

Physiological responses to drought stress and recovery reflect differences in leaf function and microanatomy among grass lineages

Seton Bachle¹, Marissa Zaricor¹, Daniel Griffith², Fan Qiu¹, Chris Still², Mark C. Ungerer¹, and Jesse Nippert¹

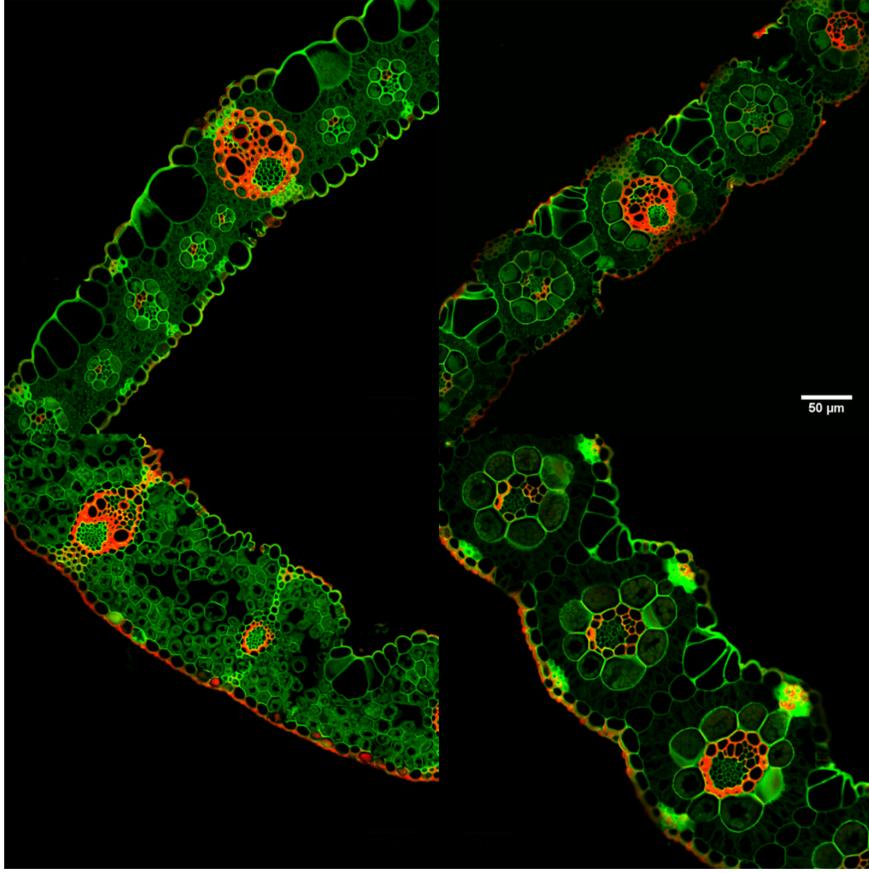
¹Kansas State University

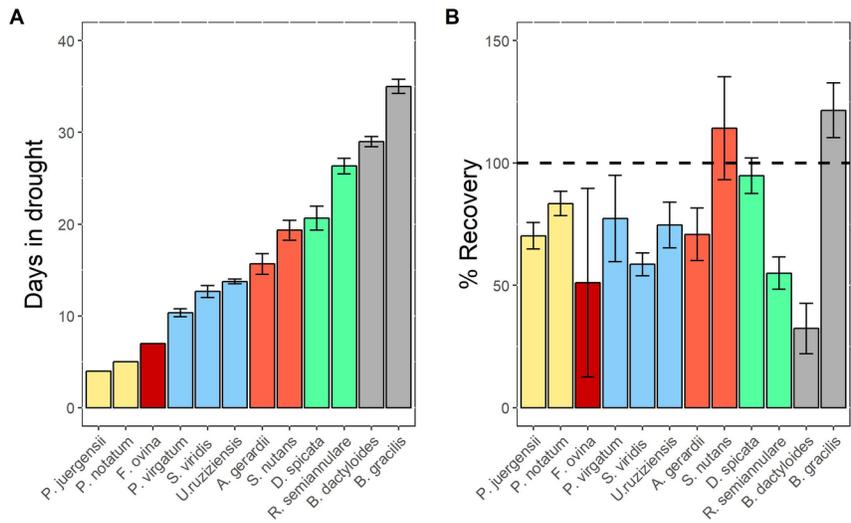
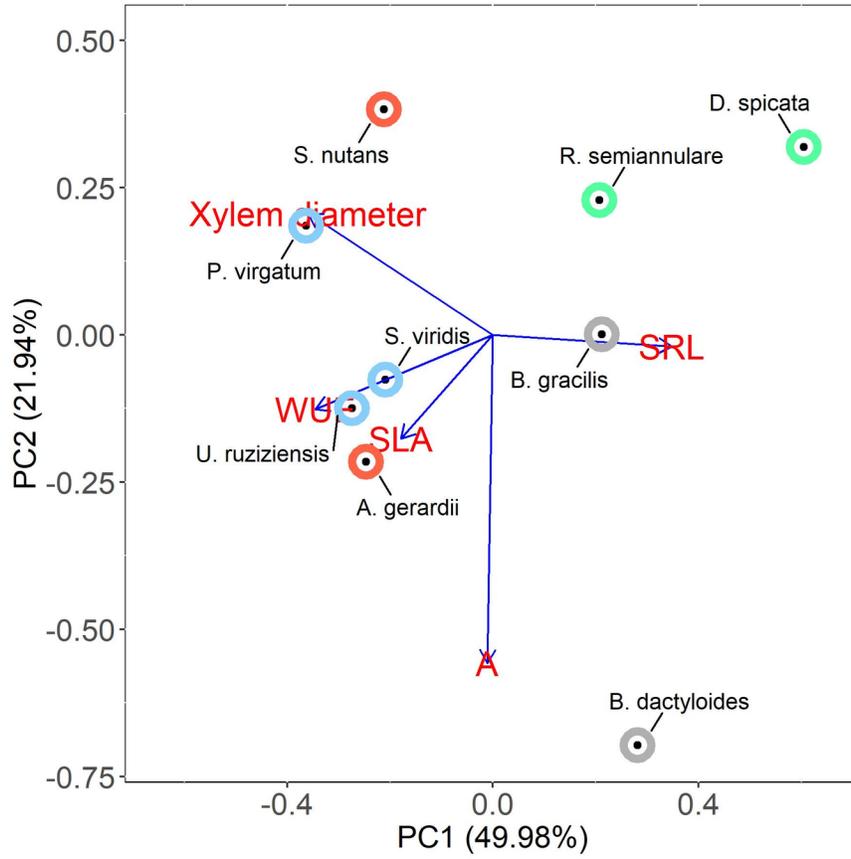
²Oregon State University

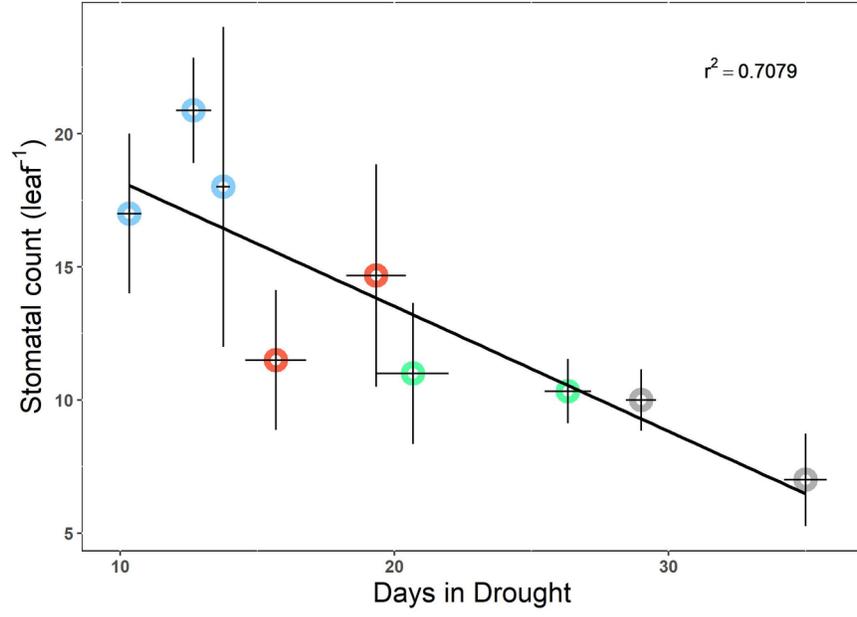
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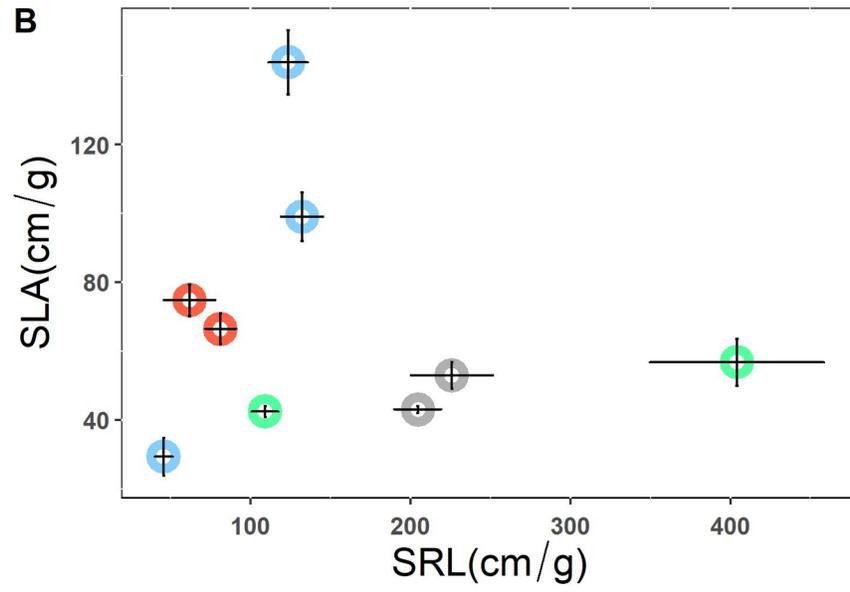
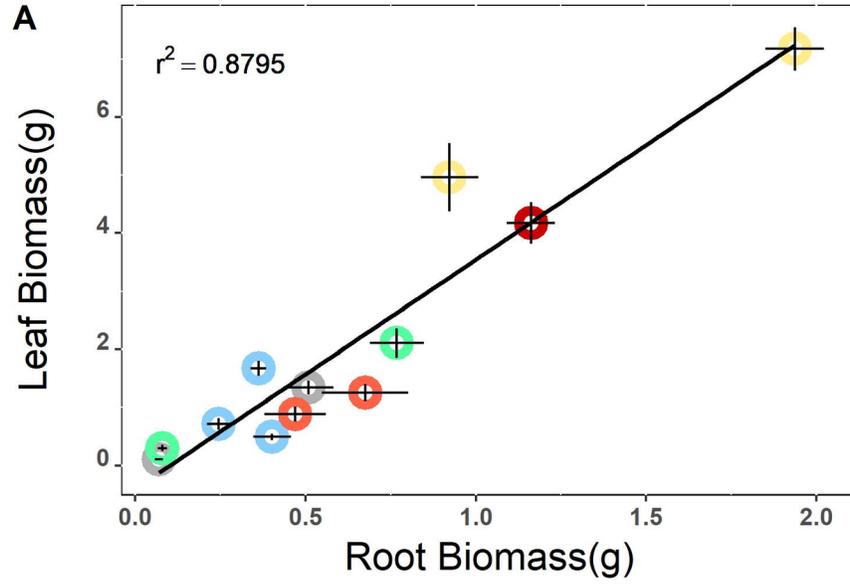
Abstract

