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HAL Authorization

1 **Experimentally altered rainfall regimes and host root traits affect grassland arbuscular**  
2 **mycorrhizal fungal communities**

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10

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12 root morphology

13

## 14 **Abstract**

15 Future climate scenarios predict changes in rainfall regimes. These changes are expected to  
16 affect plants via effects on the expression of root traits associated with water and nutrient  
17 uptake. Associated microorganisms may also respond to these new precipitation regimes,  
18 either directly in response to changes in the soil environment or indirectly in response to  
19 altered root trait expression. We characterised arbuscular mycorrhizal (AM) fungal  
20 communities in an Australian grassland exposed to experimentally altered rainfall regimes.  
21 We used Illumina sequencing to assess the responses of AM fungal communities associated  
22 with four plant species sampled in different watering treatments and evaluated the extent to  
23 which shifts were associated with changes in root traits. We observed that altered rainfall  
24 regimes affected the composition but not the richness of the AM fungal communities, and we  
25 found distinctive communities in the increased rainfall treatment. We found no evidence of  
26 altered rainfall regime effects via changes in host physiology because none of the studied  
27 traits were affected by changes in rainfall. However, specific root length was observed to  
28 correlate with AM fungal richness, while concentrations of phosphorus and calcium in root  
29 tissue and the proportion of root length allocated to fine roots were correlated to community  
30 composition. Our study provides evidence that climate change and its effects on rainfall may  
31 influence AM fungal community assembly, as do plant traits related to plant nutrition and  
32 water uptake. We did not find evidence that host responses to altered rainfall drive AM  
33 fungal community assembly in this grassland ecosystem.

34

## 35 **Introduction**

36 Grasslands cover one-fifth of Earth's terrestrial surface (Leith 1975), exhibit high  
37 biodiversity, and provide many ecosystem services including forage production, carbon

38 storage and soil stabilization (Sala & Paruelo 1997). Both the diversity and productivity of  
39 these ecosystems are highly influenced by rainfall regimes (Campbell et al. 1997) and are,  
40 therefore, sensitive to changes in the seasonality, frequency and intensity of rainfall events  
41 predicted by climate models (IPCC 2014). The response of plant communities to altered  
42 rainfall has been extensively studied with demonstrated effects on productivity (Knapp *et al.*  
43 2001; Fay *et al.* 2003) and community composition (Grime *et al.* 2000; Morecroft *et al.*  
44 2004), and has focused on aspects relating to environmental filtering of species as well as  
45 trait variation within species (Larson & Funk 2016). Plasticity of root traits related to  
46 resource acquisition might have an important role to predict species response to  
47 environmental stress (Padilla *et al.* 2013).

48 While most research has focused on plant community responses, less is known with respect  
49 to how altered rainfall in grasslands will influence belowground plant-associated organisms.  
50 This is particularly true for an important plant symbiont, the arbuscular mycorrhizal (AM)  
51 fungi (Miller *et al.* 2012). AM fungi form symbiotic associations with the majority of plant  
52 species and depend on the photosynthetic carbon (C) provided by the plant (Smith & Read  
53 2008). In return, they can enhance plant fitness, primarily via improved nutrient uptake  
54 (Smith & Smith 2011), but also by contributing to defence against pathogens (Borowicz  
55 2001) and tolerance of environmental stresses such as drought (Augé 2001). The mechanisms  
56 by which AM fungi can increase drought-tolerance are poorly understood but include  
57 maintained nutritional status (Augé 2001), increased access to water (Marulanda *et al.* 2003;  
58 Diaz-Zorita *et al.* 2002) and altered host physiology affecting water use efficiency and root  
59 hydraulic conductivity (Sánchez-Blanco *et al.* 2004; Wu *et al.* 2006). The fact that these  
60 responses can vary among plant species (Augé 2001) and AM fungal species (Marulanda *et*  
61 *al.* 2003) contributes uncertainty to the outcomes of interactions between AM fungi and  
62 plants in grasslands, as does the possibility that AM fungi respond to altered rainfall

63 independently of the requirements of the host. Therefore, an improved understanding of how  
64 AM fungal communities are likely to respond to altered rainfall may aid predictions of how  
65 grassland ecosystems will be affected by climate change.

66 Most of the studies that have addressed the question of AM fungal responses to altered  
67 rainfall focus on interactions between one plant and its associated fungi in pot experiments  
68 (reviewed by Augé 2001), although a few exceptions exist. Five to seven years of alterations  
69 of rainfall in field studies have resulted in effects on root colonization, abundance of  
70 arbuscules (involved in nutrient exchange between partners (Peterson *et al.* 2004)), vesicles  
71 (involved in energy storage (Peterson *et al.* 2004)), and the extraradical mycelium (Martínez-  
72 García *et al.* 2012; Staddon *et al.* 2003). Two recent studies have found that manipulating soil  
73 water conditions can affect the composition of AM fungi communities in roots (Deepika &  
74 Kothamasi 2015; Li *et al.* 2015). In general, effects on AM fungal responses are largely  
75 inferred in relation to changes in plant community composition (Staddon *et al.* 2003; Li *et al.*  
76 2015), and the direct effects of reduced soil moisture (Deepika & Kothamasi 2015).  
77 However, considering the effects that rainfall regimes have on host physiology (Larson &  
78 Funk 2016; Padilla *et al.* 2013), it is surprising that researchers have not studied whether  
79 changes in host traits in response to altered rainfall might be responsible for effects on AM  
80 fungi.

81 Examining plasticity in root traits in response to rainfall manipulation and in relation to AM  
82 fungal communities represents a promising opportunity to evaluate the indirect effects of  
83 rainfall on AM fungi via host physiology. Root morphology has been hypothesized to be an  
84 indicator of the capacity of plants to forage for resources and their dependency on AM fungi  
85 (Brundrett 2002), although a meta-analysis by Maherali (2014) found no relationship between  
86 the root architecture and the plant growth benefits from the AM fungi. A few studies have  
87 shown that plants with a high proportion of thick roots, low specific root length (SRL) and

88 low branching rates tend to have high rates of AM fungal colonization (Eissenstat *et al.* 2015;  
89 Comas *et al.* 2014) and it has also been suggested that roots with large diameter can support  
90 more AM fungi because they provide a larger cortical volume for colonization (Brundrett  
91 2002). Under altered precipitation regimes, these root morphological traits can be affected,  
92 although the response varies among plant species and depending on drought intensity  
93 (reviewed in Franco *et al.* 2011). Low soil moisture also limits soil nutrient availability and,  
94 thus, root chemistry, while these chemical traits (particularly the concentrations of  
95 phosphorus, nitrogen and potassium) have been demonstrated to affect levels of AM fungal  
96 root colonization (Nouri *et al.* 2014).

