleosporales* sp.
August	0 mm–control	3	222	74.0	7.0	10	8	1.67	4.62	*Paraphaeosphaeria* sp. *Pleosporales* sp. *Monosporascus* sp.
August	5 mm	5	267	53.4	30.4	14	10	2.12	6.38	*Fusarium* sp. *Paraphaeosphaeria* sp. *Moniliophthora* sp.
August	20 mm	5	358	71.6	14.3	18	20	2.33	10.7	*Paraphaeosphaeria* sp. *Monosporascus* sp. *Fusarium* sp.
August	Overall	13	847	65.2	21.9	31	26	2.81	13.8	*Paraphaeosphaeria* sp. *Fusarium* sp. *Pleosporales* sp.
July and August	Overall	26	1813	69.7	17.6	51	42	3.09	20.6	*Paraphaeosphaeria* sp. *Fusarium* sp. *Pleosporales* sp.

† Number of plants (n) sampled in each plot in July and resampled again in August.
‡ At least two similar or identical sequences based on 97% similarity and 40 base overlap using Sequencher.

transformed values, $F_{2,23} = 2.39$; $P = 0.11$; Fig. 5a), with most of the differences among the groups explained by the disparity in the abundance of hyaline hyphae: a mean proportion of 0.08 (SD = 0.12), 0.26 (SD = 0.23) and 0.23 (SD = 0.27) of intercepts crossed by hyaline hyphae in images of roots from 0, 5, and 20 mm plots, respectively. Statistical differences in fungal loads among the different groups disappeared by August, when the roots were collected again from the same plants ($F_{2,23} = 0.81$; $P = 0.46$; Fig. 5b).

A post-hoc paired t-test using the same data and comparing the fungal loads of plant roots between the July and August samples (within the same plants) revealed that the overall ($t = 5.23$, $n = 26$, $P < 0.0001$), DSE ($t = 3.45$, $n = 26$, $P = 0.002$) and hyaline ($t = 6.64$, $n = 26$, $P < 0.0001$) fungal loads had increased significantly in all groups (Fig. 5). By the second collection date, roots in the 0 mm plots had been exposed to nearly 40 mm of precipitation (from rain) while the 5 and 20 mm plots had received an additional 40 mm (or a total of 80 mm) of water.

Adding 40 mm of tap water to five additional plants at a nearby site during 2010 had a similar effect of increasing fungal loads, though because the sample size was smaller, the statistical effect was only marginally significant (Fig. 6; paired $t = 2.33$, $n = 5$, $P_{1\text{-tailed}} = 0.04$). As in 2009, although DSE increased (near statistical significance, paired $t = 1.52$, $n = 5$, $P_{1\text{-tailed}} = 0.10$) more of the increase in fungal load was explained by post-watering increases in hyaline hyphae (paired $t = 2.25$, $n = 5$, $P_{1\text{-tailed}} = 0.044$).

Discussion

Addition of water to *S. cryptandrus* changed the structure of the RAF community and caused a responsive increase in the abundance of some fungal groups. Chief amongst these was the most common OTU in our dataset (*Paraphaeosphaeria*