Grasses are cosmopolitan, existing in many biome and climate types from xeric to tropical. Traits that control physiological responses to drought vary strongly among grass lineages, suggesting that tolerance strategies may differ with evolutionary history. Here, we withheld water from 12 species representing 6 tribes of grasses to compare how tolerant and intolerant species respond to drought in different grass lineages. We measured physiological, morphological, and microanatomical traits. Dominant lineages from tropical savannas, like Andropogoneae, tolerated drought due to above and belowground morphological traits, while temperate grasses utilized conservative leaf physiology (gas exchange) and microanatomy. Increased intrinsic water-use efficiency (iWUE) coincided with a larger number of stomata, resulting in greater water loss (with inherently greater carbon gain) and increased drought sensitivity. Inherent leaf and root economic strategies impacting drought response were observed in all species, resulting in either high SLA or SRL, but not both. Our results indicate that grasses subjected to severe drought were influenced by microanatomical traits (e.g., number of stomata and xylem area) which were shared within lineages. In addition, grasses recovered at least 50% of physiological functioning across all lineages and 92% within Andropogoneae species, illustrating how drought can influence functional responses across diverse grass lineages.









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4 **Authors:** Seton Bachle^{1,2,*}, Marissa Zaricor^{1,3}, Daniel Griffith⁴, Fan Qui¹, Christopher J Still⁴,
5 Mark C Ungerer¹, Jesse B Nippert¹

6

7 ¹ *Division of Biology, Kansas State University, Manhattan KS, 66506, USA*

8 ² *Department of Forest and Rangeland Stewardship, Colorado State University, Fort Collins CO,*
9 *80523, USA*

10 ³ *Division of Biological Sciences, University of Missouri, Columbia MO, 65211, USA*

11 ⁴ *Department of Forest Ecosystem and Society, Oregon State University, Corvallis OR, 97331,*
12 *USA*

13

14 ***Corresponding Author:** Email (sbachle@colostate.edu)

15

16 **ABSTRACT**

17 Grasses are cosmopolitan, existing in many biome and climate types from xeric to tropical. Traits
18 that control physiological responses to drought vary strongly among grass lineages, suggesting
19 that tolerance strategies may differ with evolutionary history. Here, we withheld water from 12
20 species representing 6 tribes of grasses to compare how tolerant and intolerant species respond to
21 drought in different grass lineages. We measured physiological, morphological, and
22 microanatomical traits. Dominant lineages from tropical savannas, like Andropogoneae, tolerated
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25 efficiency (*iWUE*) coincided with a larger number of stomata, resulting in greater water loss
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27 economic strategies impacting drought response were observed in all species, resulting in either
28 high *SLA* or *SRL*, but not both. Our results indicate that grasses subjected to severe drought were
29 influenced by microanatomical traits (e.g., number of stomata and xylem area) which were
30 shared within lineages. In addition, grasses recovered at least 50% of physiological functioning
31 across all lineages and 92% within Andropogoneae species, illustrating how drought can
32 influence functional responses across diverse grass lineages.

33

34 **Key words:** Poaceae, Drought Response, Phylogeny, Plant Functional Traits, Microanatomy,
35 Ecophysiology, Leaf Economic Spectrum.

36 INTRODUCTION

37 Grasslands play a major role in regional carbon sequestration and water cycling because
38 grasses invest in extensive rooting systems and storage organs (Pendall *et al.* 2018; Veldman *et*
39 *al.* 2019). Carbon dynamics are highly influenced by water availability in grassland systems,
40 evident in drought years that result in decreased productivity (Fay, Carlisle, Knapp, Blair &
41 Collins 2003; Hoover & Rogers 2016; Carroll *et al.* 2021). Grasslands experiencing extreme
42 droughts can have reduced physiological functioning (Cook, Ault & Smerdon 2015; Hoover,
43 Duniway & Belnap 2015), increased invasibility from non-native species (Linder, Lehmann,
44 Archibald, Osborne & Richardson 2018), disruption of fire intervals (Wilcox *et al.* 2020), and
45 loss of ecosystem functioning (i.e. productivity & species composition) (Eters, Tarks &
46 Hernandez 2014; Mainali *et al.* 2014; Knapp *et al.* 2020). While many grass species in grassland
47 ecosystems have evolved in the context of an inherently variable climate, future climate
48 projections emphasize large shifts in water availability, resulting in extreme drought and deluge
49 events within the coming century (Stocker *et al.* 2013; Cook *et al.* 2015; Griffin-Nolan *et al.*
50 2019; Post & Knapp 2019; Knapp *et al.* 2020). While it is widely accepted that grasslands will
51 vary in drought response (ability to withstand shifts from equilibrium) and drought recovery
52 (ability to regain equilibrium), modifications in precipitation seasonality and amount will have
53 sizable and diverse impacts on ecosystem function (Hoover, Knapp & Smith 2014; Volaire 2018;
54 Maurer, Hallmark, Brown, Sala & Collins 2020).

55 Biophysical factors determining drought sensitivity in individual plant species include
56 precipitation and temperature variability (Knapp *et al.* 2015), while biotic factors such as plant
57 productivity, species richness (Burri, Niklaus, Grassow, Buchmann & Kahmen 2018), and
58 potentially dominant species with associated functional traits, also play an important role (Avolio
59 *et al.* 2019). Furthermore, the history of different drought exposure in plant lineages is likely to
60 frame future drought responses within those lineages. For example, lineages of plant species
61 from arid and semi-arid regions have functional traits (narrow leaves, strict stomatal regulation,
62 absorptive rooting systems) that allow them to acquire and conserve water (Ocheltree *et al.*
63 2020), whereas lineages from tropical regions may have wider leaves and altered stomatal traits
64 that result in distinct water-use strategies (Liu *et al.* 2018; Buckley 2019). These evolutionary
65 tradeoffs have shaped functional differences across lineages and directly impact ecological
66 dynamics (Griffith *et al.* 2020). However, the extent of such evolutionary tradeoffs has not been

67 utilized to identify lineage-specific trait responses to extreme drought conditions. Even more
68 uncommon are investigations that combine physiology, microanatomy, morphology, and
69 structural data from grass species spanning several Poaceae tribes.

70 Large interannual variation in precipitation is a feature of many grassland ecosystems
71 and, in combination with CO₂ and temperature, has played a major role in the evolution and
72 biogeographic history of major grass lineages (Osborne 2008; Cleland *et al.* 2013; Cotton,
73 Cerling, Hoppe, Mosier & Still 2016). Importantly, the varying evolutionary histories of
74 grasslands have driven the evolution of different functional traits across the Poaceae phylogeny,
75 likely accounting for differences in drought responses (Ocheltree *et al.* 2020; Knapp *et al.* 2020).
76 For example, leaf-level microanatomical trait variation and convergent evolution has resulted in
77 spatially separated photosynthetic tissues allowing for C₄ photosynthesis, which is heavily
78 expressed in Poaceae, and provides a physiological advantage that increases carbon assimilation
79 while reducing water loss via stomatal regulation (Taylor *et al.* 2010; Zhou, Helliker, Huber,
80 Dicks & Akçay 2018). While it is recognized that C₄ species are not inherently more drought
81 tolerant than C₃ species (Ehleringer 2005; Nippert, Fay & Knapp 2007; Knapp *et al.* 2020), there
82 is evidence that increased *WUE* (water-use efficiency), inherent to C₄ species, can be
83 advantageous when water is limiting (Lambert, Baer & Gibson 2011; Kimball, Gremer, Angert,
84 Huxman & Venable 2012; Leakey *et al.* 2019). For example, native species in the arid American
85 southwest, have the ability to initially tolerate the negative consequences of drought by
86 maintaining physiological functioning for prolonged periods of time (Thomey, Collins, Friggens,
87 Brown & Pockman 2014; Skelton, West & Dawson 2015). The ability of some species to
88 maintain physiological functioning despite drying soils may be due to increased cuticle
89 thickness, decreased stomatal size and densities, less negative turgor loss point, and more
90 conservative growth strategies (specific leaf area, *SLA*; specific root length, *SRL*) (Reich 2014;
91 Habermann *et al.* 2019; Bertolino, Caine & Gray 2019; Ocheltree *et al.* 2020). Alternatively, the
92 production of cheaper leaves and roots (higher carbon to nitrogen ratio) and tight stomatal
93 regulation is associated with the ability to avoid desiccation and quickly recover once drought
94 breaks (Poorter, Niinemets, Poorter, Wright & Villar 2009; Lin *et al.* 2015; Garbowski *et al.*
95 2020). The ability to quickly resume pre-drought physiological function via rapid recovery may
96 or may not be associated with the ability to tolerate drought in the first place (Hoover *et al.* 2014;
97 Volaire 2018).