97 Here, we studied the response of AM fungal communities associated with roots of four plant  
98 species to experimentally altered rainfall patterns in replicated field plots established within  
99 an Australian grassland. Specifically, we estimated the effects of altered rainfall and/or host  
100 species on AM fungal communities, the extent to which effects occurred via changes in root  
101 traits, and the importance of root morphological and chemical traits in mediating these  
102 responses. To do this, we characterized AM fungal communities, using Illumina MiSeq, and  
103 morphological and chemical root traits from the same individual plants. We hypothesized that  
104 altered rainfall regimes would affect AM fungal communities. We expect this effect to be  
105 partly because of changes in root traits of their host as a function of changing host plant  
106 resource requirements under altered rainfall. If so, we expected that traits responsive to  
107 altered rainfall regimes would also shape AM fungal communities in the roots.

## 108 **Materials and methods**

### 109 **Site and experimental design**

110 The ‘Drought and Root Herbivore Impacts on Grasslands’ (DRI-Grass) experimental  
111 platform was established in 2013 at the Hawkesbury Campus of Western Sydney University

112 in Richmond, NSW, Australia. The average annual precipitation at the site is 806 mm, and it  
113 is characterized by a high inter-annual variability (between 500 mm and over 1400 mm in the  
114 last 30 years; Australian Government Bureau of Meteorology), with winter being generally  
115 the driest and summer the wettest season. The soil at the site has a sand or loamy sand  
116 texture, with water holding capacity of 20-22%. Full details are described in Power *et al.*  
117 (2016) but, briefly, DRI-Grass was established to study the effect of drought and root  
118 herbivory on grassland community structure and ecosystem function. Here, we describe only  
119 the sampled plots. The experiment consists of 2 x 2 m plots covered with shelters that  
120 exclude natural rainfall and have automated, controlled application of water underneath the  
121 shelters. Water is applied to the plots at 1h00 each morning based on the amount of rainfall in  
122 the previous 24h and according to the following watering treatments: 1) control (same  
123 amount as ambient rainfall), 2) reduced (50% less rainfall than ambient), 3) increased rainfall  
124 (50% more rainfall than ambient), 4) summer drought (total rainfall exclusion from  
125 December to March) and 5) altered frequency (ambient rainfall, applied only once every three  
126 weeks) (Fig. S1). Treatments were selected to represent model predictions of reductions in  
127 the amount and frequency of rainfall, alongside an extreme summer drought, and a  
128 contrasting increase in rainfall to represent well-watered conditions. All treatments have six  
129 replicates and are arranged in a randomized block design.

### 130 **Root sampling**

131 Roots were collected from individual plants harvested from 30 plots in September 2015, 28  
132 months after the experiment was established. We targeted four plant species that were  
133 observed in plots within all of the watering treatments. *Microlaena stipoides* and *Paspalum*  
134 *dilatatum* are C3 and C4 grasses, respectively, while *Hypochaeris radicata* and *Senecio*  
135 *madagascariensis* are two C3 forb species displaying a tap root system and a fibrous root  
136 system, respectively.

137 Two subplots, each measuring 10 x 25 cm in area were excavated to a depth of 10cm in each  
138 plot. Plants were not present in every subplot, resulting in the sampling of 82 individual  
139 plants obtained by disentangling the roots from soil and other plants, and collecting all  
140 attached lateral roots. We sampled 20 *H. radicata*, 23 *M. stipoides*, 27 *P. dilatatum* and 12 *S.*  
141 *madagascariensis* individuals. The latter species was less represented than the others because  
142 it was less frequently observed across the plots. For each individual root system, we sampled  
143 two root fragments of approximately 2 cm in length to allow morphological root traits to be  
144 measured on the same root system. Each root fragment was processed separately, resulting in  
145 164 root fragments that were then stored at -80°C for analysis of AM fungal communities,  
146 before pooling them by plant individual for Illumina sequencing. The rest of the root system  
147 was dried at 60°C prior to trait analysis.

#### 148 **Analysis of root traits**

149 Several root traits related to morphology and chemical content were evaluated. The  
150 morphological traits were estimated by scanning the rehydrated root samples with  
151 WinRHIZO 2015Pro (Regent Instruments Inc., Quebec, Canada) to obtain the root length, in  
152 multiple size classes (based on diameter) within each sample. We then calculated the specific  
153 root length (SRL; length (cm) / dry weight of the roots (mg)), ratio between the length of the  
154 fine roots (<1mm diameter) and coarse roots (>1mm diameter), and average diameter of the  
155 root system (mm).

156 Subsamples of lateral roots from each individual were selected randomly, pooled, dried at  
157 40°C to facilitate the grinding of the root subsample to a fine powder using stainless steel  
158 beads in a TissueLyser (Qiagen, Germany). Concentrations of zinc (Zn), phosphorus (P),  
159 manganese (Mn), magnesium Mg, potassium (K), calcium Ca (ppm) and silicon (Si; %) were  
160 determined by X-ray fluorescence spectrometry using a Epsilon 3 EDXRF (PANalytical, The

161 Netherlands), using 150 mg of loose, powdered tissue on foil. The concentration of carbon  
162 (C) and nitrogen (N; ppm) were determined using an Elementar vario EL cube, CHNOS  
163 Elemental analyser (Hanau, Germany), using between 5-6 mg ground samples mixed with  
164 tungsten oxide for combustion. In total, we processed 78 plants, however five samples had  
165 poor nutrient estimates and were excluded from further analysis.

#### 166 **Extraction and amplification of AM fungal DNA**

167 DNA was extracted from each root sample using a PowerSoil DNA isolation kit (MO BIO,  
168 Carlsbad, USA) following the manufacturer's instructions. An additional step involving root  
169 tissue disruption was conducted prior to the extraction, using 5 mm stainless steel beads for 1  
170 min at 30 Hz in a TissueLyser (Qiagen, Germany). Extracted DNA was quantified using a  
171 NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, Wilmington, USA), and then  
172 diluted to 2 ng/ $\mu$ L in 10 $\mu$ L prior to polymerase chain reaction (PCR) amplification. All PCRs  
173 were performed in PTC-200 thermal cyclers (Bio-Rad, California, USA).

174 DNA samples were amplified using PCR primers described in Krüger *et al.* (2009), which  
175 target specifically AM fungi and provide coverage of all AM fungal lineages. Each reaction  
176 used 5  $\mu$ L KAPA mix (Kapa Biosystem, Wilmington, USA), 0.4 $\mu$ L LSU\_Ar (10 $\mu$ M), 0.4 $\mu$ L  
177 SSU\_Af (10 $\mu$ M) and 4.2 $\mu$ L of diluted DNA template. Thermocycling conditions for this first  
178 step were: 95°C for 3 min, 30 cycles of 98°C for 20 s, 60°C for 30 s and 72°C for 50 s,  
179 followed by 72°C for 2 min. In the second PCR round, each reaction contained 9  $\mu$ L of  
180 master mix and 1  $\mu$ L diluted PCR product (2  $\mu$ L DNA / 98  $\mu$ L water). The master mix  
181 contained: 5  $\mu$ L KAPA mix, 0.4  $\mu$ L LSU\_Br (10 $\mu$ M), 0.4  $\mu$ L SSU\_Cf (10 $\mu$ M), 3.2  $\mu$ L water.  
182 Thermocycling conditions for this first step were: 95°C for 3 min, 30 cycles of 98°C for 20 s,  
183 60°C for 30 s and 72°C for 50 s, followed by 72°C for 2 min.

