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HAL Authorization

1 **Temporal dynamics of mycorrhizal fungal communities and co-associations with**
2 **grassland plant communities following experimental manipulation of rainfall**

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13

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15

16 **Abstract**

- 17 1. Climate models project overall a reduction in rainfall amounts and shifts in the timing
18 of rainfall events in mid-latitudes and sub-tropical dry regions, which threatens the
19 productivity and diversity of grasslands. Arbuscular mycorrhizal fungi may help
20 plants to cope with expected changes but may also be impacted by changing rainfall,
21 either via the direct effects of low soil moisture on survival and function or indirectly
22 via changes in the plant community.
- 23 2. In an Australian mesic grassland (former pasture) system, we characterised plant and
24 arbuscular mycorrhizal (AM) fungal communities every six months for nearly four
25 years to two altered rainfall regimes: i) ambient, ii) rainfall reduced by 50% relative to
26 ambient over the entire year and iii) total summer rainfall exclusion. Using Illumina
27 sequencing, we assessed the response of AM fungal communities sampled from
28 contrasting rainfall treatments and evaluated whether variation in AM fungal
29 communities was associated with variation in plant community richness and
30 composition.
- 31 3. We found that rainfall reduction influenced the fungal communities, with the nature
32 of the response depending on the type of manipulation, but that consistent results were
33 only observed after more than two years of rainfall manipulation. We observed
34 significant co-associations between plant and AM fungal communities on multiple
35 dates. Predictive co-correspondence analyses indicated more support for the
36 hypothesis that fungal community composition influenced plant community
37 composition than vice versa. However, we found no evidence that altered rainfall
38 regimes were leading to distinct co-associations between plants and AM fungi.
- 39 Overall, our results provide evidence that grassland plant communities are intricately
40 tied to variation in AM fungal communities. However, in this system, plant responses

41 to climate change may not be directly related to impacts of altered rainfall regimes on
42 AM fungal communities.

43 4. Synthesis. Our study shows that AM fungal communities respond to changes in
44 rainfall but that this effect was not immediate. The AM fungal community may
45 influence the composition of the plant community. However, our results suggest that
46 plant responses to altered rainfall regimes at our site may not be resulting via changes
47 in the AM fungal communities.

48

49 **Keywords:** arbuscular mycorrhizal fungi, climate change, community assembly, driver
50 hypothesis, drought, passenger hypothesis, plant-microbe interactions.

51

52 **Introduction**

53 Grasslands are highly diverse ecosystems that provide numerous ecosystem services,
54 including forage production, soil stabilization and carbon storage (Lemaire, Hodgson, &
55 Chabbi, 2011). The diversity and productivity of these ecosystems are threatened by the
56 reduction in rainfall amounts and shifts in rainfall frequency, as predicted by climate models
57 (IPCC, 2014), because they are highly responsive to changes in rainfall regimes (Fay,
58 Carlisle, Knapp, Blair, & Collins, 2003; Grime et al., 2000). While the effect of altered
59 rainfall regimes on vegetation has been extensively studied, with known effects on the
60 composition and richness of plant communities (e.g. Grime *et al.* 2000; Morecroft *et al.*
61 2004), less focus has been given to belowground communities. Altered rainfall regimes may
62 influence both above and belowground communities as well as the interactions between these
63 communities. Altered rainfall regimes can limit the exchange of resources between plants and

64 soil communities via nutrient immobilisation in the soil (Compant, Van Der Heijden, &
65 Sessitsch, 2010) and competition for resources may shift interactions from mutualistic to
66 parasitic (N. C. Johnson, Wilson, Wilson, Miller, & Bowker, 2015).

67 Arbuscular mycorrhizal (AM) fungi are an important element of the belowground community
68 in grasslands (Miller, Wilson, & Johnson, 2012) and can promote drought tolerance (Augé,
69 2001). These fungi form symbiotic associations with the majority of plant species and are
70 dependent on the carbon provided by their host (Smith & Read, 2008). In return, they
71 contribute to plant nutrition (Smith & Smith, 2011) and tolerance to environmental stress,
72 including drought (Augé, 2001). AM fungi can help their host cope with changes in rainfall
73 in different ways, such as maintaining the nutritional status of the plant (Augé, 2001),
74 facilitating access to water (Díaz-Zorita *et al.* 2002; Marulanda *et al.* 2003) and impacting
75 host physiology, affecting water-use efficiency and root hydraulic conductivity (Sánchez-
76 Blanco, Ferrández, Morales, Morte, & Alarcón, 2004; Wu, Zou, & Xia, 2006). The extent to
77 which AM fungi contribute to drought-tolerance not only varies among plant species (Augé,
78 2001) but also among AM fungal species (Marulanda *et al.*, 2003). The differential role of
79 AM fungal species and the fact that AM fungal communities can affect plant diversity,
80 composition and productivity (Powell & Rillig, 2018; van der Heijden *et al.*, 1998) suggests
81 that changes in the AM fungal community under altered rainfall regimes will likely affect
82 associated plant communities, with outcomes that may be difficult to precisely predict.

83 Numerous studies have evaluated the response of plant-AM fungal interaction to altered
84 water conditions in pot experiments involving one plant and its associated fungi (reviewed by
85 Augé 2001). In a greenhouse experiment using a single plant species, Deepika & Kothamasi
86 (2015) found that reductions in soil moisture resulted in a reduction in the diversity of the
87 AM fungal community and changes in its composition, but knowledge gaps remain about
88 how AM fungal communities may respond to long-term altered rainfall under field

89 conditions. Previous research under field conditions has revealed that changes in rainfall
90 amounts and distribution between five and nine years resulted in changes in root colonisation,
91 abundance of vesicles (involved in storage of nutrients), arbuscules (involved in nutrient
92 exchange between partners), extraradical mycelium and spore density (Gao et al., 2016;
93 Martínez-García, de Dios Miranda, & Pugnaire, 2012; Staddon et al., 2003). Few studies
94 have evaluated how altered rainfall regimes will affect AM fungal community assembly. Two
95 recent field studies found that increasing rainfall amounts during six to seven years
96 influenced AM fungal community richness and composition in soil and roots (Gao et al.,
97 2016; Li et al., 2015), while another study found that two years of altered rainfall amounts
98 and frequency did not affect AM fungal richness but influenced the community composition
99 (Deveautour, Donn, Power, Bennett, & Powell, 2018). Knowledge gaps remain on how AM
100 fungal communities will respond in a longer term to the reduction in rainfall amounts that are
101 expected with climate change.