98 During the evolutionary development of Poaceae, separate lineages have evolved
99 different suites of traits, including fairly different water use strategies (Osborne 2008; Edwards,
100 Osborne, Stromberg & Smith 2010). For instance, the two most abundant monophyletic groups
101 of C₄ grasses - Andropogoneae (water spenders) and Chloridoideae (water savers) - vary in
102 water-use strategies because of distinct biogeographic histories (Taub 2000; Grass Phylogeny
103 Working II 2012; Griffith *et al.* 2020). Species in these lineages occupy warmer climates but
104 vary in global distribution as a function of precipitation availability: high in Andropogoneae and
105 low in Cynodonteae (Williams, Wilsey, Mcnaughton & Banyikwa 1998; Liu & Osborne 2015;
106 Lehmann *et al.* 2019). There are many characteristics impacting water-use and drought response
107 associated with this ecological sorting, and they include morphological, physiological, and
108 anatomical traits. Morphological strategies and traits associated with water relations include the
109 production of fine roots to increase water absorption (McCormack *et al.* 2015; Roumet *et al.*
110 2016; Iversen *et al.* 2017), leaf rolling to decrease irradiance (Cardoso, Pineda, Jiménez, Vergara
111 & Rao 2015), and variations in growth form (caespitose and rhizomatous) (Blair, Nippert &
112 Briggs 2014; Ott & Hartnett 2015). These traits are often related in terms of economics,
113 reflecting plant investment of carbon and nitrogen in both leaf and root structures (Pérez-
114 Harguindeguy *et al.* 2013). More specifically, these morphological traits are framed by
115 underlying structures at the microanatomical level in leaf and root tissues (John *et al.* 2017).
116 Microanatomical leaf traits within and across families in Poaceae also have been observed to
117 influence physiological responses most often associated with hydraulics (xylem area/diameter;
118 resistance to cavitation) (Hacke, Sperry, Pockman, Davis & McCulloh 2001; Bachle & Nippert
119 2018, 2021). However, the aforementioned physiological, morphological, and anatomical traits
120 may not convey equal benefits in drought response or recovery across and within Poaceae
121 lineages. For these reasons, it is increasingly important to understand how diverse lineages of
122 grass species that vary in climate niches and evolutionary histories will respond to extreme
123 drought conditions

124 Here, we conducted a robust assessment of physiological and anatomical traits from
125 multiple grass lineages in response to and following recovery from drought. The species under
126 investigation were selected based on divergent drought responses within lineages. We performed
127 a dry-down experiment to impose severe drought on 12 species of grasses across 6 tribes within
128 the Poaceae lineage. We withheld water in order to assess various physiological, morphological,

129 and microanatomical trait responses to drought, as well as (above and belowground) productivity
130 data, to capture both drought response and recovery. We hypothesized that: (1) species within
131 tribes will exhibit a similar response to drought sensitivity (duration in drought), based on similar
132 evolutionary histories and drought traits specific to withstanding long periods of low water
133 availability; (2) species within tribes will also exhibit similar responses in drought recovery,
134 based on shared evolutionary histories and functional traits that serve to quickly utilize resources
135 when available; and (3) leaf-level microanatomical traits would best describe species (within and
136 across tribes) response to, and recovery from drought due to the constraints of structures that
137 influence water transport and availability.

138

139 MATERIALS AND METHODS

140 Twelve grass species from six tribes were grown from seeds obtained from the USDA
141 Germplasm Resources Information Network or locally sourced from the Konza Prairie
142 Biological Station. Species include: *Paspalum juergensii*, *Paspalum notatum*, *Festuca ovina*,
143 *Panicum virgatum*, *Setaria viridis*, *Urochloa ruziziensis*, *Andropogon gerardii*, *Sorghastrum*
144 *nutans*, *Danthonia spicata*, *Rytidosperma semiannulare*, *Bouteloua dactyloides*, and *Bouteloua*
145 *gracilis* (accession information in Supplemental table 1). Species were selected to represent
146 different major lineages of the family *Poaceae* (Cynodonteae, Andropogoneae, Paniceae,
147 Danthoneae, Poeae, and Paspaleae), and included both C₃ (BEP and PACMAD clades) and a
148 range of C₄ species. In addition, we intentionally chose species (within the same tribe) that were
149 previously reported to have varying responses (tolerant and sensitive) to low soil moisture. Seeds
150 were germinated in 868.5 cm³ size pots with a mix of potting soil and general-purpose sand with
151 a ratio of 2:1 soil to sand and placed in a Kansas State University greenhouse under ambient
152 conditions and raised to maturity throughout 2016 – 2018. Each pot was inoculated with a
153 handful of Konza Prairie soil. After reaching maturity, the samples were subjected to 100%
154 water reduction (referred to ‘dry-down’), simulating an extreme drought, as previously described
155 (Qiu, Bachle, Nippert & Ungerer 2020b; Qiu *et al.* 2020a). During the dry-down, samples were
156 monitored daily and placed into categorical conditions based on their physiological state:
157 “Initial”, “Stressed”, and “Recovery”. Physiological leaf traits were monitored daily and
158 included: leaf-level net photosynthetic rates (A_n ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s ; mol m^{-2}
159 s^{-1}), transpiration (E ; $\text{mmol m}^{-2} \text{s}^{-1}$), and instantaneous water use efficiency ($iWUE$; A_n/E)

160 calculated as the ratio between A_n and E). Data was collected with a LI-6400 system (LiCOR
161 Biosciences Inc., Lincoln, NE, USA) equipped with an LED light source (light intensity
162 maintained at $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$) CO_2 concentration at $400 \mu\text{mol mol}^{-1}$, and relative humidity at
163 ambient levels (35-50%). Physiological states were determined by relative rates of A_n . The
164 condition: “Initial” was measured on Day 1 (first day of drought after being watered the previous
165 day) in order to avoid biased measurements from saturated soils. When samples reached near
166 stomatal closure and extremely low photosynthetic rates ($A_n < 25\%$) of Day 1 A_n (“Initial”), they
167 were categorized into the new condition “Stressed”. At this point, water was re-applied to soil
168 saturation after the pertinent data were collected. Plants were allowed two days to recover before
169 post-drought physiological data was collected (“Recovery”).

170

171 Economic trait measurements

172 After physiological data were collected in the “Recovery” period, above and
173 belowground tissues were harvested to determine productivity of all species and samples that
174 were subjected to dry-down conditions. Leaf-level economic and microanatomical data were
175 collected from samples that included all non-droughted individuals but excluded *P. juergensii*, *P.*
176 *notatum*, or *F. ovina* due to the lack of samples. The leaf tissue data included: Leaf area (LA ;
177 cm^2), specific leaf area (SLA , leaf area divided by dry mass; $\text{cm}^2 \text{g}^{-1}$), and leaf dry-matter content
178 ($LDMC$, fresh leaf mass divided by dry mass; g g^{-1}). SLA and $LDMC$ were analyzed with the
179 standardized rehydration method (Garnier, Shipley, Roumet & Laurent 2001; Pérez-
180 Harguindeguy *et al.* 2013), while LA data were obtained by processing images in ImageJ
181 (Rasband 1997). Roots were washed and cleaned of debris for digital root imaging; analysis of
182 root images was completed with a root imaging software (WinRhizo; Regent Instruments, Inc.,
183 Nepean, Ontario, Canada). Root imaging provided the following traits: total root length (cm),
184 root diameter (mm), and specific root length (SRL , root length divided by dry mass; cm g^{-1}).
185 After scans were completed, above and belowground biomass samples were dried for 48 hours at
186 65°C and weighed for productivity comparisons.

187

188 Microanatomy trait measurements

189 The newest mature leaf was used for microanatomical analysis prior to the initiation of
190 drought from the following species: *Setaria viridis*, *Urochloa ruziziensis*, *Danthonia spicata*,

191 *Rytidosperma semiannulare*, *Bouteloua dactyloides*, and *Bouteloua gracilis*. *Sorghastrum*
192 *nutans*, *Andropogon gerardii*, and *Panicum virgatum* were collected from parent populations in
193 the field at peak physiological performance. *Festuca ovina*, *Paspalum notatum*, and *Paspalum*
194 *juergensii* samples were not included in these analyses due to sample loss. Microanatomical
195 samples, roughly 30 mm in length, were collected (4 - 8 samples per species; n = 33) by clipping
196 leaf tissue and placing them into a fixative FAA (10% formalin/ 5% glacial acetic acid / 50%
197 ethanol (use 95% EtOH) / 35% DI water) under a vacuum. Tissues were then cut (cross
198 sectioned) to 4 μ m in thickness with a Leica RM2135 microtome (Leica Biosystems, Newcastle,
199 UK), and mounted in paraffin at Kansas State's College of Veterinary Medicine Histopathology
200 lab. Tissue was stained with Safranin-O and Fast Green (Ruzin 2000), cover slipped, and imaged
201 on a Zeiss 880 confocal microscope (Carl Zeiss, Walldorf, Germany) at 10X and 20X when
202 necessary with a multitrack configuration, digital dual-bypass filters and a GaAsP detector (Fig.
203 1). Microanatomical data were collected using IMAGEJ software (Rasband 1997) by analyzing
204 two tissue regions from either side of the midrib between two major vascular bundles which
205 were then averaged together from each leaf sample (Bachle & Nippert 2018, 2021). Here, the
206 total subsampled area is referred to as the cross - sectional area or the area between two major
207 vascular bundles (CSA). Microanatomical traits collected from subsamples include: xylem area
208 (X_{area} ; μm^2), xylem diameter ($X_{diameter}$; μm), t/b (xylem wall thickness/ $X_{diameter}$; xylem resistance
209 to cavitation; $\mu\text{m}\mu\text{m}^{-1}$); while stomatal count (S_{count}) was collected from the whole-leaf cross
210 section. In this study, we did not collect stomatal densities - as that would entail epithelial peels
211 or impressions, therefore we do not equate density measurements and interpretations with the
212 S_{count} . Instead, we utilized S_{count} to inform how many stomata are serving major and minor
213 vascular bundles within the whole-leaf cross section.