#### 184 **Characterisation of AM fungal communities**

185 The PCR products were purified using Agencourt AMPure XP system (Beckman Coulter,  
186 Lane Cove, NSW, Australia) and diluted with PCR-grade water to 5 ng/μL in 20 μL. Samples  
187 were pooled by plant individual by mixing 10μL of each sample. The ITS2 region was  
188 sequenced by Illumina MiSeq at the Ramaciotti Centre for Genomics (NSW, Australia),  
189 using fITS7 (5'-GTGARTCATCGAATCTTTG-3'; (Ihrmark *et al.* 2012) and ITS4 (5'-  
190 TCCTCCGCTTATTGATATGC-3'; White *et al.* 1990), and genomic libraries were prepared  
191 using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Paired-end (2 x 251 bases)  
192 sequencing was performed on the Illumina MiSeq platform.

193 The DNA sequencing data were processed using the approach described by Bissett *et al.*  
194 (2016) with a few modifications; the following is a brief description. Contigs were generated  
195 from paired-end reads using the 'make.contigs' command in *mothur* (version 1.36.1) (Schloss  
196 *et al.* 2009). Initial quality filtering removed DNA sequences containing any ambiguous  
197 bases and/or a homopolymer greater than eight bases in length. De novo operational  
198 taxonomic units (OTUs) at 97% sequence similarity were initially picked using numerically  
199 dominant sequences (observed at least four times) using the '-cluster\_otus' command in  
200 *USEARCH* (version v8.1.1803) (Edgar 2013). All quality-filtered sequences were mapped at  
201 97% sequence similarity against representative sequences of these OTUs using the '-  
202 usearch\_global' command in *VSEARCH* (version v2.3.4) (Rognes *et al.* 2016). Non-mapped  
203 sequences were subjected to a second round of de novo OTU picking, as above but only  
204 using sequences observed at least two times. All initially non-mapped sequences were then  
205 mapped against these newly picked OTUs, as above. Non-mapped sequences at this step  
206 represent singleton OTUs and were excluded from further analysis.

207 Putative taxonomic identities for fungal OTUs were generated using the 'classify.seqs'  
208 command in *mothur* (method='wang', cutoff=60) on representative sequences for each OTU,  
209 using a reference database of fungal ITS sequences and taxonomic annotations obtained from

210 UNITE (version 7.0) (Abarenkov *et al.* 2010). In addition, OTUs identified as significant  
211 indicator taxa (see below) were classified based on submitting representative sequences  
212 against the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST;  
213 Altschul *et al.* 1990).

## 214 **Statistical analysis**

215 All analyses were performed in R version 3.2.5 (R Core Development Team 2016).

216 The analyses were performed with the 73 individuals that had both DNA successfully  
217 amplified and corresponding root trait data: 18 *H. radicata*, 18 *M. stipoides*, 25 *P. dilatatum*  
218 and 12 *S. madagascariensis* individuals All root trait variables were standardized to a  
219 constant mean and variance using the ‘decostand’ function (‘vegan’; Oksanen *et al.* 2017).

220 We estimated the effects of plant species, watering treatment and their interaction on  
221 variation of root traits using permutational multivariate analysis of variance (PerMANOVA)  
222 with the ‘adonis’ function (‘vegan’). For all statistical analyses involving trait measurements,  
223 we examined the residuals for each trait variable and square root- or log-transformation was  
224 applied when appropriate.

225 For fungal community analyses, normalization of sequence reads across all samples was not  
226 performed because rarefaction curve (‘rarecurve’ function in ‘vegan’) indicated that  
227 sequencing effort for the majority of the samples was sufficient to reach saturation (Fig. S2;  
228 range = 5253 to 32975 reads/sample) and, when normalization was performed, we observed  
229 similar patterns (not shown here). Weiss *et al.* (2015) observed that lack of normalisation  
230 could impact the detection of effects when sequencing depth varied among samples and when  
231 effect sizes were small, but their simulations were relevant for communities with lower  
232 diversity and lower sequencing depth. We used a Jaccard distance based on presence/absence  
233 data, rather than relative abundances, as the nested nature of the PCRs required for AM

234 fungal characterisation may have introduced biases to the estimates of relative abundances  
235 among taxa. We tested the effect of plant species and watering treatment on the fungal  
236 community composition with PerMANOVA using the ‘adonis’ function (‘vegan’). We  
237 estimated significant indicator OTUs using ‘indval’ function (‘labdsv’; Roberts 2016).  
238 Indicator OTUs (OTUs that are characteristic of an environment) were identified when  
239 comparing samples from the increased rainfall treatment with those from the reduced, altered  
240 frequency and summer rainfall treatments because we found the largest difference in  
241 composition among these treatment groups (see results). Indicators values (IV) range from 0  
242 to 1, where highest values are associated with stronger indicators of an environment. OTUs  
243 with  $IV > 0.3$  and  $P < 0.05$  (raw, not corrected for multiple testing) were considered good  
244 indicators (Dufrene and Legendre 1997).

245 We then estimated fungal richness for each sample using the Chao index with ‘estimateR’  
246 function (‘vegan’). We assessed the response of fungal richness to plant species and watering  
247 treatment as well as their interaction in linear mixed effect models using ‘lmer’ function  
248 (‘lme4’; Bates *et al.* 2015), modelling ‘subplot’ nested in ‘plot’ as random effects; statistical  
249 significance was determined using ANOVA (Analyses of Variance) based on Kenward-  
250 Roger approximated degrees of freedom, calculated using ‘Anova’ from the ‘car’ package  
251 (Fox & Weisberg 2011). Multiple mean comparisons using Turkey’s test was used to  
252 determine how AM fungal richness differed among plant species, with ‘glht’ function  
253 (‘multcomp’; Hothorn *et al.* 2008).

254 To study the effect of root traits on expected fungal richness, we tested different types of  
255 traits (chemical, morphology) by fitting four linear mixed effect models including 1) all traits  
256 as predictors (‘Global model’), 2) only chemical traits N, P, C and K as predictors (‘Chemical  
257 model’), 3) only morphological traits as predictors (‘Morphological model’), and 4) no traits  
258 as predictors (‘Intercept-only model’). In order to account for the experimental design and the

259 variation in traits explained by plant species, all the models included ‘subplot’ nested in  
260 ‘plot’, and ‘plant species’ as random effects; ‘plant species’ was considered a random effect  
261 in this particular analysis because we were mainly interested in accounting for interspecific  
262 differences in root traits, not in the effects of the species themselves. The adequacy of the  
263 models was determined by visually examining the residuals for normality and  
264 homoscedasticity. We then evaluated the relative importance of the morphological and  
265 chemical traits of the roots on AM fungal richness by model selection based on an  
266 information-theoretic approach. The four models described above were compared using the  
267 second order Akaike Information Criterion (AICc), which corrects for small sample sizes,  
268 using the function ‘sem.model.fits’ (‘MuMIn’; Barton, 2016). The total variance explained by  
269 the fixed effects (marginal  $R^2$ ) was obtained using the function ‘r.squaredGLMM’  
270 (‘MuMIn’). We further explored the importance of individual morphological traits (and not  
271 chemical traits, see results) as predictors of the fungal richness using a multimodel inference  
272 and model averaging approach. We used the ‘dredge’ function (‘MuMIn’) on the  
273 ‘Morphological model’ to generate several models containing random subsets of variables,  
274 followed by ‘model.avg’ (‘MuMIn’) to obtain the relative variable importance and the  
275 confidence interval of each individual parameter.