102 Altered rainfall regimes may also indirectly affect the AM fungal community via changes in
103 the plant community. Staddon *et al.* (2003) found that the effect of altered rainfall regimes on
104 AM fungal density in both roots and soil was correlated both to soil moisture and to plant
105 diversity, while Martínez-García *et al.* (2012) related changes in AM fungal abundance to a
106 decrease in photosynthetic rates of the host. Changes in the vegetation via altered rainfall
107 regimes may also indirectly affect the AM fungal community assembly. Li *et al.* (2015)
108 found that changes in the composition and richness of the AM fungal community were in part
109 resulting from shifts in the biomass of particular plant functional groups. Changes in rainfall
110 regimes are expected to alter plant community composition (Grime et al., 2000; Morecroft et
111 al., 2004), which has been observed to affect the AM fungal community (Johnson et al.,
112 2004), suggesting that the indirect effect of changes in rainfall via plant species composition
113 should be further explored.

114 The majority of these studies evaluated the response of the community at one time-point but
115 AM fungal community structure and interactions with their hosts can be very dynamic
116 (Dumbrell et al., 2011). Taking these temporal dynamics into account is important to consider
117 as grassland community responses to climate change can take time to manifest and may even
118 show reversals over time (Reich, Hobbie, Lee, & Pastore, 2018). Here, we studied the
119 response of AM fungal communities to altered rainfall regimes over a period of almost four
120 years and evaluated whether changes in AM fungal communities were associated with plant
121 community richness and composition. During this time, we characterised the AM fungal
122 communities every six months, using Illumina sequencing, in replicated field plots
123 established in an Australian grassland. We expected that altered rainfall regimes would
124 reduce AM fungal richness and that different watering treatments would result in distinct
125 communities. We estimated the effect of altered rainfall regimes on the AM fungal
126 community, and evaluated whether the response varied over time. We then assessed whether
127 variation in AM fungal communities was associated with variation in plant communities and
128 whether these co-associations were influenced by rainfall manipulation. We hypothesised that
129 these communities would co-vary due to some plant-fungal associations having specificity,
130 leading to the presence of species of one community being associated with the presence of
131 species from the other community. We also hypothesised that changes in rainfall would result
132 in selection for or against particular associations between certain plants species and AM
133 fungi. We hypothesised that this co-variation between communities would be the result of
134 one community influencing the richness and composition of the other community in one of
135 two ways: AM fungal communities influencing plant communities (*Driver hypothesis*) or
136 vice versa (*Passenger hypothesis*) (Hart et al. 2001). Alternatively, AM fungal and plant
137 communities may covary as a result of each responding similarly but independently along our
138 environmental gradients (*Habitat hypothesis*, Zobel & Öpik 2014).

139

140 **Materials and methods**

141 **Site and experimental design**

142 The 'Drought and Root Herbivore Impacts on Grasslands' (DRI-Grass) experimental
143 platform was established in 2013 at the Hawkesbury Campus of Western Sydney University
144 in Richmond, NSW, Australia. The average annual precipitation at the site is 806 mm, and it
145 is characterized by a high inter-annual variability (between 500 mm and over 1400 mm in the
146 last 30 years; Australian Government Bureau of Meteorology, 2018), with summer being
147 generally the wettest season. The soil at the site has a sand or loamy sand texture, with water
148 holding capacity of 20-22%. Full details are described in Power et al. (2016) but, briefly,
149 DRI-Grass was established to study the effect of drought and root herbivory on grassland
150 community structure and ecosystem function. Here, we describe only the sampled plots. The
151 experiment is situated over a former pasture grassland (grazing stopped in 1995) and consists
152 of 2 x 2 m plots covered with shelters that exclude natural rainfall and have automated,
153 controlled application of water underneath the shelters. Water is applied to the plots at 1h00
154 each morning based on the amount of rainfall in the previous 24h and according to the
155 following watering treatments: 1) control (same amount as ambient rainfall), 2) reduced (50%
156 less rainfall than ambient) and 3) summer drought (total rainfall exclusion from December to
157 March, reducing the total annual rainfall amount by 32% to 67% depending on the year).
158 Treatments were selected to represent climatic projections of reduction in rainfall amounts
159 and a summer drought to simulate longer drought periods between rainfall events expected
160 for the region (CSIRO and Australian Government Bureau of Meteorology, 2016). All
161 treatments have six replicates and are arranged in a randomized block design. Rainfall
162 manipulation started on 21 June 2013.

163 **Plant and soil sampling**

164 Soil samples were collected approximately every six months between 2014 and 2017 (Table
 165 1). On each date, eight soil cores of 2 cm diameter, to a depth of 10 cm, were collected
 166 adjacent to the central 1 m² (two cores on each side) for each plot and composited before
 167 storing at -80°C for analysis of AM fungal communities. A total of 18 samples were collected
 168 on each sampling date. For characterisation of plant communities, the vegetation was cut to
 169 ground level within the central 1 m² of each plot and a sub-sample of harvest material
 170 (representing 25-40% of the biomass) was sorted to the species level. Samples were dried for
 171 48h at 80 °C and weighed to obtain the biomass of each plant species per plot (list of plant
 172 species in Table S1), before being scaled to the plot (1m²) level. Plant communities were
 173 assessed within ten days of soil sampling, with the exception of Autumn 2014 when soil
 174 samples were collected five weeks after plant communities were assessed (Table 1). Plant
 175 composition data was not available for spring 2015 because biomass was not sorted to species
 176 level at that date (Table 1).

177

178 Table 1. Sampling dates and richness of both the plant and AM fungal communities.

Sampling date	AM fungal community		Plant community	
	Specific dates	Richness (number of OTUs)	Specific dates	Richness (number of species)
Autumn 2014	31 March	433	24-28 February	15
Spring 2014	29 September	520	3-8 October	25
Autumn 2015	1 April	463	1-7 April	22

Spring 2015	13-14 October	515	-	-
Autumn 2016	30-31 March	1264	21-24 March	20
Spring 2016	26-27 September	1241	23-27 September	16
Autumn 2017	8-10 March	955	6-10 March	19

179

180 **Extraction, amplification and sequencing of AM fungal DNA**

181 DNA was extracted from 250 mg of soil for each sample using PowerSoil DNA isolation kit
 182 (MO BIO, Carlsbad, USA) as the manufacturer's instruction. Extracted DNA was quantified
 183 using NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, Wilmington, USA), and
 184 then diluted to 5ng/μL in 20μL prior to polymerase chain reaction (PCR) amplification. All
 185 PCRs were performed in PTC-200 thermal cyclers (Bio-Rad, California, USA).

186 DNA samples were amplified using PCR primers that target AM fungi and provide coverage
 187 for all AM fungal lineages, described in Krüger *et al.* (2009). Each reaction contained 9μL of
 188 master mix and 1μL diluted DNA template. The master mix contained: 5μL KAPA mix
 189 (Kapa Biosystem, Wilmington, USA), 0.4μL LSU_Ar (10μM), 0.4μL SSU_Af (10μM),
 190 3.2μL water. Thermocycling conditions for this first step were: 95°C for 3min, 30 cycles of
 191 98°C for 20s, 60°C for 30s and 72°C for 50s, followed by 72°C for 2 min. In the second PCR
 192 round, each reaction contained 18μL of master mix and 2μL diluted PCR product (2μL DNA
 193 / 98μL water). The master mix contained: 10μL KAPA mix, 0.8μL LSU_Br (10μM), 0.8μL
 194 SSU_Cf (10μM), 6.4μL water. Thermocycling conditions for this second step were: 95°C for
 195 3min, 30 cycles of 98°C for 20s, 63°C for 30s and 72°C for 50s, followed by 72°C for 2 min.