214

215 Data analysis

216 The selected traits were averaged by species and separated into the three physiological
217 'stages' (Initial, Stressed, and Recovery) based on physiological responses. We included tribe as
218 a factor to investigate differences among lineages. We considered using a phylogenetic
219 generalized linear mixed model (PGLMM), however, our goal was to not control for phylogeny,
220 but rather to determine if lineages with different traits have evolved different drought tolerance
221 strategies. All data were checked to meet assumptions of normality before analyses began.

222 Comparisons among tribes and dry-down ‘stages’ were analyzed using mixed-effect model
223 ANOVA with physiological data used as the response variables and tribe and condition as
224 predictor variables. Tests were performed with the lmer function within the lme4 package (Bates,
225 Maechler, Bolker & Walker 2015). To assess bivariate relationships between plant functional
226 traits, we performed simple regression analyses (using the ‘lm’ function). Non-parametric data
227 were analyzed via Kruskal-Wallis rank sum test paired with a post hoc pairwise Wilcox test. We
228 also used Akaike’s information criterion, adjusted for small sample size AICc model selection to
229 determine the most impactful trait parameters determining drought response using the “MuMIn”
230 package (Grueber, Nakagawa, Laws & Jamieson 2011; Bartoń 2018). All data were analyzed in
231 the statistical program R V3.5.3 (R Core Team 2020). In order to summarize the relationships
232 and range of physiological, functional, and microanatomical diversity represented in our dry-
233 down sample, we conducted a Principal Component Analysis (PCA) using the “prcomp”
234 function within the “stats” library on the mean trait data across species, which cumulatively
235 explained 72% of the variation in traits (Fig. 2). Not all traits were measured for every species,
236 and so we focused on key traits coming from each of the data types we measured. The purpose of
237 the PCA was to visually explore the multivariate relationships among species in multivariate
238 space, and only includes species that had all functional trait data (excluding *P. notatum*, *P.*
239 *juergensii*, and *F. ovina*).

240

241 **RESULTS**

242 Drought responses differed among species to the experimentally induced dry-down.
243 Physiological viability, defined as maintaining at least 25% of the initial photosynthetic rate,
244 ranged from 4-33 days (Fig. 3A). Drought duration (days in drought before re-watering) was
245 similar among species within tribes, but varied significantly across tribes (Fig. 3A, $P < 0.001$).
246 Species within the tribe Cynodonteae (*B. dactyloides* & *B. gracilis*) were physiologically viable
247 for the longest time during the dry-down, reaching the “stressed” stage after 30 days in drought
248 (Fig. 3A, Supporting Table S2), whereas the most drought-sensitive species were within
249 Paspaleae and Poae. These tribes were similar ($P > 0.05$), reaching the ‘stressed’ stage more
250 than 20 days before the Cynodonteae tribe (Fig. 3A, Supporting Table S2). The recovery of grass
251 species and tribes following re-watering displayed a more variable response (Fig. 3B). There
252 were no statistical differences among tribes in their recovery dynamics ($P > 0.05$), though

253 significant differences were observed among species and within tribes ($P < 0.05$). *S. nutans* and
254 *B. gracilis* were the only species that that exceeded pre-drought photosynthesis levels after
255 recovery (114% and 121% of initial A_n , respectively - Fig. 3B). *Festuca ovina* and *B. dactyloides*
256 were the only species that did not regain at least 50% of ‘Initial’ physiological measurements.
257 Several species within Paspaleae, Paniceae, Danthoneae, and Andropogoneae lineages did not
258 fully recover (100%) to ‘Initial’ physiological levels within the experimental timeframe.
259 However, all recovered to at least 50% of Day 1 measurements (Fig. 3B). Andropogoneae
260 species displayed the highest drought recovery, regaining on average over 92% of physiological
261 functioning while the only species measured in Poeae (*F. ovina*) was the least resilient, with only
262 42% recovery of pre-drought A_n following re-watering.

263 Leaf economic trait data were collected at the conclusion of the dry-down when recovery
264 data was collected for each individual sample. Production of aboveground biomass was observed
265 to vary significantly at both tribe and species level (Supporting Table S4, S5; $P < 0.001$). *SLA*
266 was statistically similar within Andropogoneae, Cynodonteae, and Danthonieae ($P > 0.05$) while
267 *SLA* within Paniceae species displayed significant variation ($P < 0.05$), ranging from $29 \text{ cm}^2 \text{ g}^{-1}$
268 (*P. virgatum*) – $143 \text{ cm}^2 \text{ g}^{-1}$ (*U. ruziziensis*). Similarly, *LDMC* was statistically similar across
269 tribes, except for two species within Paniceae ($P < 0.05$) (*S. viridis* and *U. ruziziensis*)
270 (Supporting Table S2). The production of fine root length (diameter $< 0.5 \text{ mm}$) differed among
271 tribe ($P < 0.0001$) and species ($P < 0.0001$). Significant differences in *SRL* were observed across
272 tribes and species as well ($P < 0.001$, Supporting Table S2). All species within their respective
273 tribes were found to have statistically similar *SRL* except for species in Paniceae ($P < 0.05$),
274 Paspaleae ($P < 0.05$), and Danthonieae ($P < 0.01$) due to the 294.99 cm g^{-1} difference in *SRL*.

275 Most microanatomical traits displayed significant differences among species across tribes
276 ($P < 0.05$) but typically had reduced variability between species within the same tribe X_{area} was
277 statistically different between tribes ($P < 0.001$) with the exception of Danthonieae and
278 Cynodonteae ($P = 0.485$). Andropogoneae ($589.308 \mu\text{m}^2$) had the largest X_{area} and was 5x larger
279 than the smallest X_{area} found in Cynodonteae ($108.957 \mu\text{m}^2$) ($P < 0.01$; Supporting Table S3).
280 While there were significant species differences found across all tribes ($P < 0.001$; Supporting
281 Table S3) there were no observable species differences within tribe ($P > 0.05$). $X_{diameter}$ reflected
282 a similar pattern to that of X_{area} : significant differences between tribes ($P < 0.001$) and
283 statistically similar values within tribes ($P > 0.15$; Supporting Table S3) (consistent with

284 phylogenetic niche conservatism). Xylem resistance to cavitation (t/b) differed significantly
285 across tribes ($P < 0.001$), species ($P < 0.001$), but not among species within a tribe ($P > 0.05$;
286 Supporting Table S3). Stomata within the subsampled area (S_{count}) showed high variation; the
287 significant differences among tribes ($P < 0.01$) are likely attributed to Paniceae, which had higher
288 S_{count} (Supporting Table S3).

289 There were few statistically significant relationships explaining drought responses and
290 recovery among tribes (Supporting Fig. S1), except for X_{area} and S_{count} . Surprisingly, given the
291 large volume of literature designating $iWUE$ as a pivotal functional trait reflecting drought
292 tolerance, there was no statistically significant correlation of $iWUE$ with drought resistance or
293 resilience ($P > 0.05$). However, stomatal number was significantly negatively correlated with
294 drought duration ($P < 0.01$) (Fig. 4), X_{area} ($P < 0.05$), and $iWUE$ ($P < 0.05$) (Supporting Fig. S1).
295 Results also indicate a differentiation between sample productivity and economic growth
296 strategies (Fig 5). A significant relationship was observed when comparing above and
297 belowground biomass (Fig 5A), yielding a tight positive relationship ($P < 0.001$; $r^2 = 0.825$). Yet,
298 when above and belowground economic strategies (SLA and SRL , respectively) were calculated,
299 a breakdown in the previous relationship was observed ($P > 0.05$; $r^2 = 0.008$) (Fig. 5B). While
300 SLA displayed no bivariate relationships with other traits, $LDMC$ correlated with t/b ($P < 0.01$)
301 and S_{count} ($P < 0.05$) (Supporting Fig. S1). The AICc model selection process indicated how the
302 selected functional traits influence both drought resistance and resilience. The model explaining
303 the greatest variation in drought sensitivity included Tribe, S_{count} , $iWUE$, $LDMC$, and SLA .
304 However, the best explanation for variation in drought resilience included a single anatomical
305 trait: S_{count} .

306

307 **DISCUSSION**

308 Historically, water use efficiency (WUE) has been used as a seminal physiological trait to
309 explain why some species persist and others succumb to drought (Fay *et al.* 2002; Leakey *et al.*
310 2019; Lavergne *et al.* 2019; Yang *et al.* 2021). However, the data presented here illustrate that
311 using this physiological trait by itself may misconstrue the interpretation of drought responses in
312 grass species (Fig. 3A; Supporting Fig. S1) (Morgan *et al.* 2011). Physiological, morphological
313 (SLA , SRL , $LDMC$), and anatomical data (xylem area, stomata number, t/b) in combination help
314 provide a more nuanced perspective on grass responses to drought, and should be integrated in

315 order to more appropriately identify mechanisms of water stress across diverse grass lineages.
316 Shared phylogenetic and biogeographic histories have resulted in unique adaptations and trait
317 development in Poaceae that are reflected in the patterns of global distribution and subsequent
318 responses to drought (Christin, Freckleton & Osborne 2010; Liu & Osborne 2015;
319 Watcharamongkol, Christin & Osborne 2018). Here, we show considerable variation in
320 functional traits among diverse species within Poaceae in response to drought and recovery. In
321 addition, these data illustrate that the traits and adaptations that confer an ability to withstand
322 extreme drought conditions are not the same as the traits and adaptations that confer an ability to
323 recover from drought, as they are driven by the coordination of different factors (Fig. 2; Fig. 3).