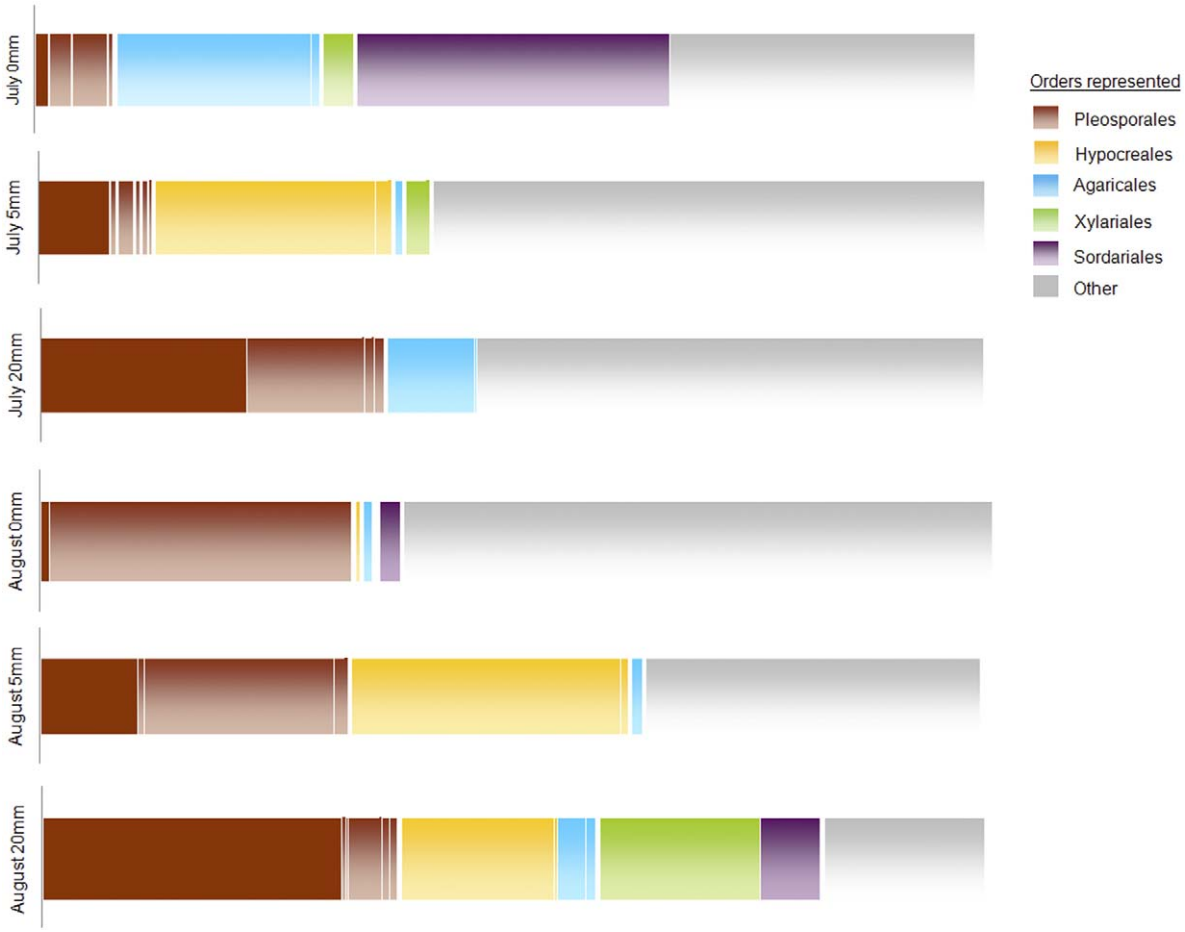

Fig. 3. Distribution of the five most common fungal groups (*Paraphaeosphaeria* spp., *Fusarium* spp., *Moniliophthora* spp., *Monosporascus* spp., and Sordariales spp.) obtained from RAF communities from *S. cryptandrus* collected within three different precipitation treatments (0, 5, and 20 mm) at the Monsoon Rainfall Manipulation Experiment (MRME) plots during July and again in August. *Paraphaeosphaeria* sp. 1 (OTU1; solid brown) was obtained from all three treatments during both collections (July and August). Identification based on ITS sequence data and BLAST searches.

sp. 1), which increased proportionately with larger inputs of water in July and August (Fig. 3). Microscopic and molecular data also support the assertion that even comparatively small amendments of water can result in more similar RAF communities (Fig. 4) and initiate increases in fungal biomass within (and surrounding) the roots. We reproduced a similar effect during the dry season (May, 2010), by disclosing that five additional plants also significantly increased their overall fungal load when we added a total of 40 mm of water over 48 h (Fig. 6). Some of this increase was attributable to DSEs (e.g., *Paraphaeosphaeria* spp.).