196 The PCR products were purified using Agencourt AMPure XP system (Beckman Coulter,
 197 Lane Cove, NSW, Australia), and diluted with PCR-grade water to 5ng/μL in 20μL. The
 198 ITS2 region was sequenced by Illumina MiSeq using FITS7 (5'-

199 GTGARTCATCGAATCTTTG-3'; Ihrmark et al., 2012) and ITS4 (5'-
200 TCCTCCGCTTATTGATATGC-3'; White, Bruns, Lee, & Taylor, 1990). Genomic libraries
201 were prepared using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Paired-end
202 (2 x 251 bases) sequencing was performed on the Illumina MiSeq platform. Samples were
203 processed, from DNA extraction to sequencing, in two batches: those collected in 2014-2015
204 (sequencing performed at the Ramaciotti Centre for Genomics; Sydney, NSW) and those
205 collected in 2016-2017 (sequenced at the Western Sydney University Next-Generation
206 Sequencing Facility; Richmond, NSW). Four samples collected in 2014 and 2015 were also
207 amplified with the second batch to evaluate the extent that 'batch' influenced the generation
208 of compositional data.

209 The DNA sequencing data were processed using the approach described by Bissett et al.
210 (2016) with a few modifications; the following is a brief description. Contigs were generated
211 from paired-end reads using the 'make.contigs' command in *mothur* (version 1.36.1) (Schloss
212 et al., 2009). Initial quality filtering removed DNA sequences containing any ambiguous
213 bases and/or a homopolymer greater than eight bases in length. De novo operational
214 taxonomic units (OTUs) at 97% sequence similarity were initially picked using numerically
215 dominant sequences (observed at least four times) using the '-cluster_otus' command in
216 *USEARCH* (version v8.1.1803) (Edgar, 2013). All quality-filtered sequences were mapped at
217 97% sequence similarity against representative sequences of these OTUs using the '-
218 usearch_global' command in *VSEARCH* (version v2.3.4) (Rognes, Flouri, Nichols, Quince, &
219 Mahé, 2016). Non-mapped sequences were subjected to a second round of de novo OTU
220 picking, as above but only using sequences observed at least two times. All initially non-
221 mapped sequences were then mapped against these newly picked OTUs, as above. Non-
222 mapped sequences at this step represent singleton OTUs and were excluded from further
223 analysis.

224 Putative taxonomic identities for fungal OTUs were generated by using BLAST (Altschul,
225 Gish, Miller, Myers, & Lipman, 1990) to compare a representative sequence for each OTU
226 against a reference database of fungal ITS sequences and taxonomic annotations obtained
227 from UNITE (version 7.0) (Abarenkov et al., 2010) and additional AM fungal ITS sequences
228 downloaded from the NCBI nucleotide database on 6 June 2018.

229

230 **Statistical analysis**

231 All the analyses were performed in R version 3.5.0 (R Core Team, 2018).

232 For fungal community analyses, normalisation of sequence reads across all samples was not
233 performed because the rarefaction curve (using ‘rarecurve’ in ‘vegan’; Oksanen et al. 2018)
234 showed that sequencing effort was sufficient to reach saturation for the majority of the
235 samples (Fig. S1, range: 262-29,269 reads/sample (Table S7)). A soil sample collected in the
236 summer drought treatment in autumn 2017 contained only 66 reads and was excluded from
237 the analyses. The tests were performed separately for samples collected in 2014-2015 and
238 2016-2017 because we found that richness (Fig. S1) and compositional (Fig. S2) data of the
239 AM fungal community were not comparable between samples processed in the two batches.

240 We estimated the fungal richness for each sample using the Chao index using ‘estimateR’
241 function (‘vegan’). We assessed the effects of the watering treatment, sampling date and their
242 interaction on the fungal richness using linear mixed-effects models to account for repeated
243 sampling at each plot with ‘lmer’ function (‘lme4’; Bates, Mächler, Bolker, & Walker, 2015).
244 We performed multiple comparisons between watering treatments using the ‘emmeans’
245 function (‘emmeans’; Lenth, 2018). We then calculated differences in AM fungal
246 composition using a Jaccard distance based on presence/absence data because nested PCRs

247 may have introduced biases in the estimates of relative abundance. We assessed the effects of
248 the watering treatment, sampling date and their interaction on the AM fungal composition
249 with permutational multivariate analysis of variance (PerMANOVA) using the ‘adonis’
250 function (‘vegan’). Multiple comparisons were performed using the ‘pairwise.adonis’
251 function (‘pairwiseAdonis’; Martinez Arbizu, 2017). We assessed significant indicator OTUs
252 (OTUs that are characteristic of an environment) using the ‘indval’ function (‘labdsv’,
253 Roberts, 2016). Indicator values (IV) range from 0 to 1, where highest values are associated
254 with stronger indicators of an environment. We also performed variation partitioning to
255 quantify the effect of spatial covariation relative to experimental manipulation of rainfall on
256 each date. We calculated Principal Coordinates of Neighbour Matrices (PCNM) using the
257 ‘pcnm’ function (‘vegan’) and selected significant PCNM axes using the ‘ordistep’ function
258 before performing variation partitioning to disentangle the role of the watering treatment and
259 spatial variables with the ‘varpart’ function (‘vegan’).

260 We assessed the effects of the watering treatment and sampling date on the plant composition
261 with PerMANOVA using the ‘adonis’ function (‘vegan’), based on ‘Bray-Curtis’ distances.
262 Before the analysis, plant abundances were square-root transformed to normalise the
263 differences in total biomass among the plant species. To assess the co-variation between the
264 composition of the plant and the AM fungal communities at each sampling date, we used
265 symmetric co-correspondence analyses (Co-CA; Ter Braak & Schaffers 2004). This method
266 quantifies the correlations between two species matrices by maximizing the covariance
267 between weighted average species scores of one community with those of the other
268 community. Square-root transformed data were used for the plant community and
269 presence/absence data were used for the AM fungal community. We then estimated the co-
270 variation between the communities using ‘coca’ (‘cocorresp’ package; Simpson, 2009),
271 specifying the ‘symmetric’ method, retaining the first four axes. The significance of the

272 model fit was based on permutation tests and we obtained the axis correlations using
273 'corAxis' ('cocorresp'). We used the plots to observe the influence of the watering treatment
274 on the co-association between communities at each sampling date. Axes of the dual plots are
275 optimized to maximize covariance between communities but can still be interpreted using
276 centroid and biplot rules (Ter Braak & Schaffers, 2004).