276 To evaluate the correlation between plant traits and AM fungal community composition, we  
277 performed a mantel test using the ‘mantel’ function (‘vegan’). The dissimilarity matrix of the  
278 plant traits was obtained using Euclidean distances of the transformed trait matrix (see above)  
279 prior to mantel test. To identify significant root traits that explain variation among AM fungal  
280 communities, we first verified whether there was multicollinearity between traits using the  
281 ‘vif.cca’ function (‘vegan’) and then performed a stepwise model selection using permutation  
282 tests with ‘ordistep’ function (‘vegan’). We assessed the amount of variation explained by the  
283 significant traits with a partial distance-based Redundancy Analysis (db-RDA) using the

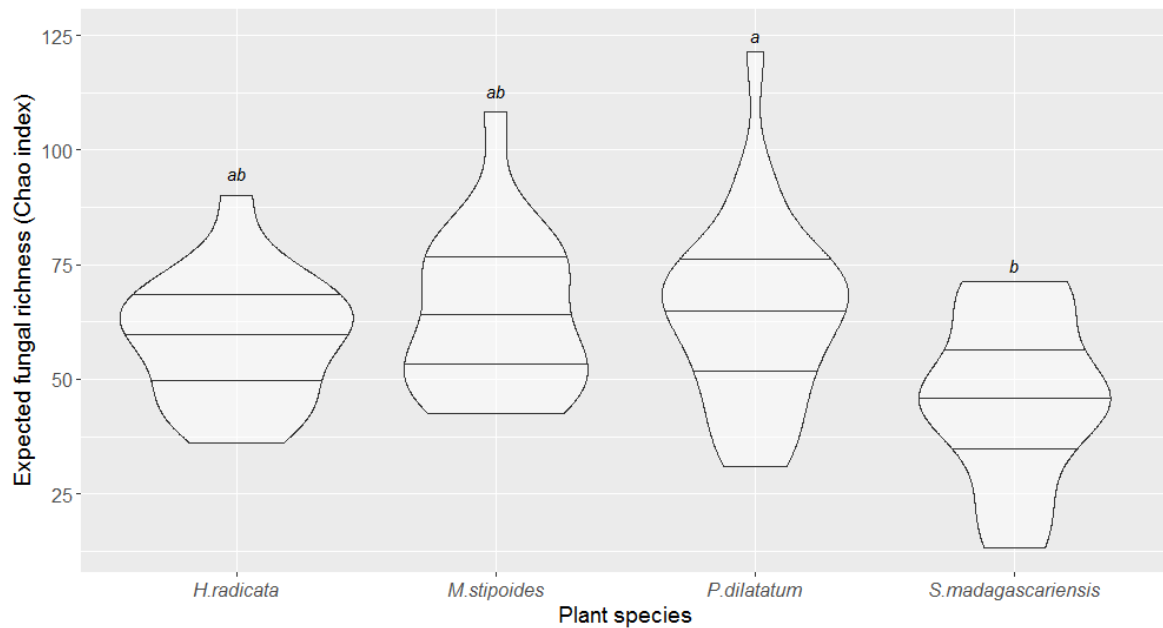
284 'capscale' function ('vegan'), accounting for watering treatment and plant species effects  
285 before testing the constraints (by including them as sources of 'conditional' variation in the  
286 analysis).

287

## 288 **Results**

### 289 **Plant species and watering treatment effects on AM fungal communities**

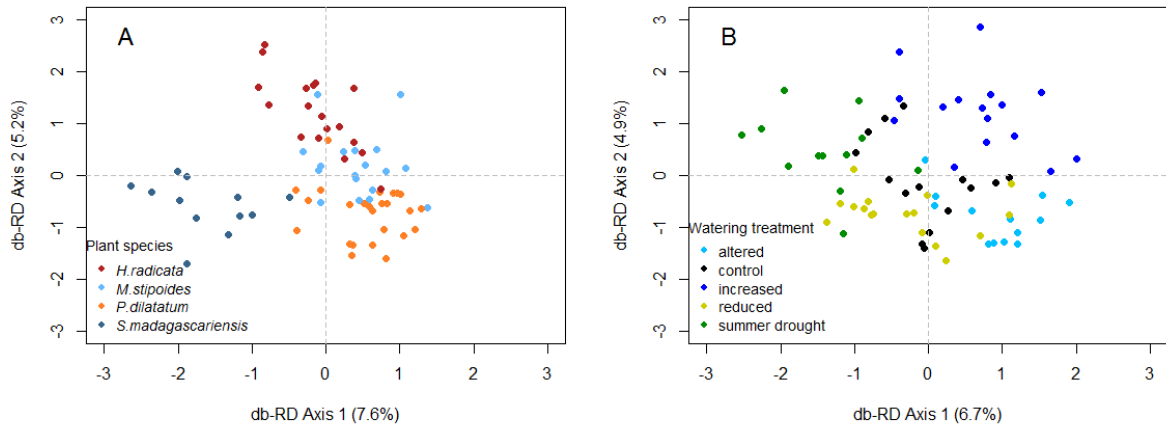
290 We evaluated the relative importance of the watering treatments and host characteristics on  
291 root-associated AM fungal communities. In total, we observed 774 OTUs across 81 plants.  
292 We first evaluated whether AM fungal community richness was influenced by the watering  
293 treatment and whether it differed among the different plant species. However, we found no  
294 evidence that the fungal richness was affected by watering treatment (linear mixed-effect  
295 model,  $P=0.20$ ; Fig. S3) nor an interaction between watering treatment and plant species  
296 (linear mixed-effect model,  $P=0.58$ ). Fungal richness was, however, found to differ  
297 significantly between plant species (linear mixed-effect model,  $P=0.02$ ; Fig. 1).



298

299 Figure 1. Violin plot showing the estimated (Chao) richness of AM fungal communities  
 300 associated with the four plant species. Horizontal lines in the violin plots represent the median,  
 301 25% and 75% quantiles, while the width of the violin plots represents data densities at the  
 302 different fungal richness. Letters indicates mean contrasts using Tukey's test, differences are  
 303 significant at  $P < 0.05$ .