324 The grass species measured here displayed variable sensitivity to the dry-down, visible in
325 both drought response and recovery (Fig. 3). The physiological responses observed during the
326 dry-down were statistically related to variability in anatomical features, mainly those influencing
327 water relations at the leaf level (Fig. 4). We also found a separation between productivity and
328 economic strategies in both above and belowground tissues of the selected Poaceae species (Fig.
329 5), reflecting a trade-off in leaf and root growth economic strategies. Differences in economic
330 traits indicated water-use strategies varied in drought responses (Funk *et al.* 2017; Reich &
331 Flores-Moreno 2017; Cui, Weng, Yan & Xia 2020). For example, plants with lower *SLA* and
332 higher *SRL* traits are likely to have lower metabolic costs and grow in resource-poor
333 environments with an increased ability to acquire resources (Cornelissen *et al.* 2003; Pérez-
334 Harguindeguy *et al.* 2013). These trait strategies should allow for sustained physiological
335 tolerance and quicker drought conditions. While evolutionary relatedness guides physiological,
336 morphological, and anatomical traits in determining drought responses, drought recovery was
337 mainly driven by S_{count} alone. The number of stomata within the subsampled cross-sectional area
338 (CSA) should not be interpreted as a stomatal density measurement because the subsampling
339 method we used was not limited to a defined leaf area. Instead, S_{count} indicates the number of
340 stomata that are supplying CO₂ to major and minor vascular bundles, which may be a more
341 direct mechanistic comparison to that of stomatal densities. Stomatal density, rather, considers a
342 leaf's surface as a uniform and homogeneous surface and has little to no consideration for
343 microanatomical traits. Such microanatomical trait measurements (i.e., mesophyll area, bundle
344 sheath area, and the diffusion distance through mesophyll) are important to consider due to their

345 influence on carbon assimilation and subsequent water loss (Esau 1953; Sack & Frole 2006;
346 Ocheltree, Nippert & Prasad 2011; Lundgren *et al.* 2019).

347 Low soil moisture negatively impacts growth, increases xylem tension, and decreases
348 carbon assimilation (Lemoine, Griffin-Nolan, Lock & Knapp 2018; Jardine, Thomas & Osborne
349 2021). The ability to mitigate and recover from drought is based on anatomical and physiological
350 traits (Taylor, Ripley, Woodward & Osborne 2011; Olson, Anfodillo, Gleason & McCulloh
351 2020). While the impacts of severe drought on the physiology of grassland species have been
352 observed in previous research, few studies combined physiological, whole-leaf, and
353 microanatomical trait data (Fry *et al.* 2013; Liu & Osborne 2015). As was previously mentioned,
354 we intentionally chose species (within the same tribe) that were previously reported to have
355 varying responses to low soil moisture. However, our results indicate that closely related grasses
356 can respond similarly to decreasing soil moisture (Fig. 3A; Fig. 4) but display variable responses
357 when water becomes available (Fig. 3B). This variability supports previous claims that drought
358 responses within a functional type (i.e. C₄ grasses) are not uniform and vary due to a myriad of
359 reasons (i.e. evolutionary histories, functional trait, etc.)(Liu & Osborne 2015; Griffith *et al.*
360 2020). The diversity in physiological responses among species has been observed to protect
361 individuals and populations while protecting ecosystem functioning from detrimental effects of
362 drought (Mori, Furukawa & Sasaki 2013; Kreyling *et al.* 2017; Roberts, Twidwell, Angeler &
363 Allen 2019).

364 Given the fundamental role that past evolutionary histories have played in shaping
365 current species distributions (Fox *et al.* 2018; Folk, Siniscalchi & Soltis 2020), species that
366 exhibit variable responses to ecosystem disturbances would benefit more than species that
367 maintain static responses (Isbell & Wilsey 2011; Isbell *et al.* 2015). For instance, species that
368 were more drought resistant (Cynodonteae) are broadly represented in the mixed and shortgrass
369 prairies of North America, regions that are known to have less rainfall and more frequent drought
370 (Carroll *et al.* 2021). In contrast, the drought-sensitive species (Paspalum) are from tropical
371 locations where moisture is typically not the most limiting resource. In addition, Cynodonteae
372 were also observed to have fewer stomata and decreased gas exchange rates compared to
373 Paniceae and Paspalum species, leading to less water loss (Fig. 4; Supporting Table S2, S3).
374 Therefore, it stands to reason that phylogenetically dissimilar species evolving under different
375 environmental constraints would exhibit disparate drought response to the imposed dry-down,

376 while more closely related species would respond more uniformly (Fig. 3A; Fig. 4). Our data
377 also displays a clear indication of an evolved plasticity in physiological responses to variable
378 climate conditions, as native grasses typically occupy regions with similar climate variability
379 (Bachle, Griffith & Nippert 2018). Grasses sampled in this experiment were severely desiccated
380 and recovered >50% of pre-drought physiological functioning and in several cases, physiological
381 rates that were 20-30% higher than the initial state (Fig. 3B), highlighting a potentially unique
382 characteristic of grasses across lineages. The ability to quickly acquire water and other nutrients
383 following drought disturbances likely facilitates grasses competing with other neighboring
384 functional types with deeper access to water (Holdo, Nippert & Mack 2018; Kulmatiski, Beard,
385 Holdrege & February 2020).

386 Water availability directly impacts plant physiological responses, which are constrained
387 by internal anatomical machinery (Fig. 1) (Christin *et al.* 2013; Bachle & Nippert 2021). For
388 example, the spatial separation of C₄ photosynthesis allows for a reduced stomatal conductance
389 and decreased water loss, leading to higher water-use efficiencies (Farquhar & Sharkey 1982;
390 Ehleringer & Monson 1993; Berry & Patel 2008; Way, Katul, Manzoni & Vico 2014). This
391 characteristic is often assumed to underlie success under conditions of increased aridity. Our
392 findings, however, do not support this claim (Supporting Fig. S1). *iWUE* was not observed to
393 directly aid in drought sensitivity or recovery of grasses in this study but it was positively related
394 to the number of stomata (Supporting Fig. S1), indicating here, that the presence of more stomata
395 is associated with higher *iWUE*. This counterintuitive result does not indicate a more drought
396 tolerant strategy, rather, it communicates higher gas exchange rates resulting in greater loss of
397 water leading to desiccation (Xu & Zhou 2008; Bertolino *et al.* 2019; Knapp *et al.* 2020).
398 However, previous research has indicated that stomatal patterning, morphology, and densities
399 can greatly influence/alter physiological responses to water stress (Nunes, Zhang & Raissig
400 2020; Harrison, Arce Cubas, Gray & Hepworth 2020). Figure 4 clearly indicates species with
401 more stomata have an increased sensitivity (decreased resistance) to drought, which may require
402 reevaluations of previously held claims regarding the functional significance of *WUE* (Condon
403 2004).

404 Xylem characteristics have also been previously shown to impact an individual's water–
405 use (Wahl & Ryser 2000; He *et al.* 2020; Olson *et al.* 2020). Xylem area is a commonly
406 measured trait because it corresponds with the amount of water that can be transported at any

407 given time. Here, our results indicate two water transport strategies. Larger xylem (X_{area})
408 decreases drought resistance while displaying a positive relationship with recovery, when
409 excluding *A. gerardii* and *S. nutans* (Supporting Fig. S1). This strategy enables individuals with
410 larger X_{area} to transport greater amounts of water, when available (seen in recovery). But,
411 drought conditions can lead to increased tension on the water column inside xylem vessels,
412 ultimately increasing the potential for embolism formation during drought conditions (McCulloh,
413 Domec, Johnson, Smith & Meinzer 2019). Previous research has highlighted how increased
414 thickness of xylem wall tissue with smaller diameter lumen (t/b) can protect from embolism
415 events in water limiting conditions (Blackman *et al.* 2018; Guérin *et al.* 2020); however, our data
416 do not corroborate such findings (Supporting Fig. S1). Anatomical traits were observed to
417 contain large variation, which we can contribute to two main factors: (1) Our sample size was
418 relatively small, due to the time - consuming nature of anatomical studies; and (2)
419 microanatomical traits are complex in nature and have large variability among individuals and
420 within grass leaves (Fig. 1) (Ocheltree *et al.* 2011; Bachle & Nippert 2021).

421 While microanatomical traits and leaf-level physiological rates provide key mechanistic
422 insights into drought sensitivity and resilience, whole-plant traits are more easily observable and
423 require less detailed scientific instrumentation and training (John *et al.* 2017; Reich & Flores-
424 Moreno 2017). Whole-plant traits illustrate broader growth strategies by the individual, such as
425 resistance or avoidance of detrimental growth conditions. Our results indicate a linear
426 relationship between above and belowground productivity (Fig. 5A), indicating a constant
427 proportional investment by the selected grasses. However, when comparing two widely utilized
428 traits within the leaf and root economic spectrum (SLA and SRL), the previous relationship
429 breaks down to reveal tradeoffs in grass growth strategies (Fig. 5B). Individuals that invest in a
430 root system designed for quick absorption of water and nutrients (high SRL) can only produce an
431 inexpensive leaf (low SLA), while more ‘expensive’ leaves (high SLA) appear to be associated
432 with a less economically efficient rooting strategy (Fig. 5A). This finding highlights the inability
433 of grasses to produce tissues at the upper limits of the leaf and root economic spectrum.
434 However, it's important to recognize and consider the potential for convoluted interpretations of
435 economic traits, as they are the amalgamation of several underlying traits (Pérez-Harguindeguy
436 *et al.* 2013; Bachle & Nippert 2021). In addition, there could be grasses, which were not
437 investigated here, that invest in high SLA and SRL . One of these complex traits is leaf dry matter

438 content (*LDMC*), which was correlated with the thickening of xylem vessel walls or xylem
439 reinforcement: *t/b*. This thickening of water transport tissues (or xylem reinforcement) increases
440 the strength at which the water column can be under tension, allowing for a more negative water
441 potential while decreasing the likelihood of cavitation, a physiological indicator of drought stress
442 (McCulloh *et al.* 2019).