277 To evaluate the extent to which, on each sampling date, variation in each of the AM fungal
278 and plant matrices could be used to *predict* variation of the other, we used the predictive Co-
279 CA (Ter Braak & Schaffers, 2004). This method differs from the symmetric Co-CA because
280 it is a regression method, equivalent to canonical correspondence analysis, but uses the
281 species composition matrix as explanatory variables. Using the plant community as the
282 predicting matrix will test the *Passenger hypothesis*, while using the AM fungal community
283 as the predicting matrix will test the *Driver hypothesis*. We used 'coca', specifying the
284 'predictive' method ('cocorresp') to fit the model. To assess the prediction accuracy, we used
285 the 'leave-one-out' cross-validation procedure with 'crossval' ('cocorresp'). This consists of
286 a partial least squares regression carried out as many times as there were plots, excluding one
287 plot for each regression and using the parameters of the model to predict the species
288 composition of the excluded plot. The predicted species composition is then compared to the
289 observed data to obtain the prediction fit.

290 We then tested whether AM fungal richness was a significant predictor of plant richness and
291 diversity. We estimated plant diversity using Shannon index with 'diversity' ('vegan'). We
292 fitted a linear-mixed effect model testing the effect of the AM fungal expected richness and
293 the sampling date as well as their interaction with 'lmer' ('lme4'). The sequencing batch was
294 included as a random effect to account for differences in the composition data between
295 samples collected in 2014-2015 and 2016-2017.

296

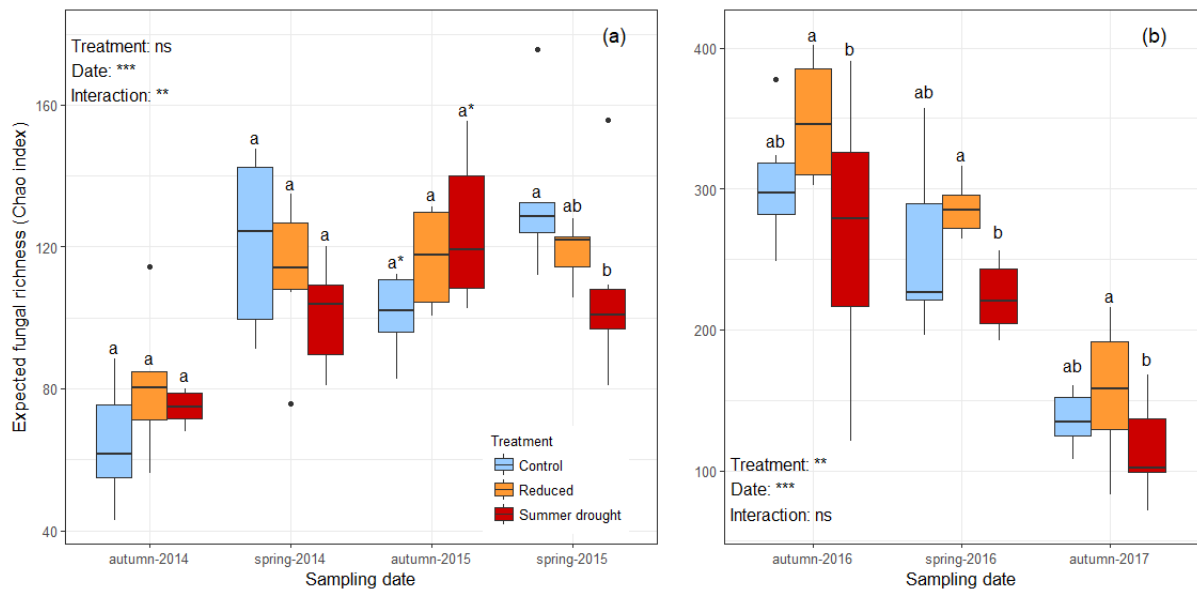
297 **Results**

298 **AM fungal community responses to altered rainfall regimes take time to appear**

299 We obtained 6,986,086 forward reads paired with reverse reads before any quality control,
300 including 3,396,239 reads from the first batch (2014-2015) and 3,589,847 from the second
301 batch (2016-2017). Initial quality filtering excluded 2,699,326 reads and removal of putative
302 chimeras and singletons removed a further 2,472,929 reads (resulting in 33% and 20% of the
303 first and second batch passing filtering steps, respectively). Following these steps, we were
304 left with 666,651 reads for the control treatment (28 % passing filtering steps), 645,825 for
305 the reduced treatment (27 % passing filtering steps) and 501,355 for the summer drought
306 treatment (23 % passing filtering steps).

307 Between 2014 and 2015, we found that the watering treatment affected AM fungal richness
308 differently on each sampling date (linear mixed-effects model_{treatment by date interaction}; $P < 0.01$)
309 and that there was no significant main effect of the watering treatment average across the
310 dates (linear mixed-effects model_{treatment}; $P = 0.68$). This was due to a marginally
311 nonsignificant effect of the watering treatment in autumn 2015 (multiple comparisons; $P =$
312 0.07), and in spring 2015 AM fungal richness differed marginally significantly between
313 control and summer drought treatments (multiple comparisons; $P = 0.04$; Fig. 1a). However,
314 the watering treatments had a consistent effect on AM fungal richness for samples collected
315 in 2016 and 2017 (linear mixed-effects model_{treatment}; $P < 0.01$; linear mixed-effects model_{treatment by date interaction}; $P = 0.92$; Fig. 1b). Soil water content, on average, was reduced in
316 autumn 2016 but not in spring 2016 nor autumn 2017 (Table S2), so consistent watering
317 treatment effects on AM fungi observed at these later dates do not correspond to a reduction
318 in soil water content compared to 2014 and 2015. We observed that fungal richness was

320 higher in the reduced watering treatment compared with the summer drought treatments
 321 (multiple comparisons; $P < 0.01$, Fig. 1b). The fungal richness varied depending on the
 322 sampling date between 2014 and 2015 (linear mixed-effects model_{date}; $P < 0.01$; Fig. 1a) and
 323 between 2016 and 2017 (linear mixed-effects model_{date}; $P < 0.01$; Fig. 1b).



324

325 Figure 1. Estimated (Chao) richness of the AM fungal communities according to the watering
 326 treatment at each sampling date. Letters indicate mean contrasts using a multiple comparison
 327 test and differences are significant at $P < 0.05$. Asterisks following the letters in panel (a)
 328 show marginally non-significant differences ($P = 0.07$) between the *control* and the *summer*
 329 *drought* treatment. Multiple comparisons between watering treatments were performed within
 330 each sampling date in panel (a) because between 2014 and 2015 we found an interaction
 331 between sampling date and watering treatment, while multiple comparisons were performed
 332 on the main effect of watering treatment in panel (b) because we found no interaction
 333 between watering treatment and sampling date for data collected between 2016 and 2017.