Our microscopic observations of *S. cryptandrus* roots and roots of other grass species (e.g., Khidir et al. 2010), confirmed the spatial intimacy of the vascular cylinder with fungal hyphae (Fig. 7). The mycelial network that extends from the cortical tissue into the soil increases the surface area available to the plant to acquire limiting resources, including water; this also opens the possibility that these hyphae serve as conduits for resources to be transported into the plant's vascular tissue. This may be important given that these grasses only have fibrous roots systems and

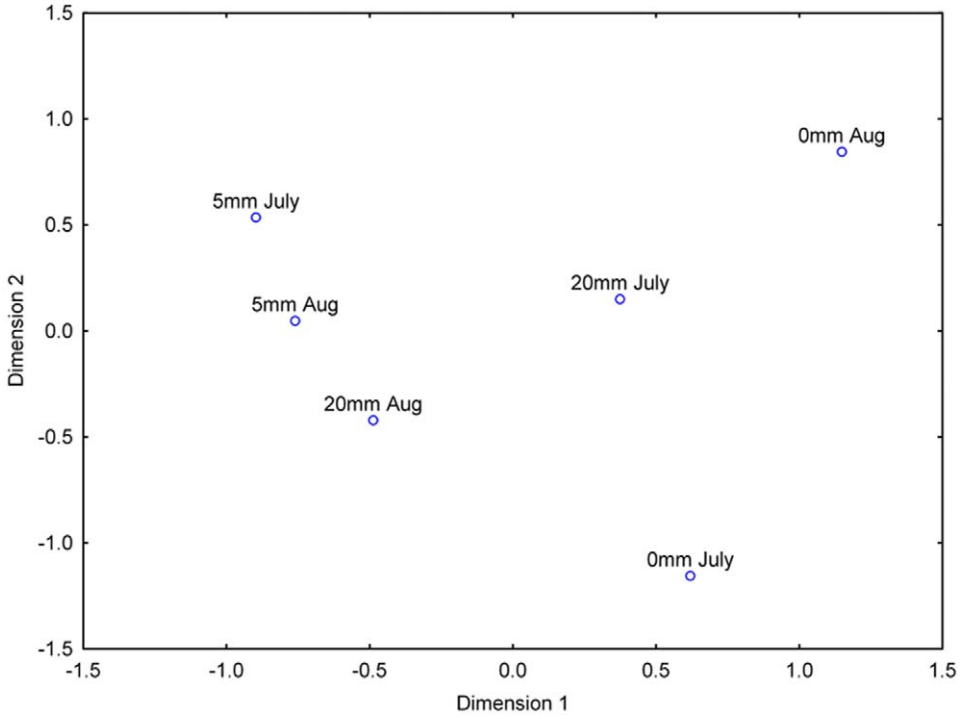

Fig. 4. Multidimensional Scaling (MDS) diagram showing relative similarity in RAF communities within *S. cryptandrus* collected from different plots within the MRME at SNWR. Fungal communities were assessed from the roots of same plants assessed in July and August. Roots were collected within plots where 5 mm of water was added at the beginning of each week (5 mm) or 20 mm added in at the beginning of the month (20 mm) or only allowed ambient precipitation (0 mm). RAF communities were analyzed utilizing Morisita-Horn similarity indices comparing OTUs created by assessing rDNA of ITS1, 5.8S, and ITS2 regions utilizing 97% similarity and 40 bp overlap in SEQUENCHER 4.8.

have few (and evanescent) opportunities to imbibe water below the top few cm of soil (Kurc and Small 2004). The fact that plants often have water as a limiting resource has suggested to some researchers that a few members of the soil microfungal community (particularly AMFs) function to absorb and transfer water to their plant hosts (Allen 2007, Allen 2009, Apple 2010, Marulanda et al. 2003). The results from this study suggest that non-AMF are also important fungal partners that may establish intimate and potentially mutualistic relationships with grasses.

An increase in fungal root pathogens after rains also may be a plausible explanation for the increases in RAF. However, we saw no evidence that these plants were suffering under the growing fungal loads. Quite the contrary, many of the plants in the 20 and 5 mm plots (with the highest RAF loads) were comparatively taller than those in the 0 mm plots and appeared asymptomatic. Moreover, the same core groups of fungi are found throughout North America in several species of grasses (Herrera et al. 2010), and so the ubiquity and intensity of the relationship with this and other grass species suggest to us that these groups are not true pathogens.