443 There is currently a dearth of available functional trait data in grasses, an
444 underrepresented functional type in trait databases given the importance of grass species for food
445 and forage and their geographical coverage. Results from this study indicate the need for
446 increased collection of grass functional traits across a diverse assemblage of species within a
447 functional type. Plant functional types are often used in ecosystem models to more easily group
448 plants by common features; however, as our results indicate, this may lead to poor
449 parameterization and model output as such functional types do not account for phylogenetic
450 relatedness. Our AICc selection indicates that microanatomical traits (specifically stomatal
451 count), physiological, economic traits, and phylogeny were essential to understanding species
452 ability to withstand drought, while stomatal count was the best explanation for recovery
453 responses. The trait data from these lineage-specific responses to drought have potential
454 consequences for how different grasslands are represented and forecast in Earth System Models
455 (Still, Cotton & Griffith 2018; Griffith *et al.* 2020).

456 The evolutionary histories of lineages within Poaceae have led to the development of
457 unique morphology (leaves and roots), anatomy (stomatal shape and photosynthetic tissue
458 arrangement), and physiology; these traits have allowed this functional type to dominate much of
459 the surface cover on every continent (excluding Antarctica). Phylogenetic divergences and
460 subsequent trait adaptations have led to contrasting responses and recovery from drought (Fig.
461 3). Anatomical traits were key in explaining physiological drought response and recovery,
462 specifically traits concerned with water usage. Surprisingly, species that exhibited increased
463 *iWUE* were more prone to quicker desiccation, which is most likely due to the same individuals
464 maintaining larger numbers of stomata and higher overall rates of gas exchange (Supporting Fig.
465 S1). Interestingly this same trait (S_{count}) was responsible for recovery from drought, which did
466 allow for faster drought recovery. This study underscores the importance of collecting a myriad
467 of in-depth trait data from several Poaceae lineages to better understand the mechanisms that
468 describe drought responses and recovery.

469

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479 **REFERENCES**

- 480 Avolio M.L., Forrester E.J., Chang C.C., La Pierre K.J., Burghardt K.T. & Smith M.D. (2019) Demystifying
 481 dominant species. *New Phytologist* **223**, 1106–1126.
- 482 Bachle S., Griffith D.M. & Nippert J.B. (2018) Intraspecific Trait Variability in *Andropogon gerardii*, a
 483 Dominant Grass Species in the US Great Plains. *Frontiers in Ecology and Evolution* **6**, 217.
- 484 Bachle S. & Nippert J.B. (2018) Physiological and anatomical trait variability of dominant C4 grasses. *Acta*
 485 *Oecologica* **93**, 14–20.
- 486 Bachle S. & Nippert J.B. (2021) Microanatomical traits track climate gradients for a dominant C4 grass
 487 species across the Great Plains, USA. *Annals of Botany* **127**, 451–459.
- 488 Bartoń K. (2018) MuMIn: Multi-Model Inference.
- 489 Bates D., Maechler M., Bolker B. & Walker S. (2015) Fitting linear mixed-effects models using lme4.
 490 *Journal of Statistical Software* **67**, 1–48.
- 491 Berry J.O. & Patel M. (2008) Kranz Anatomy and the C4 Pathway. *Encyclopedia of life sciences (ELS)*, 1–6.
- 492 Bertolino L.T., Caine R.S. & Gray J.E. (2019) Impact of stomatal density and morphology on water-use
 493 efficiency in a changing world. *Frontiers in Plant Science* **10**, 225.
- 494 Blackman C.J., Gleason S.M., Cook A.M., Chang Y., Laws C.A. & Westoby M. (2018) The links between
 495 leaf hydraulic vulnerability to drought and key aspects of leaf venation and xylem anatomy among
 496 26 Australian woody angiosperms from contrasting climates. *Annals of Botany* **122**, 59–67.
- 497 Blair J., Nippert J.B. & Briggs J.M. (2014) Grassland Ecology. In *The Plant Sciences - Ecology & The*
 498 *Environment*, Springer r. (ed R.K. Monson), pp. 389–423. Springer, Springer-Verlag Berlin
 499 Heidelberg.
- 500 Buckley T.N. (2019) How do stomata respond to water status? *New Phytologist* **224**, 1–13.
- 501 Burri S., Niklaus P.A., Grassow K., Buchmann N. & Kahmen A. (2018) Effects of plant productivity and
 502 species richness on the drought response of soil respiration in temperate grasslands. *PLoS ONE* **13**,
 503 e0209031.
- 504 Cardoso J.A., Pineda M., Jiménez J. de la C., Vergara M.F. & Rao I.M. (2015) Contrasting strategies to
 505 cope with drought conditions by two tropical forage C4 grasses. *AoB Plants* **7**, plv107.
- 506 Carroll C.J.W., Slette I.J., Griffin R.J., Lauren N., Ava E.B., Denton E.M., ... Knapp A.K. (2021) Is a drought a
 507 drought in grasslands? Productivity responses to different types of drought. *Oecologia*.
- 508 Christin P.A., Freckleton R.P. & Osborne C.P. (2010) Can phylogenetics identify C4 origins and reversals?
 509 *Trends in Ecology and Evolution* **25**, 403–409.
- 510 Christin P.A., Osborne C.P., Chatelet D.S., Columbus J.T., Besnard G., Hodkinson T.R., ... Edwards E.J.
 511 (2013) Anatomical enablers and the evolution of C4 photosynthesis in grasses. *Proceedings of the*
 512 *National Academy of Sciences of the United States of America* **110**, 1381–6.
- 513 Cleland E.E., Collins S.L., Dickson T.L., Farrer E.C., Gross K.L., Gherardi L.A., ... Suding K.N. (2013)
 514 Sensitivity of grassland plant community composition to spatial vs. temporal variation in
 515 precipitation. *Ecology* **94**, 1687–1696.
- 516 Condon A.G. (2004) Breeding for high water-use efficiency. *Journal of Experimental Botany* **55**, 2447–
 517 2460.
- 518 Cook B.I., Ault T.R. & Smerdon J.E. (2015) Unprecedented 21st century drought risk in the American
 519 Southwest and Central Plains. *Science Advances* **1**, e1400082–e1400082.
- 520 Cornelissen J.H.C., Lavorel S., Garnier E., Díaz S., Buchmann N., Gurevich D.E., ... Poorter H. (2003) A
 521 handbook of protocols for standardised and easy measurement of plant functional traits
 522 worldwide. *Australian Journal of Botany* **51**, 335–380.
- 523 Cotton J.M., Cerling T.E., Hoppe K.A., Mosier T.M. & Still C.J. (2016) Climate, CO₂, and the history of
 524 North American grasses since the Last Glacial Maximum. *Science Advances* **2**, e1501346.

525 Cui E., Weng E., Yan E. & Xia J. (2020) Robust leaf trait relationships across species under global
526 environmental changes. *Nature Communications* **11**, 1–9.

527 Edwards E.J., Osborne C., Stromberg C. & Smith S. (2010) The Origins of C4 Grasslands: Integrating
528 Evolutionary and Ecosystem Science. *Science* **328**, 587–590.

529 Ehleringer J.R. (2005) The Influence of Atmospheric CO₂, Temperature, and Water on the Abundance of
530 C3/C4 Taxa. In *A History of Atmospheric CO₂ and Its Effects on Plants, Animals and Ecosystems*.
531 (eds J.R. Ehleringer & T.E. Cerling), pp. 185–213. Springer, New York.

532 Ehleringer J.R. & Monson R.K. (1993) Evolutionary and ecological aspects of photosynthetic pathway
533 variation. *Annual Review of Ecology and Systematic*, 411–439.

534 Esau K. (1953) *Plant Anatomy*, 1st ed. John Wiley, New York.

535 Eters D., Tarks P. & Hernandez M. (2014) Functional response of U.S. grasslands to the early 21st-century
536 drought. *Ecology* **95**, 2121–2133.

537 Farquhar G.D. & Sharkey T.D. (1982) Stomatal Conductance and Photosynthesis. *Annual Review of Plant*
538 *Physiology* **33**, 317–345.

539 Fay P.A., Carlisle J.D., Knapp A.K., Blair J.M. & Collins S.L. (2003) Productivity responses to altered rainfall
540 patterns in a C4-dominated grassland. *Oecologia* **137**, 245–251.

541 Fay Philip., Carlisle J.D., Danner B.T., Lett M.S., McCarron J.K., Stewart C., ... Collins S.L. (2002) Altered
542 rainfall patterns, gas exchange, and growth in grasses and forbs. *International Journal of Plant*
543 *Sciences* **163**, 549–557.

544 Folk R.A., Siniscalchi C.M. & Soltis D.E. (2020) Angiosperms at the edge: Extremity, diversity, and
545 phylogeny. *Plant Cell and Environment* **43**, 2871–2893.

546 Fox D.L., Pau S., Taylor L., Strömberg C.A.E., Osborne C.P., Bradshaw C., ... Still C.J. (2018) Climatic
547 controls on C4 grassland distributions during the Neogene: A model-data comparison. *Frontiers in*
548 *Ecology and Evolution* **6**, 1–19.

549 Fry E.L., Manning P., Allen D.G.P., Hurst A., Everwand G., Rimmler M. & Power S.A. (2013) Plant
550 Functional Group Composition Modifies the Effects of Precipitation Change on Grassland
551 Ecosystem Function. *PLoS ONE* **8**, e57027.

552 Funk J.L., Larson J.E., Ames G.M., Butterfield B.J., Cavender-Bares J., Firn J., ... Wright J. (2017) Revisiting
553 the Holy Grail: Using plant functional traits to understand ecological processes. *Biological Reviews*
554 **92**, 1156–1173.

555 Garbowski M., Avera B., Bertram J.H., Courkamp J.S., Gray J., Hein K.M., ... Brown C.S. (2020) Getting to
556 the root of restoration: considering root traits for improved restoration outcomes under drought
557 and competition. *Restoration Ecology* **28**, 1384–1395.

558 Garnier E., Shipley B., Roumet C. & Laurent G. (2001) A standardized protocol for the determination of
559 specific leaf area and leaf dry matter content. *Functional Ecology* **15**, 688–695.

560 Grass Phylogeny Working II G. (2012) New grass phylogeny resolves deep evolutionary relationships and
561 discovers C4 origins. *New Phytologist* **193**, 304–312.

562 Griffin-Nolan R.J., Blumenthal D.M., Collins S.L., Farkas T.E., Ava M., Mueller K.E., ... Knapp A.K. (2019)
563 Shifts in plant functional composition following long-term drought in grasslands. *Journal of Ecology*
564 **107**, 2133–2148.