334

335 Glomeraceae was the most frequently observed AM fungal taxon at every sampling date, and
336 within each date, the abundance of Claroideoglomeraceae and Diversisporaceae appeared to
337 vary the most between watering treatments (Fig. S3). We identified specific OTUs associated
338 with the watering treatment and we found 24 significant OTUs which had an indicator value
339 > 0.5 . The majority of these OTUs belonged to the Glomeraceae, with indicator OTUs
340 belonging to Claroideoglomeraceae and Gigasporaceae observed mostly at later dates (in
341 2016 and 2017, Table S3).

342 We tested the compositional data of AM fungi from each sampling run separately as i)
343 sampling run effect was very large and obscured all other patterns (Fig. S1 and S2) and ii) it
344 was not possible to account for this effect using random effects in these analyses. Between
345 2014 and 2015, we found that the community composition varied according to the sampling
346 date ($R^2 = 0.08$, $P < 0.01$, Table 2). We observed no effect of the watering treatment on the
347 community composition ($R^2 = 0.03$, $P = 0.09$) and, in the ordination, we observed a trend
348 towards differentiation by treatment but also substantial overlap along both axes, indicating
349 that the watering treatment had, at most, a weak effect on the structuring of AM fungal
350 communities (Fig. 2a). We found no evidence that the watering treatments influenced AM
351 fungal communities differently depending on the sampling date ($P = 0.99$). Treatment effects
352 were stronger for samples collected in 2016 and 2017. For these dates, we found a small but
353 significant effect of the watering treatment ($R^2 = 0.05$, $P < 0.01$, Table 2) and sampling date
354 ($R^2 = 0.08$, $P < 0.01$) on the community composition but no significant interaction between
355 the watering treatment and the sampling date ($P = 0.99$). We observed that between 2016 and
356 2017, the watering treatments formed distinctive communities (Fig. 2b), with the largest
357 difference between the control and the summer drought treatment (multiple comparisons; R^2
358 $= 0.05$, $P < 0.01$, in Fig. 2b) followed by the control and reduced treatment, which was
359 marginally non-significant (multiple comparisons; $R^2 = 0.04$, $P = 0.09$, in Fig. 2b). Watering

360 treatment effects observed at these later dates do not correspond to a reduction in soil water
 361 content at these dates compared to 2014 and 2015 (Table S2). Variance explained by
 362 watering treatment was small overall, never exceeding 3% on any date, and was highest in
 363 autumn 2017 (Table S4). In addition, the proportion of variation explained by PCNM axes
 364 tended to be lower in later years than in earlier years (Table S4).

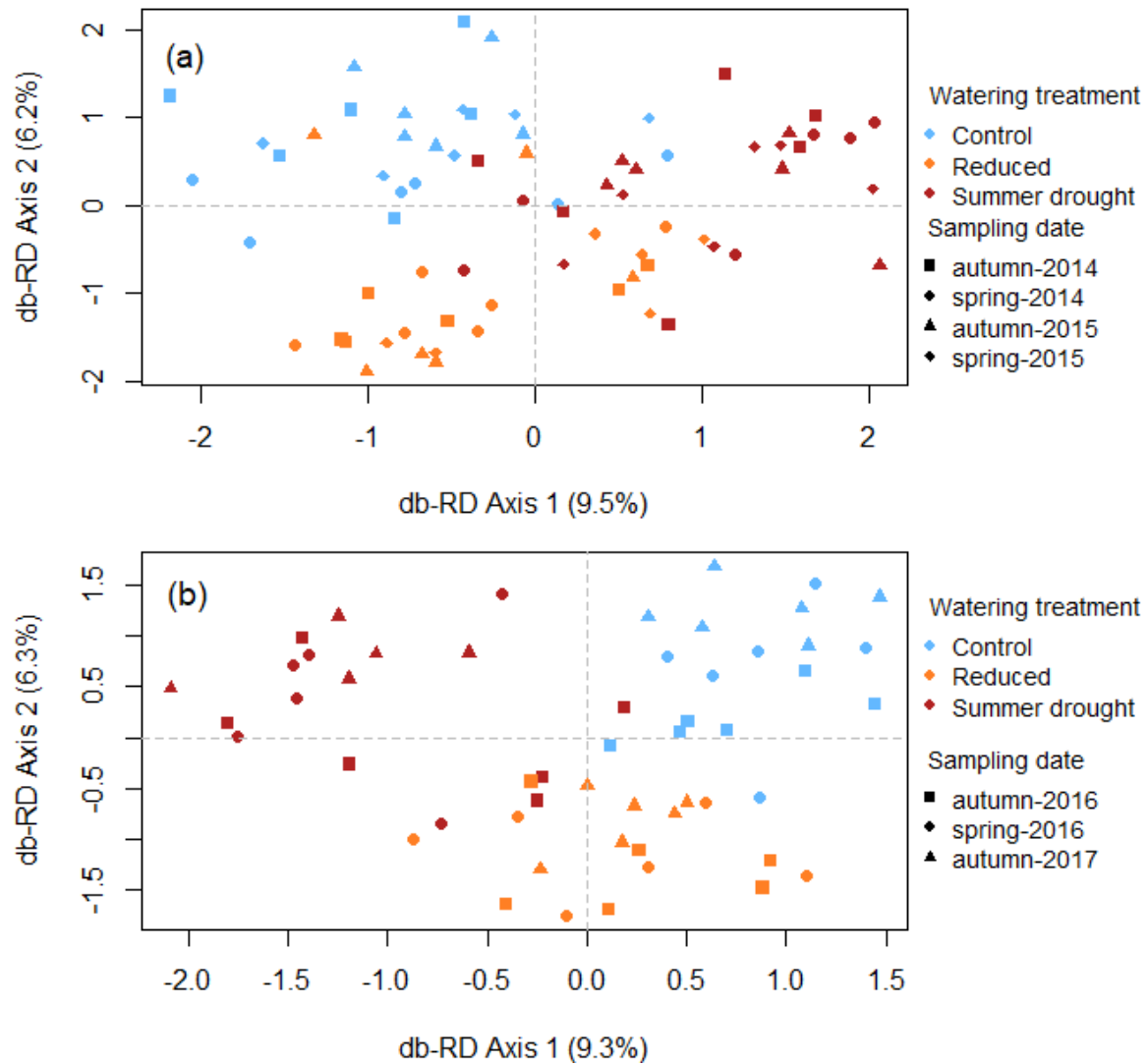
365

366

367 Table 2. PerMANOVA results when testing the effect of the watering treatment, sampling
 368 date as well as their interaction on the AM fungal community richness for samples collected
 369 in 2014-2015 and 2016-2017.

Effect	2014-2015			2016-2017		
	<i>F</i> -value	R ²	<i>P</i> -value	<i>F</i> -value	R ²	<i>P</i> -value
Watering treatment	1.19	0.03	0.09	1.38	0.05	<0.01
Sampling date	1.85	0.08	<0.01	2.05	0.08	<0.01
Watering treatment x sampling date	0.76	-	0.99	0.79	-	0.99

370



371

372

373 Figure 2. Distance-based redundancy analysis (db-RDA) ordination of AM fungal

374 communities sampled between 2014 and 2015 (a) and between 2016 and 2017 (b),

375 constrained by watering treatment.