Based on both the 2009 and 2010 datasets it appears that hyaline hyphae increase a bit more than the dark septate hyphae. That said, our observations of *Paraphaeosphaeria* spp. cultures in the laboratory indicate that many of the isolates start out unpigmented and only accrue pigmentation as they age. Other studies using species of RAF have suggested that pigmentation in culture or in situ change based on several environmental variables (e.g., Eliahu et al. 2007, Lewis 1974). Therefore, we cannot preclude the possibility that

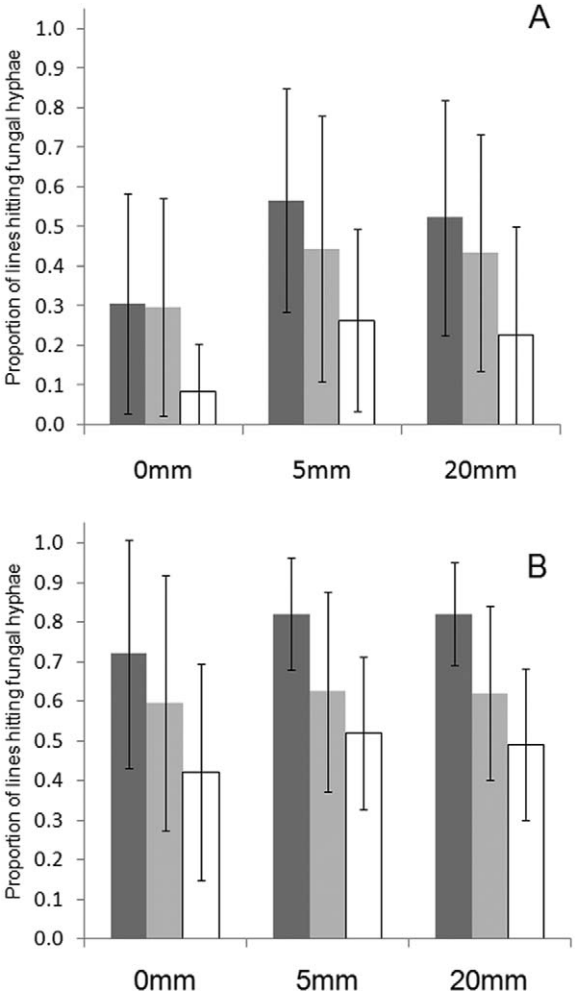

Fig. 5. Mean (± SD) proportion of line intercepts hitting any (Overall; dark bars), dark septate (DSE, gray bars), and hyaline (white bars) fungal hyphae on images taken of *S. cryptandrus* roots from the Monsoon Rainfall Manipulation Experiment (MRME) plots at Sevilleta National Wildlife Refuge in July (A) and August (B) from the same plants. 0 mm plots received ambient precipitation, while 5 mm and 20 mm plots had ambient precipitation and 5 mm of water added at the beginning of each week (5 mm plots) or 20 mm added in at the beginning of the month (20 mm plots).

some of the increases in hyaline hyphae noted in our microscopic observations may eventually manifest themselves as increased DSE loads as the fungi mature. Alternatively, light-colored endophytic species (e.g., *Fusarium* spp.) may be more responsive to water inputs at our site, though this conclusion could not be supported by the molecular data as was the case with the dark *Paraphaeosphaeria* spp.

Although the results need to be examined carefully and standard cautions regarding cloning and PCR bias are appropriate here, it appears that at least one dominant member of the RAF community responds quickly (within 72 h) to increases in water availability. Because water availability at SNWR decreases sharply within two or three days after summer rains (Kurc and Small 2007), it seems that grasses respond rapidly (e.g., greater increases in soil water potential below grassland canopies compared to shrub canopies; Pockman and Small 2010) to take advantage of this short-lived resource. We posit that certain fungal groups (e.g., *Paraphaeosphaeria* OTU1) increase in abundance when monsoonal rains fall and help the plant increase surface area-to-volume ratios and functional efficiency of their roots, although admittedly, we need to examine this possibility experimentally.