565 Griffith D.M., Osborne C.P., Edwards E.J., Bachle S., Beerling D.J., Bond W.J., ... Still C.J. (2020) Lineage-
566 based functional types: characterising functional diversity to enhance the representation of
567 ecological behaviour in Land Surface Models. *New Phytologist* **228**, 15–23.

568 Grueber C.E., Nakagawa S., Laws R.J. & Jamieson I.G. (2011) Multimodel inference in ecology and
569 evolution: Challenges and solutions. *Journal of Evolutionary Biology* **24**, 699–711.

570 Guérin M., von Arx G., Martin-Benito D., Andreu-Hayles L., Griffin K.L., McDowell N.G., ... Gentine P.
571 (2020) Distinct xylem responses to acute vs prolonged drought in pine trees. *Tree physiology* **40**,
572 605–620.

573 Habermann E., San Martin J.A.B., Contin D.R., Bossan V.P., Barboza A., Braga M.R., ... Martinez C.A.
 574 (2019) Increasing atmospheric CO₂ and canopy temperature induces anatomical and physiological
 575 changes in leaves of the C₄ forage species *Panicum maximum*. *PLoS ONE* **14**, e0212506.
 576 Hacke U.G., Sperry J.S., Pockman W.T., Davis S.D. & McCulloh K.A. (2001) Trends in wood density and
 577 structure are linked to prevention of xylem implosion by negative pressure. *Oecologia* **126**, 457–
 578 461.
 579 Harrison E.L., Arce Cubas L., Gray J.E. & Hepworth C. (2020) The influence of stomatal morphology and
 580 distribution on photosynthetic gas exchange. *Plant Journal* **101**, 768–779.
 581 He P., Gleason S.M., Wright I.J., Weng E., Liu H., Zhu S., ... Ye Q. (2020) Growing-season temperature and
 582 precipitation are independent drivers of global variation in xylem hydraulic conductivity. *Global
 583 Change Biology* **26**, 1833–1841.
 584 Holdo R.M., Nippert J.B. & Mack M.C. (2018) Rooting depth varies differentially in trees and grasses as a
 585 function of mean annual rainfall in an African savanna. *Oecologia* **186**, 269–280.
 586 Hoover D., Duniway M. & Belnap J. (2015) Pulse-drought atop press-drought: unexpected plant
 587 responses and implications for dryland ecosystems. *Oecologia* **179**, 1211–1221.
 588 Hoover D., Knapp A. & Smith M. (2014) Resistance and resilience of a grassland ecosystem to climate
 589 extremes. *Ecology* **95**, 2646–2656.
 590 Hoover D.L. & Rogers B.M. (2016) Not all droughts are created equal: The impacts of interannual
 591 drought pattern and magnitude on grassland carbon cycling. *Global Change Biology* **22**, 1809–
 592 1820.
 593 Isbell F., Craven D., Connolly J., Loreau M., Schmid B., Beierkuhnlein C., ... Eisenhauer N. (2015)
 594 Biodiversity increases the resistance of ecosystem productivity to climate extremes. *Nature* **526**,
 595 574–577.
 596 Isbell F.I. & Wilsey B.J. (2011) Increasing native, but not exotic, biodiversity increases aboveground
 597 productivity in ungrazed and intensely grazed grasslands. *Oecologia* **165**, 771–781.
 598 Iversen C.M., McCormack M.L., Powell A.S., Blackwood C.B., Freschet G.T., Kattge J., ... Violle C. (2017) A
 599 global Fine-Root Ecology Database to address below-ground challenges in plant ecology. *New
 600 Phytologist* **215**, 15–26.
 601 Jardine E.C., Thomas G.H. & Osborne C.P. (2021) Traits explain sorting of C₄ grasses along a global
 602 precipitation gradient. *Ecology and Evolution*, 1–12.
 603 John G.P., Scoffoni C., Buckley T.N., Villar R., Poorter H. & Sack L. (2017) The anatomical and
 604 compositional basis of leaf mass per area. *Ecology Letters* **20**, 412–425.
 605 Kimball S., Gremer J.R., Angert A.L., Huxman T.E. & Venable D.L. (2012) Fitness and physiology in a
 606 variable environment. *Oecologia* **169**, 319–329.
 607 Knapp A.K., Carroll C.J.W., Denton E.M., La Pierre K.J., Collins S.L. & Smith M.D. (2015) Differential
 608 sensitivity to regional-scale drought in six central US grasslands. *Oecologia* **177**, 949–957.
 609 Knapp A.K., Chen A., Griffin-Nolan R.J., Baur L.E., Carroll C.J.W., Gray J.E., ... Smith M.D. (2020) Resolving
 610 the Dust Bowl paradox of grassland responses to extreme drought. *Proceedings of the National
 611 Academy of Sciences of the United States of America* **117**, 22249–22255.
 612 Kreyling J., Dengler J., Walter J., Velev N., Ugurlu E., Sopotlieva D., ... Jentsch A. (2017) Species richness
 613 effects on grassland recovery from drought depend on community productivity in a multisite
 614 experiment. *Ecology Letters* **20**, 1405–1413.
 615 Kulmatiski A., Beard K.H., Holdrege M.C. & February E.C. (2020) Small differences in root distributions
 616 allow resource niche partitioning. *Ecology and Evolution* **10**, 9776–9787.
 617 Lambert A.M., Baer S.G. & Gibson D.J. (2011) Intraspecific Variation in Ecophysiology of Three Dominant
 618 Prairie Grasses Used in Restoration: Cultivar Versus Non-Cultivar Population Sources. *Restoration
 619 Ecology* **19**, 43–52.

620 Lavergne A., Graven H., De Kauwe M.G., Keenan T.F., Medlyn B.E. & Prentice I.C. (2019) Observed and
621 modelled historical trends in the water-use efficiency of plants and ecosystems. *Global Change*
622 *Biology* **25**, 2242–2257.

623 Leakey A.D.B., Ferguson J.N., Pignou C.P., Wu A., Jin Z., Hammer G.L. & Lobell D.B. (2019) Water use
624 efficiency as a constraint and target for improving the resilience and productivity of C3 and C4
625 Crops. *Annual Review of Plant Biology* **70**, 781–808.

626 Lehmann C.E.R., Griffith D.M., Simpson K.J., Anderson T.M., Archibald S., Beerling D.J., ... Osborne C.P.
627 (2019) Functional diversification enabled grassy biomes to fill global climate space. *bioRxiv*,
628 583625.

629 Lemoine N.P., Griffin-Nolan R.J., Lock A.D. & Knapp A.K. (2018) Drought timing, not previous drought
630 exposure, determines sensitivity of two shortgrass species to water stress. *Oecologia* **188**, 965–
631 975.

632 Lin Y.S., Medlyn B.E., Duursma R.A., Prentice I.C., Wang H., Baig S., ... Wingate L. (2015) Optimal stomatal
633 behaviour around the world. *Nature Climate Change* **5**, 459–464.

634 Linder H.P., Lehmann C.E.R., Archibald S., Osborne C.P. & Richardson D.M. (2018) Global grass (Poaceae)
635 success underpinned by traits facilitating colonization, persistence and habitat transformation.
636 *Biological Reviews* **93**, 1125–1144.

637 Liu C., He N., Zhang J., Li Y., Wang Q., Sack L. & Yu G. (2018) Variation of stomatal traits from cold
638 temperate to tropical forests and association with water use efficiency. *Functional Ecology* **32**, 20–
639 28.

640 Liu H. & Osborne C.P. (2015) Water relations traits of C4 grasses depend on phylogenetic lineage,
641 photosynthetic pathway, and habitat water availability. *Journal of Experimental Botany* **66**, 761–
642 773.

643 Lundgren M.R., Mathers A., Baillie A.L., Dunn J., Wilson M.J., Hunt L., ... Fleming A.J. (2019) Mesophyll
644 porosity is modulated by the presence of functional stomata. *Nature Communications* **10**, 2825.

645 Mainali K.P., Heckathorn S.A., Wang D., Weintraub M.N., Frantz J.M. & Hamilton E.W. (2014) Impact of a
646 short-term heat event on C and N relations in shoots vs. roots of the stress-tolerant C4 grass,
647 *Andropogon gerardii*. *Journal of Plant Physiology* **171**, 977–985.

648 Maurer G.E., Hallmark A.J., Brown R.F., Sala O.E. & Collins S.L. (2020) Sensitivity of primary production to
649 precipitation across the United States. *Ecology Letters* **23**, 527–536.

650 McCormack M.L., Dickie I.A., Eissenstat D.M., Fahey T.J., Fernandez C.W., Guo D., ... Zadworny M. (2015)
651 Redefining fine roots improves understanding of below-ground contributions to terrestrial
652 biosphere processes. *New Phytologist* **207**, 505–518.

653 McCulloh K.A., Domec J.C., Johnson D.M., Smith D.D. & Meinzer F.C. (2019) A dynamic yet vulnerable
654 pipeline: Integration and coordination of hydraulic traits across whole plants. *Plant Cell and*
655 *Environment* **42**, 2789–2807.

656 Morgan J.A., Lecain D.R., Pendall E., Blumenthal D.M., Kimball B.A., Carrillo Y., ... West M. (2011) C4
657 grasses prosper as carbon dioxide eliminates desiccation in warmed semi-arid grassland. *Nature*
658 **476**, 202–205.

659 Mori A.S., Furukawa T. & Sasaki T. (2013) Response diversity determines the resilience of ecosystems to
660 environmental change. *Biological Reviews* **88**, 349–364.

661 Nippert J.B., Fay P.A. & Knapp A.K. (2007) Photosynthetic traits in C3 and C4 grassland species in
662 mesocosm and field environments. *Environmental and Experimental Botany* **60**, 412–420.

663 Nunes T.D.G., Zhang D. & Raissig M.T. (2020) Form, development and function of grass stomata. *The*
664 *Plant Journal* **101**, 780–799.