376

377 **Plant-AM fungal community co-associations are not influenced by altered rainfall**

378 **regimes**

379 Plant communities were also responsive to watering treatments (PerMANOVA; $F_{2,98} = 3.8$,
380 $R^2 = 0.05$, $P < 0.01$; Fig. S4) and differed between sampling dates (PerMANOVA; $F_{5,98} =$
381 6.4 , $R^2 = 0.05$, $P < 0.01$). To determine whether watering treatments were influencing co-
382 associations between plant and fungal communities, we evaluated the co-variation among
383 species from the plant and the AM fungal communities at each sampling date for which we
384 had assessed both trophic groups. Co-variation fit varied between 26.7 % and 31.4 %
385 according to the sampling date, with the strongest co-variation observed in spring 2014
386 (Table 3). The co-variation between plants and AM fungi was marginally non-significant at
387 the first sampling date (autumn 2014, $P = 0.07$) and was non-significant in autumn 2017 ($P =$
388 0.87).

389 We expected that the co-associations between the plant and the AM fungal communities
390 would be influenced by the watering treatment. At the different sampling dates, we observed
391 some separation in the sample loadings along the first axes but we observed that overall the
392 species loadings were not strongly associated with particular plots (but a few exceptions are
393 described below) (Fig. 3). Loadings associated with the samples from each watering
394 treatment overlapped (Fig. 3), suggesting that the co-associations did not differ depending on
395 the watering treatments. In addition, we observed that indicator OTUs were generally not
396 among those with the strongest loadings on either axis (Fig. 3), suggesting that OTUs
397 strongly associated to a watering treatment were not strongly co-associated with a particular
398 plant.

399 On some of the sampling dates, individual plots and species were identified as extreme
400 outliers, suggesting stronger co-associations between particular plant species and AM fungal
401 OTUs. The association between plant and AM fungal communities in one of the summer
402 drought treatment plots differed in relation to the other sampling plots in autumn 2015 (along
403 the first axis, Fig. 3e, f) and in autumn 2016 (along the second axis, Fig. 3g, h), not in autumn

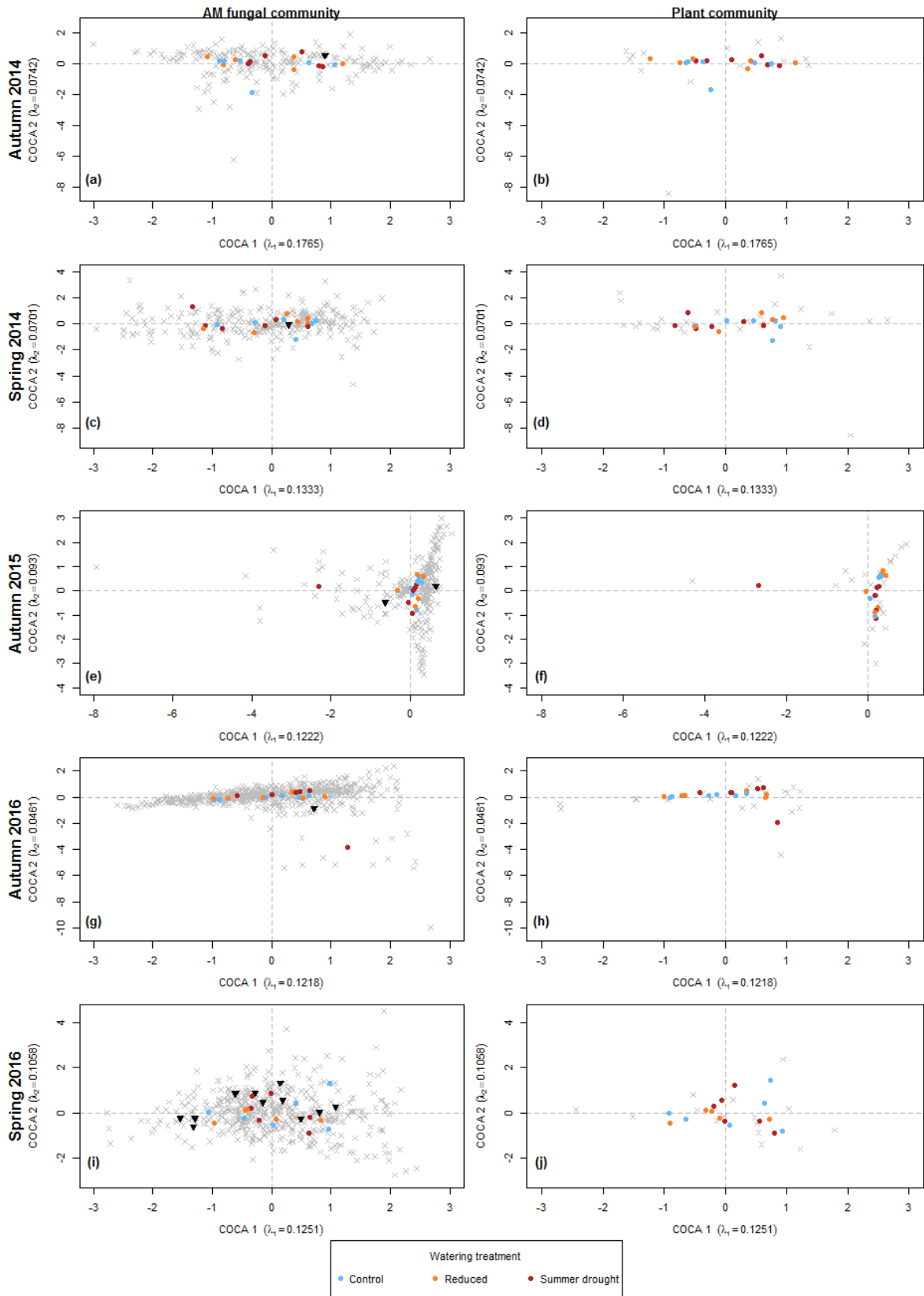
404 2014 (Fig. 3a, b). *Sida rhombifolia* was the most abundant plant species in this divergent plot
 405 in autumn 2015 (strongly loaded along first axis; Fig. 3e, f), and 2016 (strongly loaded along
 406 the second axis; Fig. 3g, h), and was absent in other plots (except in autumn 2017, when we
 407 observed no significant co-association). We observed that nine OTUs associated with *S.*
 408 *rhombifolia* in autumn 2015 were also associated with that plant in autumn 2016 (Table S5).
 409 *Commelina cyanea* also formed distinctive associations with AM fungal OTUs in 2014
 410 (species outlier along the second axes in Fig 3a, b and 3c, d) and 2015 (along the second axis
 411 in Fig. 3e, f). We observed that four OTUs associated with *C. cyanea* in autumn 2014 were
 412 also associated with that plant in spring 2014, but associated OTUs in 2015 were different
 413 from the previous sampling dates (Table S6). This plant species was also present in autumn
 414 2016 but was positioned near the origin in the dual plot, suggesting less strong associations
 415 with particular AM fungal OTUs at that sampling date (Fig. 3g, h).