304 We then evaluated the influence of watering treatment and plant species on AM fungal  
 305 community composition. We found a significant but small effect of both watering treatment  
 306 (PerMANOVA,  $F_{4,53}=1.40$   $R^2=0.07$ ;  $P<0.01$ ) and plant species (PerMANOVA,  $F_{3,53}=1.19$ ,  
 307  $R^2=0.05$ ;  $P=0.05$ ) on the community composition, but no significant interaction between  
 308 watering treatment and plant species (PerMANOVA,  $F_{12,53}=0.88$   $P=0.99$ ). We observed that  
 309 *S. madascariensis* and *H. radicata* had distinctive communities along the first and second  
 310 axes, respectively, of the 'plant species'-constrained db-RDA (Fig. 2a). For the 'watering  
 311 treatment'-constrained db-RDA, we observed some overlap between communities sampled  
 312 from the control treatment and those exposed to some type of drought (reduced, altered  
 313 frequency and summer drought treatments), while the communities from the increased  
 314 rainfall treatment were distinctly different (Fig. 2b).



315

316 Figure 2. Distance based-Redundancy Analysis (db-RDA) ordination of AM fungal  
 317 communities constrained by plant species (A) and by watering treatment (B) based on Jaccard  
 318 distances. Each point represents a fungal community described from samples pooled at the  
 319 level of an individual root system.

320

321 To identify specific OTUs associated with environments experiencing either any type of  
 322 drought or experiencing increased rainfall, we estimated OTU indicator values in  
 323 comparisons between these two groups ('reduced', 'summer' and 'reduced frequency'  
 324 treatments; 'increased' treatment). We found 24 significant indicator OTUs, of which 10 had  
 325 an indicator value > 0.3 and were all identified as Glomerales (Table 1; Fig. S4).

326

327 Table 1. Indicator OTUs associated with either reduced, altered, summer treatments or  
 328 increased treatment. The table includes the name of the OTU, indicator value (IV), treatments  
 329 associated, and, based on the sequence comparison using BLAST, the taxonomy, percentage  
 330 of sequence covered (query cover), percentage of aligned residues (ID %) and accession  
 331 number of the best matched database sequence. Indicators values (IV) range from 0 to 1,  
 332 where highest values are associated with stronger indicators of an environment.

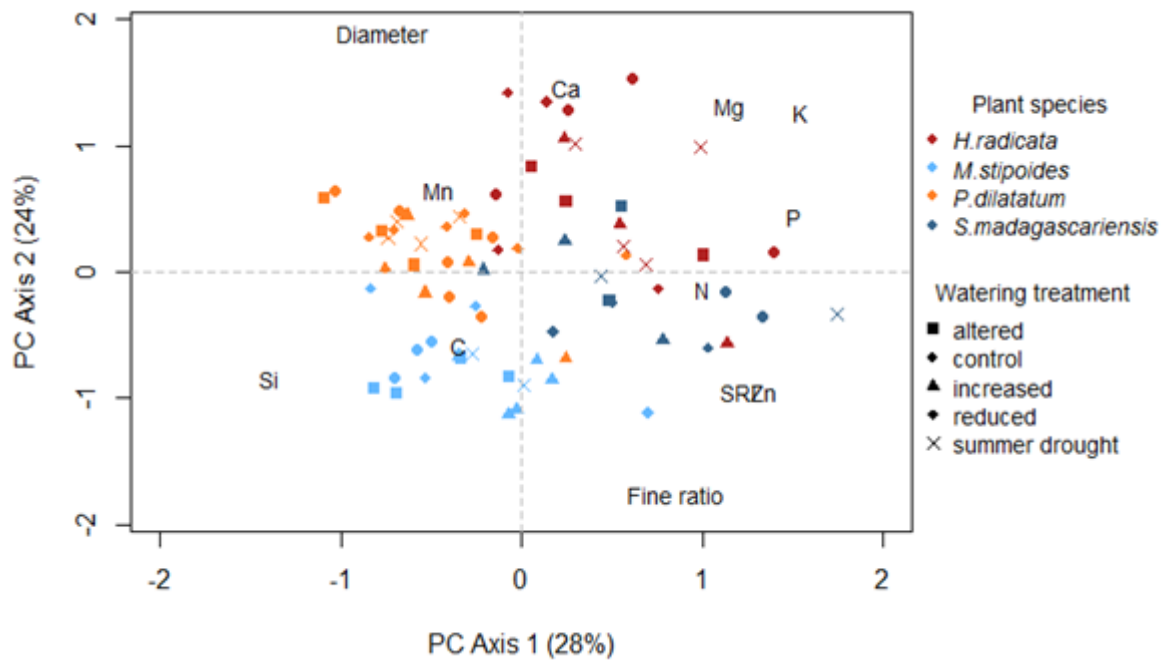
OTU ID	IV	P	Treatments	Best matched taxa using BLAST			
				Taxonomy	Query cover	ID %	Accession
ITSall_ OTUa_ 562	0.65	0.001	Reduced, altered, summer	<i>Glomus custos</i>	97%	93%	GQ205073.1
ITSall_ OTUb_ 10	0.62	0.002	Reduced, altered, summer	<i>Glomus intraradices</i>	100%	92%	AF185685.1
ITSall_ OTUa_ 8591	0.59	0.014	Reduced, altered, summer	<i>Glomus intraradices</i>	100%	92%	AF185685.1
ITSall_ OTUa_ 862	0.40	0.037	Reduced, altered, summer	<i>Glomus intraradices</i>	100%	93%	AF185686.1
ITSall_ OTUa_ 2876	0.38	0.050	Reduced, altered, summer	<i>Claroideoglomus claroideum</i>	100%	83%	GQ388713.1
ITSall_ OTUa_ 491	0.48	0.001	Increased	<i>Glomus</i> sp.	100%	98%	FJ769290.1
ITSall_ OTUa_ 4807	0.34	0.029	Increased	<i>Rhizoglomus</i> sp.	100%	93%	KY555056.1
ITSall_ OTUb_ 195	0.34	0.030	Increased	<i>Glomus</i> sp.	100%	90%	AJ504633.1
ITSall_ OTUa_ 423	0.33	0.012	Increased	<i>Dominikia bernensis</i>	100%	88%	HG938301.1
ITSall_ OTUa_ 4067	0.31	0.011	Increased	<i>Rhizophagus</i> sp.	100%	85%	KY362438.1

333

334 **Interactions between root traits and AM fungal communities**

335 We first evaluated the extent that the measured root traits differed among the plant species  
 336 and whether the watering treatments influenced these traits by estimating trait variation

337 between plants, based on Euclidean distances. We observed significant interspecific variation  
 338 in the measured root traits (PerMANOVA:  $F_{3,53} = 17.02$ ,  $R^2=0.45$ ,  $P<0.01$ ; Fig. 3), but there  
 339 was no evidence that the set traits were affected by the watering treatment (PerMANOVA:  
 340  $F_{4,53} = 0.03$ ,  $R^2=0.02$ ,  $P=0.81$ ; Fig. 3) or by the interaction between species and watering  
 341 treatment (PerMANOVA:  $F_{12,53} = 1.02$ ,  $R^2=0.10$ ,  $P=0.36$ ; Fig. 3).