665 Ocheltree T.W., Mueller K.M., Chesus K., LeCain D.R., Kray J.A. & Blumenthal D.M. (2020) Identification
666 of suites of traits that explains drought resistance and phenological patterns of plants in a semi-
667 arid grassland community. *Oecologia*, 55–66.

668 Ocheltree T.W., Nippert J.B. & Prasad P.V. V (2011) Changes in stomatal conductance along grass blades
669 reflect changes in leaf structure. *Plant, cell & environment* **35**, 1040–9.

670 Olson M.E., Anfodillo T., Gleason S.M. & McCulloh K.A. (2020) Tip-to-base xylem conduit widening as an
671 adaptation: causes, consequences, and empirical priorities. *New Phytologist*.

672 Osborne C.P. (2008) Atmosphere, ecology and evolution: what drove the Miocene expansion of C4
673 grasslands? *Journal of Ecology* **96**, 35–45.

674 Ott J.P. & Hartnett D.C. (2015) Bud-bank and tiller dynamics of co-occurring C3 caespitose grasses in
675 mixed-grass prairie. *American Journal of Botany* **102**, 1462–1471.

676 Pendall E., Bachelet D., Conant R.T., Masri B. El, Flanagan L.B., Knapp A.K., ... Schaffer S.M. (2018)
677 Grasslands. In *Second State of the Carbon Cycle Report (SOCCR2): A Sustained Assessment Report*.
678 (eds N. Cavallaro, G. Shrestha, R. Birdsey, M.A. Mayes, R.G. Najjar, S.C. Reed, ... Z. Zhu), pp. 399–
679 427. Washington, DC, USA.

680 Pérez-Harguindeguy N., Diaz S., Garnier E., Lavorel S., Poorter H., Jaureguiberry P., ... Cornelissen J.H.C.
681 (2013) New handbook for standardized measurement of plant functional traits worldwide.
682 *Australian Journal of Botany* **61**, 167–234.

683 Poorter H., Niinemets Ü., Poorter L., Wright I.J. & Villar R. (2009) Causes and consequences of variation
684 in leaf mass per area (LMA): A meta-analysis. *New Phytologist* **182**, 565–588.

685 Post A.K. & Knapp A.K. (2019) Plant growth and aboveground production respond differently to late-
686 season deluges in a semi-arid grassland. *Oecologia* **191**, 673–683.

687 Qiu F., Bachle S., Estes R., Duvall M.R., Nippert J. & Ungerer M. (2020a) Transcriptional responses to
688 water stress and recovery in a drought-tolerant fescue wild grass (*Festuca ovina*; Poaceae).
689 *Genome* **64**, 1–13.

690 Qiu F., Bachle S., Nippert J.B. & Ungerer M.C. (2020b) Comparing control options for time-series RNA
691 sequencing experiments in nonmodel organisms: An example from grasses. *Molecular Ecology*
692 *Resources* **20**, 681–691.

693 R Core Team (2020) R: A Language and Environment for Statistical Computing.

694 Rasband W. (1997) ImageJ. *U.S National Institutes of Health*.

695 Reich P.B. (2014) The world-wide “fast-slow” plant economics spectrum: A traits manifesto. *Journal of*
696 *Ecology* **102**, 275–301.

697 Reich P.B. & Flores-Moreno H. (2017) Peeking beneath the hood of the leaf economics spectrum. *New*
698 *Phytologist* **214**, 1395–1397.

699 Roberts C.P., Twidwell D., Angeler D.G. & Allen C.R. (2019) How do ecological resilience metrics relate to
700 community stability and collapse? *Ecological Indicators* **107**, 105552.

701 Roumet C., Birouste M., Picon-Cochard C., Ghestem M., Osman N., Vrignon-Brenas S., ... Stokes A. (2016)
702 Root structure-function relationships in 74 species: Evidence of a root economics spectrum related
703 to carbon economy. *New Phytologist* **210**, 815–826.

704 Ruzin S. (2000) Microtechnique: Plant microtechnique and microscopy. *New Phytologist* **148**, 57–58.

705 Sack L. & Frole K. (2006) Leaf structural diversity is related to hydraulic capacity in tropical rain forest
706 trees. *Ecology* **87**, 483–491.

707 Skelton R.P., West A.G. & Dawson T.E. (2015) Predicting plant vulnerability to drought in biodiverse
708 regions using functional traits. *Proceedings of the National Academy of Sciences* **112**, 5744–5749.

709 Still C.J., Cotton J.M. & Griffith D.M. (2018) Assessing earth system model predictions of C4 grass cover
710 in North America: From the glacial era to the end of this century. *Global Ecology and Biogeography*
711 **28**, 1–13.

712 Stocker T.F., Qin D., Plattner G.-K., Tignor M., Allen S.K., Boschung J., ... Midgley P.M. (2013) *Climate*
713 *Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment*
714 *Report of the Intergovernmental Panel on climate Change*.

715 Taub D.R. (2000) Climate and the U.S. distribution of C4 grass subfamilies and decarboxylation variants
716 of C4 photosynthesis. *American Journal of Botany* **87**, 1211–1215.

717 Taylor S.H., Hulme S.P., Rees M., Ripley B.S., Ian Woodward F. & Osborne C.P. (2010) Ecophysiological
718 traits in C3 and C4 grasses: A phylogenetically controlled screening experiment. *New Phytologist*
719 **185**, 780–791.

720 Taylor S.H., Ripley B.S., Woodward F.I. & Osborne C.P. (2011) Drought limitation of photosynthesis
721 differs between C3 and C4 grass species in a comparative experiment. *Plant, Cell and Environment*
722 **34**, 65–75.

723 Thomey M.L., Collins S.L., Friggens M.T., Brown R.F. & Pockman W.T. (2014) Effects of monsoon
724 precipitation variability on the physiological response of two dominant C4 grasses across a
725 semiarid ecotone. *Oecologia* **176**, 751–762.

726 Veldman J.W., Aleman J.C., Alvarado S.T., Anderson T.M., Archibald S., Bond W.J., ... Zaloumis N.P.
727 (2019) Comment on “The global tree restoration potential.” *Science* **366**.

728 Volaire F. (2018) A unified framework of plant adaptive strategies to drought: crossing scales and
729 disciplines. *Global Change Biology* **24**, 2929–2938.

730 Wahl S. & Ryser P. (2000) Root tissue structure is linked to ecological strategies in grasses. *New*
731 *Phytologist* **148**, 459–471.

732 Watcharamongkol T., Christin P.A. & Osborne C.P. (2018) C4 photosynthesis evolved in warm climates
733 but promoted migration to cooler ones. *Ecology Letters* **21**, 376–383.

734 Way D.A., Katul G.G., Manzoni S. & Vico G. (2014) Increasing water use efficiency along the C3 to C4
735 evolutionary pathway: A stomatal optimization perspective. *Journal of Experimental Botany* **65**,
736 3683–3693.

737 Wilcox K.R., Koerner S.E., Hoover D.L., Borkenhagen A.K., Burkepile D.E., Collins S.L., ... Smith M.D.
738 (2020) Rapid recovery of ecosystem function following extreme drought in a South African savanna
739 grassland. *Ecology* **101**, 1–9.

740 Williams K.J., Wilsey B.J., Mcnaughton S.J. & Banyikwa F.F. (1998) Temporally Variable Rainfall Does Not
741 Limit Yields of Serengeti Grasses. *Oikos* **81**, 463–470.

742 Xu Z. & Zhou G. (2008) Responses of leaf stomatal density to water status and its relationship with
743 photosynthesis in a grass. *Journal of Experimental Botany* **59**, 3317–3325.

744 Yang S., Zhang J., Han J., Wang J., Zhang S., Bai Y., ... Rong Y. (2021) Evaluating global ecosystem water
745 use efficiency response to drought based on multi-model analysis. *Science of the Total Environment*
746 **778**, 146356.

747 Zhou H., Helliker B.R., Huber M., Dicks A. & Akçay E. (2018) C4 photosynthesis and climate through the
748 lens of optimality. *Proceedings of the National Academy of Sciences of the United States of America*
749 **115**, 12057–12062.

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752 **Figure Legends**

753 **Figure 1:** Leaf cross-sections of each major grass tribe stained with Safranin Red and Fast
754 Green. Top left, Andropogoneae; top right, Cynodonteae; bottom left, Danthoneiae; bottom right,
755 Paniceae. Image taken with a Zeiss 880 confocal microscope.

756 **Figure 2:** Principal components analysis (PCA) of mean trait values (in red text) of species in
757 the dry down phase of the experiment. This PCA provides a summary of species in multivariate
758 trait space using the first two PC axes, which together account for 72% of the trait variation. PC1
759 was most associated with variation in water use and rooting strategies whereas PC2 was
760 primarily associated with photosynthetic rate. Information concerning PCA axes importance and
761 subsequent loadings are located in Supporting Table 6. Andropogoneae (light red), Cynodonteae
762 (grey), Danthoneiae (green), and Paniceae (blue); each point is a species mean.

763 **Figure 3:** A) Number of days each species and tribe lasted before stomatal closure and
764 rewatering occurred. B) The physiological recovery (A_n) compared to Day 0 or Initial physiology
765 (measured here as a percent). Dashed line signifies a complete 100% recovery of physiological
766 function (i.e., A_n at or above its initial value). Andropogoneae (light red), Cynodonteae (grey),
767 Danthoneiae (green), Paniceae (blue), Paspaleae (yellow), Poeae (red); each point is a species
768 mean and \pm SE.

769 **Figure 4.** Relationship between stomatal count per entire leaf cross-section and days in drought
770 before “Recovery”. Andropogoneae (light), Cynodonteae (grey), Danthoneiae (green), and
771 Paniceae (blue); each point is a species mean and \pm SE.

772 **Figure 5:** A) The relationship between leaf biomass and root biomass. B) Specific leaf area
773 against specific root length. Andropogoneae (light red), Cynodonteae (grey), Danthoneiae
774 (green), Paniceae (blue), Paspaleae (yellow), Poeae (red); each point is a species mean and \pm SE.

775

776 **Conflict of interest statement**

777 We declare that the submitted work was not carried out in the presence of any personal,
778 professional or financial relationships that could potentially be construed as a conflict of interest.

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