416 Table 3. Co-variation between plant and AM fungal communities at each sampling date. This
 417 table shows the correlation coefficients between plant-derived and AM fungi-derived site
 418 scores of the first four axes, the percentage of fit and associated p-value depicting the inertia
 419 in the AM fungal community associated with variability in the plant community based on the
 420 four first axes.

Sampling date	Correlation between axes				Percentage of fit	P-value
	1	2	3	4		
Autumn 2014	0.98	0.97	0.97	0.98	26.7	0.07
Spring 2014	0.96	0.96	0.94	0.94	31.4	< 0.01
Autumn 2015	0.99	0.98	0.97	0.98	28.5	0.02
Autumn 2016	0.95	0.99	0.97	0.96	28.9	< 0.01
Spring 2016	0.97	0.98	0.99	0.95	27.2	0.03
Autumn 2017	-	-	-	-	24.1	0.87

421

422



423

424

425 Figure 3. Co-associations among species from the AM fungi and plant communities. Dual
426 plots resulting from the symmetric CO-CA showing species (crosses) and sample (points)
427 weighted average scores for the AM fungal community (left panel) and the plant community
428 (right panel), and indicator OTUs characteristic of a watering treatment (triangles on left
429 panels). AM fungi and plant species in corresponding positions with respect to the origin in
430 the dual plots are positively associated. Stronger co-associations between species are found
431 the further they are from the origin and the sample scores show proximity to the species they
432 are likely to contain. Dual plots showing co-associations in autumn 2017 were not included
433 because co-variation between communities was non-significant at that date.

434

435 **AM fungal communities predict plant community composition**

436 We hypothesized that the co-variations between those two communities were the result of
437 one community influencing the composition of the other. Using predictive co-correspondence
438 analysis on all dates except autumn 2017 (no significant co-association), we found stronger
439 evidence for AM fungal community composition as a predictor of plant community
440 composition than as a response to plant community composition. In the latter case, only 1.2%
441 of variation was predicted in spring 2014 and all other dates had negative estimates,
442 indicating poor cross-validators fits (Table 4). The predictive power of the AM fungal
443 community varied among sampling dates: 18.5% of the variation was predicted in the plant
444 community at the first sampling date (Table 4). Prediction levels were between 7.7% and
445 11.2% on further dates, with negative estimates only observed in spring 2016 (Table 4).

446

447 Table 4. Prediction levels of the composition of one community on the composition of the
 448 other community. Table show sampling date, prediction levels (based on cross-validators fit;
 449 values above 0 validates the model) and the number of axes to obtain maximum prediction
 450 level. Prediction levels in autumn 2017 were not included because co-variation between
 451 communities was non-significant at that date.

Sampling date	AM fungi predicting plants		Plants predicting AM fungi	
	Axes	Prediction level (%)	Axes	Prediction level (%)
Autumn 2014	1	18.5	-	-1.5
Spring 2014	7	8.8	1	1.2
Autumn 2015	4	7.7	-	-0.3
Autumn 2016	2	11.2	-	-2.5
Spring 2016	-	-1.3	-	-3.5

452

453

454 While we observed the above associations between AM fungal and plant community
 455 composition, we found no evidence for relationships between AM fungal and plant diversity.
 456 This was the case when looking at main effects, with no effect of fungal richness on plant
 457 richness (linear mixed-effect model; $P = 0.99$) or plant diversity (linear mixed-effect model;
 458 $P = 0.59$) observed. In addition, plant richness (linear mixed-effect model; $P_{\text{richness by date}}$
 459 $\text{interaction} = 0.29$) or plant diversity (linear mixed-effect model; $P_{\text{richness by date interaction}} = 0.35$)
 460 was not observed to be influenced by the fungal richness depending on the sampling date
 461 (Fig. S5).

462

463 Discussion

464 **AM fungal community responses to altered rainfall regimes take time to appear**

465 We found that altered rainfall regimes affected AM fungal community composition and
466 richness, and that the AM fungal community response depended on the watering treatment
467 applied to the grassland plots. Three years after rainfall manipulation began, *summer drought*
468 and *reduced* treatments resulted in distinct communities differing in richness and
469 composition. Differences in community responses to changes in rainfall may be due to
470 varying intensity and timing of water limitation. Rainfall exclusion in summer resulted in a
471 larger shift in composition, possibly because *summer drought* simulated an intense drought
472 associated with warmer temperatures and resulted in more extreme stress. In addition,
473 summer is generally the wettest season at our site (Power et al., 2016); therefore, some AM
474 fungal species found at this site may be poorly adapted to water exclusion in the summer.
475 Altogether, these results indicate that AM fungal responses to climate change will depend on
476 the specific future rainfall conditions that arise.

477 Once AM fungal communities responded to the *summer drought* treatment, the response
478 persisted throughout the year. We found that AM fungal composition still differed from
479 *ambient* treatment in spring, six to seven months after water limitation is removed. Others
480 have found that summer drought applied for seven years affected AM fungal abundance in
481 roots and soil, and that these changes also persisted throughout the year (Staddon et al.,
482 2003). This suggests that particularly longer dry periods, as projected with climate change,
483 will result in shifts in AM fungal communities that are not expected to reverse after rainfall
484 events occur in autumn or winter.

485 The response of AM fungal communities to altered rainfall regimes varied over time. In a
486 study using a temporal approach to observe fungal responses to changes in rainfall, Hawkes
487 *et al.* (2011) found that the changes in fungal communities were rapid and reversible. In
488 contrast, we observed a consistent response of the AM fungal community three years after
489 rainfall manipulation began. AM fungal communities at our site are exposed to low annual

490 rainfall amount and high inter- and intra-annual rainfall variability (Power et al., 2016),
491 possibly shaping their response to changes in rainfall regimes. AM fungal species at our site
492 may possess adaptive traits to survive those highly variable rainfall patterns for multiple
493 years. In addition, the delayed response of the AM fungal community may also be a result of
494 frequent fungal dispersal among plots. At our experimental site, sampling plots are 2 m
495 distance minimum from each other, it is therefore possible that the AM fungi were able to
496 disperse to nearby plots regardless of their watering treatment. We did observe a spatial effect
497 on the AM fungal community composition, which became weaker at later sampling dates
498 suggesting that the mixing of the communities may have masked the effect of altered rainfall
499 during the first years of the experiment. Alternatively, it may have been that spatial variation
500 decreased as environmental filtering due to rainfall manipulation became more important in
501 later years.