342  
 343 Figure 3. Principal Component Analysis (PCA) ordination of plant individuals based on  
 344 Euclidean distances. Each point represents measurements taken on an individual plant. The text  
 345 indicates the loadings associated with each root trait: Specific Root Length ('SRL'), average  
 346 diameter of the roots ('Diameter'), fine (<1mm diameter) to coarse (>1mm diameter) roots  
 347 length ratio ('Fine ratio') and elemental composition Nitrogen ('N'), Carbon ('C'), Phosphorus  
 348 ('P'), Silicon ('Si'), Zinc ('Zn'), Potassium ('K'), Magnesium ('Mg'), Calcium ('Ca'),  
 349 Manganese ('Mn').

350  
 351 We hypothesized that variation in root traits, both within and among host plant species, was  
 352 responsible for variation in fungal richness, so we used model selection to evaluate the  
 353 relative importance of morphological and chemical root trait effects. We found that  
 354 morphological traits were better predictors of fungal richness than chemical traits, based on

355 the lower Akaike weights of models including chemical variables ('Global model' and  
 356 'Chemical model'; Table 2). However, low explanatory power (Marginal  $R^2=0.13$ ) and  
 357 Akaike weight ( $w_i=0.68$ ) was observed for the model including morphological traits as  
 358 predictors and the model fit was marginally better than the model excluding all variables  
 359 ('Intercept only model';  $\Delta AIC_c=1.86$ ), suggesting that either morphological traits, in general,  
 360 were not strong predictors of fungal richness or that only a subset of the morphological traits  
 361 included in the model were useful.

362 Table 2. Model fit statistics for the 'Global model' (including both morphological and chemical  
 363 variables), models including either morphological or chemical variables, and the model with  
 364 intercept only, testing the effect of the root traits on AM fungal richness. Table includes the  
 365 number of estimated parameters ( $K$ ), proportion of variance explained by fixed factors  
 366 (Marginal  $R^2$ ),  $AIC_c$  value, the  $AIC_c$  difference between the best fitted model and the  
 367 corresponding model ( $\Delta AIC_c$ ) and 'Akaike weight' ( $w_i$ ) for each model. Models are ranked by  
 368 increasing  $AIC_c$  value. All linear mixed-effect models include plot and species as random  
 369 effects.

Model	Variables	$K$	Marginal $R^2$	$AIC_c$	$\Delta AIC_c$	Akaike weight ( $w_i$ )
	SRL					
'Morphological traits'	Fine to coarse root length ratio	4	0.13	248.9	0	0.68
	Diameter					
'Intercept-only'	None	1	0	250.7	1.86	0.27
'Global model'	All	8	0.17	254.9	6	0.03
	N					
	P					
'Chemical traits'	K	5	0.05	256.5	7.58	0.02
	C					

370

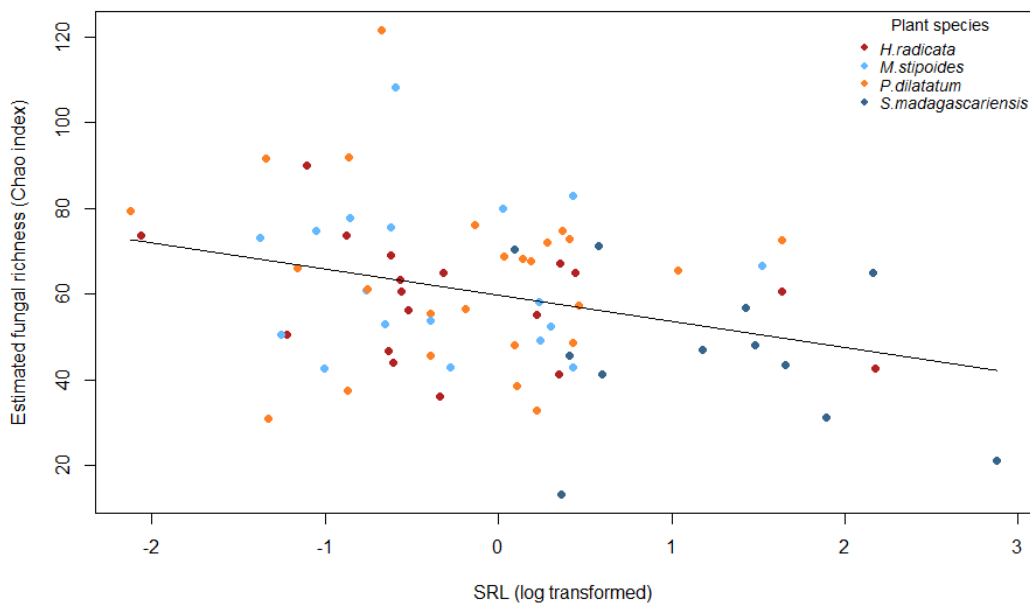
371 When comparing models containing all combinations of the measured morphological traits as  
 372 predictors, SRL was the best predictor of fungal richness (Table 3) with moderate importance  
 373 (sum of Akaike weights = 0.79). To quantify this relationship while also accounting for  
 374 variation among plant species, we fitted a new model including SRL as the only explanatory  
 375 variable with plot and plant species as random effects and observed a negative relationship

376 between SRL and fungal richness (linear mixed-effects model;  $P < 0.01$ , Marginal  $R^2 = 0.11$ ,  
 377 Fig. 4). This result may indicate that the relationship between SRL and fungal richness  
 378 represents a sampling effect, with smaller diameter roots containing less cortical tissue and,  
 379 therefore, less fungal biomass for a given length.

380 Table 3. Relative importance of morphological root traits variables for predicting variation in  
 381 AM fungal richness in roots. Table includes the relative variable importance (corresponds to  
 382 the sum of ‘Akaike weights’ across models including each variable) and the model-averaged  
 383 confidence interval for each estimated parameter. Linear mixed model includes ‘plot’ and  
 384 ‘species’ as random effects.

Variable	Relative variable importance	Confidence intervals	
		2.5%	97.5%
SRL	0.79	-0.68	-0.10
Diameter	0.38	-0.18	0.64
Fine to coarse root length ratio	0.26	-0.44	0.36

385

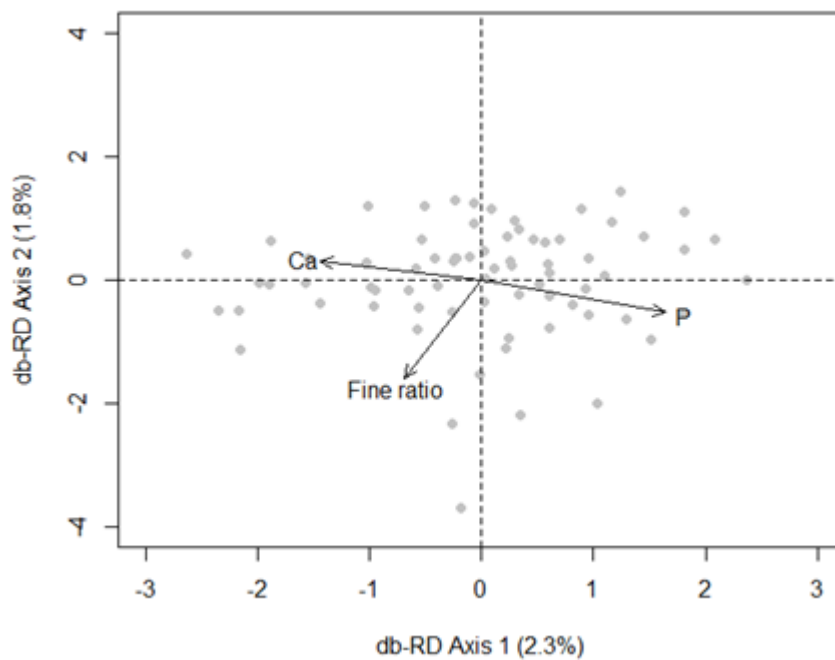


386

387 Figure 4. Correlation between (natural log transformed) SRL and estimated (Chao) AM fungal  
 388 richness. The relationship was evaluated in a linear mixed model including subplot nested in  
 389 plot and plant species as random effects.

