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Seasonal soil moisture variability, not drought, drives differences in photosynthetic physiology of two C₄ grass species

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Abstract Seasonal changes in soil moisture drive the phenology of grassland plants during the growth period, yet we do not understand the biochemical processes underlying seasonal changes in grass photosynthesis. This lack of understanding at least partially stems from the paucity of information describing the metabolic and stomatal responses of dominant C₄ grass species to drought. Here, we characterized seasonal patterns in plant physiology, including stomatal and non-stomatal limitations of photosynthesis, for two dominant C₄ grass species, *Bouteloua curtipendula* and *Schizachyrium scoparium*. We also tested how rainfall reduction might modify seasonal patterns in photosynthesis for both species. Specifically, we predicted that drought would reduce carboxylation (V_{cmax}) and electron transport (J_{max}), thereby limiting net CO₂ assimilation (A) and suppressing biomass for *Bouteloua curtipendula* and *Schizachyrium*

scoparium. We tested these predictions using the first in situ drought experiment to measure the impact of drought on C₄ physiology. Our results demonstrate that photosynthesis of co-occurring, dominant C₄ grasses is primarily limited by RuBP regeneration. Interestingly, J_{max} was not reduced by drought for either *B. curtipendula* or *S. scoparium*, enabling both species to maintain constant A under drought. Seasonal changes in soil moisture did decrease J_{max} , which in turn reduced A , for *S. scoparium*. Photosynthesis of *B. curtipendula*, on the other hand, remained stable throughout the growing season. That two common C₄ species possess such different biochemical and photosynthetic responses to soil moisture highlights the physiological variability inherent within plant functional groups, and underscores the need for more field studies of C₄ biochemistry.

Keywords V_{cmax} · J_{max} · Primary production · Grasslands · Rubisco · RuBP

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Introduction

Seasonal changes in soil moisture drive the phenology of grassland plants throughout the growth period. Water availability is often highest during cool, wet months in the spring (Knapp et al. 2001; Cherwin and Knapp 2012; Hoover et al. 2014). As temperature rises, increased evapotranspiration rates reduce soil moisture by mid to late summer (Knapp et al. 2001;

Cherwin and Knapp 2012; Hoover et al. 2014). Interestingly, photosynthesis of dominant C_4 grasses often shows the same seasonal pattern. Per-area foliar photosynthesis rates are highest during the cool summer months and decline by roughly 50% during the mid-summer (Dietrich and Smith 2016). This phenological shift in leaf-level photosynthesis appears to be driven by seasonal reductions in soil moisture (Hoover et al. 2014), meaning that mid-summer months are when grass productivity is most water-limited (Knapp et al. 2001). Yet no study has examined the leaf biochemical mechanisms by which seasonal changes in soil moisture drive grass photosynthesis, nor tested how rainfall reductions throughout the growing season might impact seasonal patterns in leaf biochemistry or gas exchange.

Tallgrass prairies are undoubtedly water-limited; rates of net CO_2 assimilation (A) and stomatal conductance of H_2O vapor (g_{sw}) of many C_3 and C_4 grass species decline when rainfall is limited (Ripley et al. 2010; Taylor et al. 2010, 2011, 2014). For this reason, drought suppresses aboveground net primary production (ANPP) in most North American grasslands (Carroll et al. 2021). Yet the magnitude of reductions in A , g_{sw} , and ANPP depends on the timing of drought relative to plant phenology. A critical climate period analysis of ANPP at Konza Prairie found that tallgrass production was most sensitive to changes in rainfall during the spring when soils were wettest (Craine et al. 2012). However, multiple experimental and observational studies at Konza Prairie and elsewhere have reported that lower precipitation during the mid to late summer months causes the most severe reduction in ANPP (Hoover et al. 2014; Dietrich and Smith 2016; Zeiter et al. 2016; Lemoine et al. 2017). Early-season hydrological droughts have little effect on leaf water potential, A , plant growth rates, or ANPP (Hamerlynck et al. 1997; La Pierre et al. 2011; Dietrich and Smith 2016; Lemoine et al. 2017), whereas mid-summer droughts exacerbate seasonal declines in A by increasing plant water stress (Dietrich and Smith 2016). Late summer droughts that occur at the end of the growing season do not affect plant physiology or ANPP because the growth period has already ended (Lemoine et al. 2018). It is likely that the seasonal variation in the impacts of rainfall reductions stem from the response of leaf biochemistry to drought over the course of the growing season, yet no study has quantitatively linked

seasonal soil moisture, leaf biochemistry, and photosynthesis of C_4 grasses using a mechanistic model.