502

503 **AM fungal community composition as a putative driver of plant community variation**

504 Under the *Passenger* or *Driver hypotheses* (Hart et al., 2001), it is assumed that changes in
505 the composition of an AM fungal community responds to or leads to, respectively, changes in
506 the plant community. Alternatively, Zobel & Öpik (2014) hypothesised that AM fungal and
507 plant communities may co-vary as a result of an environmental gradient and not because both
508 communities were casually linked (*Habitat hypothesis*). Overall, our results support the
509 *Driver hypothesis* playing a larger role in this system. While we found no evidence that the
510 richness of the AM fungal community influenced the richness and diversity of the plants, AM
511 fungal community composition was a significant predictor of plant community composition.
512 In addition, we found that the plant community composition was a very poor predictor of the
513 AM fungal community composition, indicating that AM fungi were not acting as passengers

514 at our site. Therefore, plant community assembly at our site may be driven in part by factors
515 influencing AM fungal communities, rather than by factors affecting only the vegetation.

516 Others have also found relationships between plants and AM fungal community assembly.
517 Davison et al. (2011) found that AM fungal composition differed according to plant
518 functional group but not plant species, and López-García et al. (2017) showed that plant traits
519 influence species composition and phylogenetic structure of the AM fungal community.

520 Although these studies show that host plants influence AM fungal community assembly, the
521 authors did not evaluate the influence of the plant community structure (but a few selected
522 plant species), nor the role of the AM fungi in determining the plant community structure.

523 Another study evaluating relationships between communities found that plant composition
524 influenced AM fungal community composition in soil but not vice versa, and that this effect
525 was weak after accounting for spatial effects (Horn, Hempel, Verbruggen, Rillig, & Caruso,
526 2017). However, this study used indirect approaches to estimate the strength and significance
527 of relationships, which are accurate only if the major pattern of variation in the predictor
528 community is also important for the response community (Ter Braak & Schaffers, 2004). In
529 contrast, in a field succession experiment, García de León et al. (2016) found a strong
530 correlation between AM fungal and plant community composition, and suggested that the
531 fungal community was driving the plant community because of the fungal pool present before
532 plants' establishment. Re-analysing these data using co-correspondence analysis has the
533 potential to determine in a direct way the direction of these relationships in field experiments
534 and improve our understanding on associations between plants and AM fungal communities.

535 However, our evidence in support of the *Driver hypothesis* is correlational in nature and it is
536 possible that biases may exist with the statistical approach that we used. For instance, the
537 levels of prediction of a community over the composition of the other community obtained in
538 this study may be due in part to differences in diversity and resolution between plant and AM

539 fungal communities. Lack of balance between communities in terms of species numbers may
540 influence prediction levels because co-correspondence analysis uses one set of variables from
541 a species matrix to predict the other set of variables in another species matrix. Assessing the
542 likelihood of this and other biases requires further evaluation of co-correspondence analysis
543 in different scenarios using, for example, a simulation approach (Caruso, Powell, & Rillig,
544 2012). To confirm or deny patterns detected from co-correspondence, an experimental
545 approach using microcosms such as van der Heijden *et al.* (1998) may ascertain whether a
546 changed AM fungal community will affect the vegetation in return. In addition, this approach
547 can also provide mechanistic insight, to understand how changes in the AM fungal
548 community composition and richness affect plant nutrition, productivity and tolerance to
549 water stress under different rainfall conditions.

550

551 **Plant-fungal co-associations and responses to rainfall are decoupled in this system**

552 Whether changes in AM fungal composition resulting from altered rainfall will result in
553 changes in the vegetation at our site remains uncertain. We did find that AM fungal
554 communities responded to altered rainfall regimes and that their composition predicted plant
555 composition. However, if altered rainfall patterns were influencing the plant community via
556 shifts in AM fungal communities, we would expect altered rainfall regimes to lead to
557 differing co-associations between AM fungal and plant communities. Our results do not show
558 this. We found that co-associations between plants and AM fungal species were not strongly
559 associated with any particular watering treatments. Rather, plots were likely to contain co-
560 associations observed in other plots as well, regardless of their watering treatment. In the few
561 cases where we did observe stronger co-associations in certain sampling plots, these were

562 related to the presence of a particular plant species in a single plot rather than being observed
563 in several plots exposed to the same watering treatment.

564 At our site, despite the observed relationships between communities, those AM fungal taxa
565 that responded to changes in rainfall may not be the same as those taxa determining outcomes
566 of plant community assembly, meaning that processes around changing rainfall regimes and
567 co-associations between plants and fungi are decoupled. We found a few fungal OTUs
568 characteristic of a watering treatment at each sampling date but none of these indicator OTUs
569 were strongly co-associated with a particular plant. It is possible that OTUs responding to
570 changes in rainfall may not provide strong benefits to plants under low soil moisture and that
571 plants are therefore less susceptible to the loss of these OTUs. Plant communities may also be
572 more responsive to shifts in AM fungal communities and their associated services as a result
573 of altered rainfall patterns in systems more limited by precipitation or in environments with
574 less rainfall variability, where water limitation is more consistent.

575 Co-associations between plant and fungal communities were less strong or even absent once
576 treatment effects on AM fungal communities were observed. It is possible that shifts in the
577 AM fungal community resulting from altered rainfall at later dates disrupted the co-
578 associations between communities. Both this observation and the delay in detecting effects of
579 rainfall manipulation on AM fungal communities highlight the importance of a long-term,
580 temporal approach to study community responses to climate change. Experiments using soils
581 collected at different times may contribute to study temporal dynamics of co-associations
582 between plants and AM fungal communities. Future studies could inoculate microcosms with
583 soils collected at different timepoints after starting rainfall manipulation to evaluate both the
584 resilience of these fungal communities when exposed to different watering treatments, and
585 the strength of their co-associations with the plant community depending on the amount of
586 time AM fungi were exposed to environmental changes.

587

588 **Conclusions**

589 In conclusion, AM fungal communities will respond to changes in rainfall regimes projected
590 with climate change. Shifts in the AM fungal communities may take time to appear and the
591 direction in which these communities are expected to respond to altered rainfall regimes will
592 depend on the precise nature of future climatic conditions. In addition, we found support for
593 the hypothesis that fungal community composition influenced plant community composition.
594 However, at our site, our results suggest that plant responses to climate change may not be
595 directly related to impacts of altered rainfall regimes on AM fungal communities.

596

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609

610 **Author contribution**

611 CD, AEB, SAP and JRP designed the study. CD and JRP performed statistical analyses,
612 interpreted results and wrote the manuscript. All authors collected and processed samples,
613 and revised this manuscript.

614 **Conflict of interest**

615 The authors have no conflict of interest to declare.

616 **Data availability**

617 All data have been archived in Figshare (<https://doi.org/10.6084/m9.figshare.8869586.v1>),
618 and scripts and data can be accessed at
619 https://bitbucket.org/Coline_Dev/coassociations_amfplants. Raw DNA sequencing data are
620 available under NCBI BioProject PRJNA551082.

621

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