390

391 We expected the AM fungal community composition to be affected by the root traits of their  
392 host. When evaluating the overall effect of the root traits, we found a significant correlation  
393 between the AM fungal community composition and all the root traits (Mantel test;  $r = 0.15$ ,  
394  $P < 0.01$ ). We then identified relevant root traits and tested their importance on the AM fungal  
395 community, after accounting for the conditional effects of the watering treatment and plant  
396 species. When comparing models including different sets of traits, we observed that  
397 phosphorus (ordistep;  $P < 0.01$ ), calcium (ordistep;  $P = 0.02$ ), and fine to coarse root length  
398 ratio (ordistep;  $P = 0.02$ , Fig. 5) were traits significantly correlated with AM fungal  
399 community composition. In total, those traits explained 5.6% of variation in community  
400 composition (partial db-RDA,  $F_{3,62} = 1.41$ ,  $P < 0.01$ ), while plant species and the water  
401 treatment together explained 12.6% of this variation.



402  
403 Figure 5. Distance based-Redundancy Analysis (db-RDA) showing root traits significantly  
404 correlated with AM fungal communities after accounting for conditional effects of host species  
405 and watering treatments. Each point represents a fungal community described from samples  
406 pooled at the level of an individual root system and the arrows and text indicate the loadings  
407 associated with each root trait: phosphorus ('P'), calcium ('Ca') and fine (<1mm diameter) to  
408 coarse (>1mm diameter) root length ratio ('Fine ratio'). Significant root traits were identified  
409 performing a forward model selection using permutation tests.

410

## 411 **Discussion**

412 We found that modified rainfall regimes affected the composition, but not the richness, of  
413 AM fungal communities in this experimental grassland. We also found that the proportion of  
414 variation associated with these effects was similar to that associated with host species. In  
415 particular, our results showed distinctive communities when grassland plots were exposed to  
416 increased rainfall, compared to those experiencing drought treatments. Other studies have  
417 observed changes in the composition and richness of AM fungal communities from semi-arid  
418 environments due to an increase in rainfall amount (Deepika & Kothamasi 2015; Li *et al.*  
419 2015). In contrast, rainfall reduction, change in frequency or summer drought did not form as  
420 distinctly divergent communities from the ambient treatment, possibly because these fungi  
421 exhibit adaptation to the high intra- and inter-annual rainfall variability that is characteristic  
422 of our site (Power *et al.* 2016). If this is the case, their frequent exposure and adaptation to  
423 low amounts of rainfall and high variability in the frequency and timing of these rainfall  
424 events may shape their response to future changes in rainfall.

425 The studied AM fungal communities also differed depending on the plant species that they  
426 were associated with, and these responses were partly due to the root traits expressed by the  
427 hosts. Others have observed relationships between plant traits and fungal communities, and  
428 have related these observations to variation in plant-derived resources available for those soil  
429 communities (Sayer *et al.* 2017; Liu *et al.* 2015; Koorem *et al.* 2017). The AM fungal  
430 communities found in roots may also be expected to be related to the plant dependency on  
431 AM fungi to acquire particular limiting nutrients (Brundrett 2002). We found that traits  
432 involved in plant nutrition and dependence on their symbionts were shaping our AM fungal  
433 communities. The best predictors of AM fungal community composition were root P and Ca  
434 concentrations, both nutrients associated with AM fungal transfer to host plants (Rhodes &

435 Gerdemann 1978). Interestingly, Ca is also an important signal molecule for plant-AM fungi  
436 communication as it is involved in the establishment of the symbiosis (reviewed by Oldroyd  
437 2013). The proportion of fine roots was also involved in shaping the community composition,  
438 while SRL influenced AM fungal richness, where plants with higher SRL had less diverse  
439 communities.

440 Despite the observed relationships between root traits and AM fungal communities, we did  
441 not find evidence for rainfall manipulation affecting the AM fungal community via effects on  
442 root traits. This is because none of the studied root traits were significantly affected by the  
443 manipulations to rainfall, which is surprising because changes in rainfall have been found to  
444 affect root traits in different species measured in other studies (Larson & Funk 2016; Padilla  
445 *et al.* 2013). The lack of effect on root traits may be a result of selecting plant species present  
446 across all watering treatments and, in this type of grassland with highly variable rainfall  
447 (Power *et al.* 2016), abundant plant species might possess traits adapted to these variable  
448 conditions and, thus, have low plasticity. Our results may also reflect the fact that our data  
449 represent a snapshot at one point in time, and additional time points might reveal more  
450 variation associated with the rainfall manipulations. Despite this, AM fungal communities are  
451 responsive to root trait variation within and between plant species, indicating that this  
452 mechanism should be considered in further studies, particularly in more responsive species  
453 and in ecosystems where plasticity may be more likely.

454 It is also possible that investigation into other root traits may provide more insight into the  
455 processes associated with AM fungal community assembly and host responses to altered  
456 rainfall. Architectural traits important for plant access to water such as rooting depth, root  
457 length density and root branching have been observed to respond to changes in rainfall  
458 regimes (Brunner *et al.* 2015) with, for example, some of the other grassland species from  
459 this local site exhibiting shallower rooting profiles when exposed to reduced rainfall inputs

460 under controlled, polytunnel conditions (Gibson-Forty et al., 2016). Reduction in rainfall has  
461 been shown to result in the accumulation of sugars, sugar alcohols, proline and proteins in  
462 plants (Farooq *et al.* 2009), which could, in turn, affect the AM fungal community. In  
463 addition, root lifespan and turnover have been observed to be affected by changes in rainfall  
464 (Brunner *et al.* 2015) and AM fungal community composition and richness can vary  
465 depending on the age of the roots (Kil *et al.* 2014; but see Donn *et al.* 2017). We could not  
466 account for most of these traits in this field study due to our focus on matching community  
467 and trait data at the individual plant level, which limited the amount of material that we could  
468 analyse and prevented measurements of traits linked to phenology.