Similar to the relationship between AMF (or ectomycorrhizae) and many plants (Simard et al. 1997, Smith and Read 1997), we suspect that the relationship between the RAF and many grasses relies on the exchange of carbon for other limiting resources, including water (Green et al. 2008). However, at SNWR most carbon assimilation by plants only occurs after substantial rains (Kurc and Small 2007, Huxman et al. 2004). The paradox is a difficult one; fungi cannot grow without carbon which can only be assimilated by plants after larger monsoonal events. Perhaps fungi are able to obtain carbon quickly from root reserves even after small rains, and in return the plants are able to sequester more water through the extended fungal network. Additional work would need to be conducted to support the assertion that fungal loads in the roots of grasses should decrease in those plants that have less carbon to share (irrespective of how much they have assimilated).

Finally, it is possible (maybe likely), that small inputs of water are bound up with small particles within the soil. The fewer the number of water molecules present, the more energy is needed to pull them out (Rundel and Jarrell 1989). Often the energy status is expressed as the total soil potential which, in turn, is an additive function of the matric, solute and gravimetric potentials. Although percolating water may reside in small

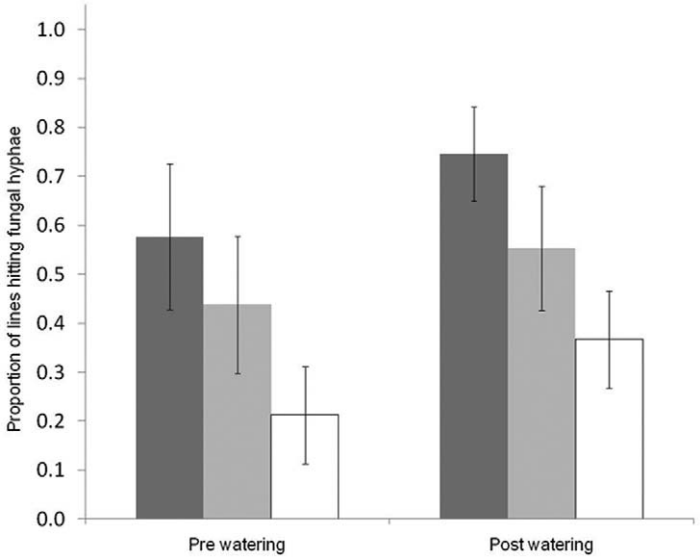

Fig. 6. Mean (± SD) proportion of line intercepts hitting any (Overall; dark bars), dark septate (DSE, gray bars), and hyaline (white bars) fungal hyphae on images taken of *S. cryptandrus* roots from SNWR before and after adding 20 mm to plants on two successive days (40 mm total). Images captured and assessed (as a single-blind study) by two undergraduates and using a variation of McGonigle et al.'s (1990) method.

Fig. 7. DSE colonizing *S. cryptandrus* secondary roots. DSE commonly observed growing along the intercellular grooves on the external surfaces of the root endodermis and well within the cortical tissue (magnification = 1000×).

quantities within the soil, it remains for the roots to pull these water molecules into the vascular cylinder. Unlike soils in more mesic environments, aridland soils are often alkaline and saline, and may provide sufficient energetic barriers to preclude plants from obtaining mois-

ture (as partially described by Allen 2007). If plant root hairs do not have the chemical or structural components to pull out the water against all of these soil potentials, responsive fungal hyphae may be small enough, have the enzymatic complexity and encompass sufficient surface area deeper in the soil horizon to overcome the structural and chemical hurdles to absorb additional water molecules that could be biochemically bartered with its plant host or traded in a larger open web that entwines the grassland ecosystem.

We are in the process of indirectly (but experimentally) testing this functional component of the fungal loop hypothesis. Namely, that hyphae increase the surface-area to volume ratio available to absorb water and in so doing provide a mutualistic basis for the relationship between some members of the RAF community and grasses (and perhaps other plants). Since *Paraphaeosphaeria* sp. 1 increases with water availability in our study, we are currently testing whether this species is able to serve as a conduit for water in an in vitro arrangement. Although the water economy in these complex ecological systems is dynamic and shifting (Smith et al. 2009), it would not be too farfetched to hypothesize that several members of the Ascomycete RAF also can be facultative brokers in this type of economy.

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Supplement

Clone library database describing all sequences (including partial taxonomic identity) obtained from roots of all 13 plants in our study (*Ecological Archives* C002-006-S1).