469 We found a few indicator OTUs distinctly associated to either drought treatments or  
470 increased rainfall, suggesting that the AM fungal communities could be more responsive in  
471 years with higher rainfall. All of the indicator OTUs belonged to the Glomerales order.  
472 Others have found that not only Glomerales spp. but also *Acaulospora* sp. (Diversisporales  
473 order) have been affected by changes in watering regime (Deepika & Kothamasi 2015). In  
474 our experiment, Glomerales spp. was the most abundant taxa, whereas *Acaulospora* spp. only  
475 represented 0.9% of the taxa detected in our samples (Table S1). It is possible that the  
476 dominance of Glomerales spp. is due to bias in the methods to characterise AM fungi (Berruti  
477 *et al.* 2017), which may be addressed by further developments in primer design and  
478 sequencing approaches. The role of particular OTUs in plant nutrition and tolerance to water  
479 stress is unknown, limiting our understanding on how plants will respond to the loss of those  
480 OTUs under altered rainfall regimes. Additionally, we observed over 700 OTUs using our  
481 sequencing and data processing approach; the loss of OTUs and their associated services due  
482 to changes in rainfall might therefore be more significant in less diverse ecosystems. In  
483 addition, grassland responses to climate change may also be associated with altered  
484 abundance of the AM fungi both inside and outside the plant instead of shifts in composition.

485 The sample limitations mentioned in relation to alternative root traits also prevented us from  
486 accurately characterising AM fungal colonisation or another measure of absolute abundance,  
487 and further work in this experimental system would be needed to answer this question.

488 A noteworthy observation is that variability among AM fungal communities was high, even  
489 among communities sampled from the same treatments, and a large proportion of the  
490 variation remained unexplained. This is similar to observations by Li *et al.* (2015), who  
491 reported that rainfall was the most important predictor of AM fungal communities in roots  
492 despite only explaining 6.7% of the variation. This high variability inherent of the AM fungal  
493 communities is increasingly being recognized as an important characteristic of their ecologies  
494 (Powell & Bennett 2016; Hart *et al.* 2016; Donn *et al.* 2017) and creates further difficulties  
495 for predicting changes in those communities and associated functions. Current research is  
496 ongoing in this experimental system and is aiming to evaluate whether consideration of  
497 variation in soil properties - both natural and that associated with rainfall treatments - and  
498 changes in plant communities in analyses of AM fungal community variation improve our  
499 understanding of their responses to altered rainfall regimes.

500 In conclusion, our results suggest that AM fungal communities in grasslands, especially those  
501 similar to the one studied here, are likely to change under future rainfall scenarios. However,  
502 the magnitude of these predicted effects is still unclear as effect sizes in this case were small  
503 relative to the high level of variation among AM fungal communities observed here.

504 Although we found no evidence of effects of altered rainfall regimes via changes in host  
505 physiology, this potential indirect effect should still be tested in further work. The effects of  
506 inter- and intraspecific root trait variation on AM fungal communities observed here provides  
507 insight into the ways that AM fungi associated with more plastic plant species might respond  
508 to variable rainfall in different systems.

509

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522

523

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### 683 **Data accessibility**

684 All data have been archived with the Western Sydney University Data Repository (doi:  
685 <http://dx.doi.org/10.4225/35/5a24cbf955391>) and the scripts and data can be accessed at  
686 [https://bitbucket.org/Coline\\_Dev/roottraits\\_amf](https://bitbucket.org/Coline_Dev/roottraits_amf). Raw DNA sequencing data are available  
687 under NCBI BioProject ID PRJNA420909.

688

689 **Author contribution**

690 CD, AEB, JRP and SP designed the study. CD, SP and SD collected and processed samples.  
691 CD and JRP analysed the data and wrote the manuscript. All the authors revised the  
692 manuscript.

693

694 **Tables and Figures**

695

696 Table 1. Indicator OTUs associated with either reduced, altered, summer treatments or  
697 increased treatment. The table includes the name of the OTU, indicator value (IV), treatments  
698 associated, and, based on the sequence comparison using BLAST, the taxonomy, percentage  
699 of sequence covered (query cover), percentage of aligned residues (ID %) and accession  
700 number of the best matched database sequence. Indicators values (IV) range from 0 to 1,  
701 where highest values are associated with stronger indicators of an environment.

702

703 Table 2. Model fit statistics for the ‘Global model’ (including both morphological and  
704 chemical variables), models including either morphological or chemical variables, and model  
705 with intercept only, testing the effect of the root traits on the AM fungal richness. Table  
706 includes the number of estimated parameters ( $K$ ), proportion of variance explained by fixed  
707 factors (Marginal  $R^2$ ),  $AIC_c$  value, the  $AIC_c$  difference between the best fitted model and the  
708 corresponding model ( $\Delta AIC_c$ ) and ‘Akaike weight’ ( $w_i$ ) for each model. Models are ranked  
709 by increasing  $AIC_c$  value. All linear mixed-effect models include plot and species as random  
710 effects.

711

712 Table 3. Relative importance of morphological root traits variables for predicting variation in  
713 AM fungal richness in roots. Table includes the relative variable importance (corresponds to  
714 the sum of ‘Akaike weights’ across models including each variable) and the model-averaged  
715 confidence interval for each estimated parameter. Linear mixed model includes ‘plot’ and  
716 ‘species’ as random effects.

717

718 Figure 1. Violin plot showing the estimated (Chao) richness of AM fungal communities in the  
719 four plant species. Horizontal lines in the violin plots represent the median, 25% and 75%  
720 quantiles, while the width of the violin plots represents data densities at the different fungal  
721 richness. Letters indicates mean contrasts using Tukey’s test, differences are significant at  $P$   
722  $<0.05$ .

723

724 Figure 2. Distance based-Redundancy Analysis (db-RDA) ordination of AM fungal  
725 communities constrained by plant species (A) and by watering treatment (B) based on  
726 Jaccard distances. Each point represents a fungal community described from samples pooled  
727 at the level of an individual root system.

728

729 Figure 3. Principal Component Analysis (PCA) ordination of plant individuals based on  
730 Euclidean distances. Each point represents measurements taken on an individual plant. The  
731 text indicates the loadings associated with each root trait: Specific Root Length ('SRL'),  
732 average diameter of the roots ('Diameter'), fine (<1mm diameter) to coarse (>1mm diameter)  
733 roots length ratio ('Fine ratio') and elemental composition Nitrogen ('N'), Carbon ('C'),  
734 Phosphorus ('P'), Silicon ('Si'), Zinc ('Zn'), Potassium ('K'), Magnesium ('Mg'), Calcium  
735 ('Ca'), Manganese ('Mn').

736

737 Figure 4. Correlation between (natural log transformed) SRL and estimated (Chao) AM fungal  
738 richness. The relationship was evaluated in a linear mixed model including subplot nested in  
739 plot and plant species as random effects.

740

741 Figure 5. Distance based-Redundancy Analysis (db-RDA) showing root traits significantly  
742 correlated with AM fungal communities after accounting for conditional effects of host  
743 species and watering treatments. Each point represents a fungal community described from  
744 samples pooled at the level of an individual root system and the arrows and text indicate the  
745 loadings associated with each root trait: phosphorus ('P'), calcium ('Ca') and fine (<1mm  
746 diameter) to coarse (>1mm diameter) root length ratio ('Fine ratio'). Significant root traits  
747 were identified performing a forward model selection using permutation tests.