In part, our lack of understanding in seasonal drought responses exists because we still do not fully understand how rainfall reduction affects non-stomatal and stomatal limitations of photosynthesis in C_4 grasses. Non-stomatal limitations include the rate of Rubisco carboxylase activity (V_{cmax} , generally reflecting CO_2 limitation) and the maximum rate of electron transport driving RuBP regeneration (J_{max} , generally reflecting light limitation). Stomatal limitation describes the extent to which stomatal closure impedes CO_2 diffusion into the mesophyll and, as a result, reduces A . In many C_3 grassland species, non-stomatal limitations intensify during droughts via reduced V_{cmax} (Signarbieux and Feller 2011) and J_{max} (Reed and Loik 2016), leading to lower A . Compared to C_3 species, however, leaf biochemistry of C_4 grasses has been understudied. A three-decade old meta-analysis had already synthesized estimates of V_{cmax} and J_{max} for 109 C_3 plants (Wullschlegel 1993), whereas a recent meta-analysis of C_4 plants found estimates of V_{cmax} and J_{max} for only 49 species, of which only 13 were not agricultural (Pignon and Long 2020). Even recent experiments quantifying the impacts of drought on leaf biochemistry of grassland species exclusively measured C_3 plants, including grasses (Signarbieux and Feller 2011). Perhaps just as problematically, some studies that did quantify V_{cmax} and J_{max} of C_4 species under drought used a C_3 biochemical model (Ulrich et al. 2019), which can yield inaccurate results. Even well-studied C_4 species such as *A. gerardii* only possess information on ambient gas exchange and not the underlying biochemical reactions. Common garden and pot experiments suggest that C_4 photosynthesis is metabolically limited under droughts, which prevents rapid recovery following the alleviation of water stress (Ripley et al. 2007, 2010; Taylor et al. 2014). This might explain why rainfall reductions during the mid-summer period can impose season-long deficits of biomass (Dietrich and Smith 2016), but the extent to which this generalizes to dominant tallgrass species is unknown because there has been no in situ field study of leaf biochemistry in C_4 grasses.

Here, we characterized seasonal patterns in plant physiology, including stomatal and non-stomatal limitations of photosynthesis, for two dominant C_4 grass species, *Bouteloua curtipendula* (PCK C_4

subtype) and *Schizachyrium scoparium* (NADP-ME C₄ subtype). We also sought to understand how rainfall reductions would affect seasonal patterns in these important photosynthetic processes, and thus potentially explain why mid-summer droughts are the most damaging to ANPP. Both *B. curtipendula* and *S. scoparium* are common throughout North American grasslands and contribute substantially to ANPP in these ecosystems. Since dominant grass species often control ecosystem function in grasslands (Smith and Knapp 2003; Smith 2011; Koerner et al. 2014), and leaf physiology drives ecosystem-level production (Walker et al. 2017), understanding how drought impacts the biochemistry of these two dominant C₄ grass species could potentially elucidate the mechanisms by which drought reduces tallgrass productivity. Specifically, we tested the following three hypotheses:

H1 Rainfall reductions will reduce *A* and *g_{sw}* in both *B. curtipendula* and *S. scoparium*. However, the effects of rainfall reduction depend on phenology. Water limitation will minimally affect gas exchange during the spring when soil moisture is highest, but intensify throughout the growing season as soil moisture deficits accrue and temperature increases. This hypothesis is based on similar observations for how phenological drought affected gas exchange in *A. gerardii* (Dietrich and Smith 2016), and statistical modeling that suggests *S. scoparium* flower production is most sensitive to mid- and late-summer droughts (Lemoine et al. 2017). Water use efficiency (WUE) should remain high throughout the growing season, as is common for C₄ plants under drought conditions (Ripley et al. 2010; Taylor et al. 2010).

H2 Despite the reduction in *g_{sw}*, metabolic limitation of photosynthesis will be the dominant mechanism by which rainfall reduction will reduce *A* for both *B. curtipendula* and *S. scoparium*. The C₄ photosynthetic pathway concentrates CO₂ into bundle sheath cells, enabling plants to maintain high *A* despite stomatal closure. As a result, rainfall reduction will induce metabolic limitations by lowering *V_{cmax}* or *J_{max}*. This hypothesis is based on observations that drought reduces both *V_{cmax}* and *J_{max}* in C₃ species (Signarbieux and Feller 2011; Reed and Loik 2016), and can induce strong metabolic limitation of C₄ grass species (Ripley et al. 2010). In our system,

we hypothesized that rainfall reduction would impede *J_{max}* more than *V_{cmax}* because C₄ species concentrate CO₂ internally and rarely experience CO₂ limitation. Further, reduced electron transport under water limitation is the dominant mechanism by which drought induces metabolic limitation of photosynthesis in some C₄ plant species (Ripley et al. 2007).

H3 Reduced *A* under rainfall reduction will suppress ANPP of both *B. curtipendula* and *S. scoparium*. Similar patterns have been observed for *A. gerardii* (Dietrich and Smith 2016), but the correlation between *A* and ANPP is not clear-cut. Many species maintain *A* during drought but suffer reduced biomass, likely because plants can reallocate biomass from shoots to roots without reducing per area CO₂ assimilation (Wilcox et al. 2017). Alternatively, diminished *A* during drought does not always reduce end-of-season biomass (Fernández and Reynolds 2000).

Materials and methods

Site description

We conducted our experiment at the University of Wisconsin – Milwaukee at Waukesha field station in Oconomowoc, Wisconsin. The field station is an approximately 40 ha reserve containing a variety of natural and restored habitats, including oak savanna, jack pine forests, maple forests, oak forests, and tallgrass prairie. The tallgrass prairie is dominated by the grasses *A. gerardii*, *B. curtipendula*, and *S. scoparium*, and the forbs *Echinacea purpurea*, *E. paradoxa*, *Dalea spp.*, and *Monarda fistulosa*. There were no C₃ grasses in our study site. The site is relatively cool and mesic. During the growing season (April–September), mean daily temperatures are around 20 °C and mean precipitation is approximately 520 mm (Fig. 1A). Rainfall occurs evenly from May–November (Fig. S1). Drought is a common feature in southern Wisconsin, with 1–2 severe droughts occurring every 30 years, a number which is predicted to double by 2100 (Sheffield and Wood 2008). Averaged from 2090 to 2099, global circulation models predict that Great Lakes will experience at least one severe

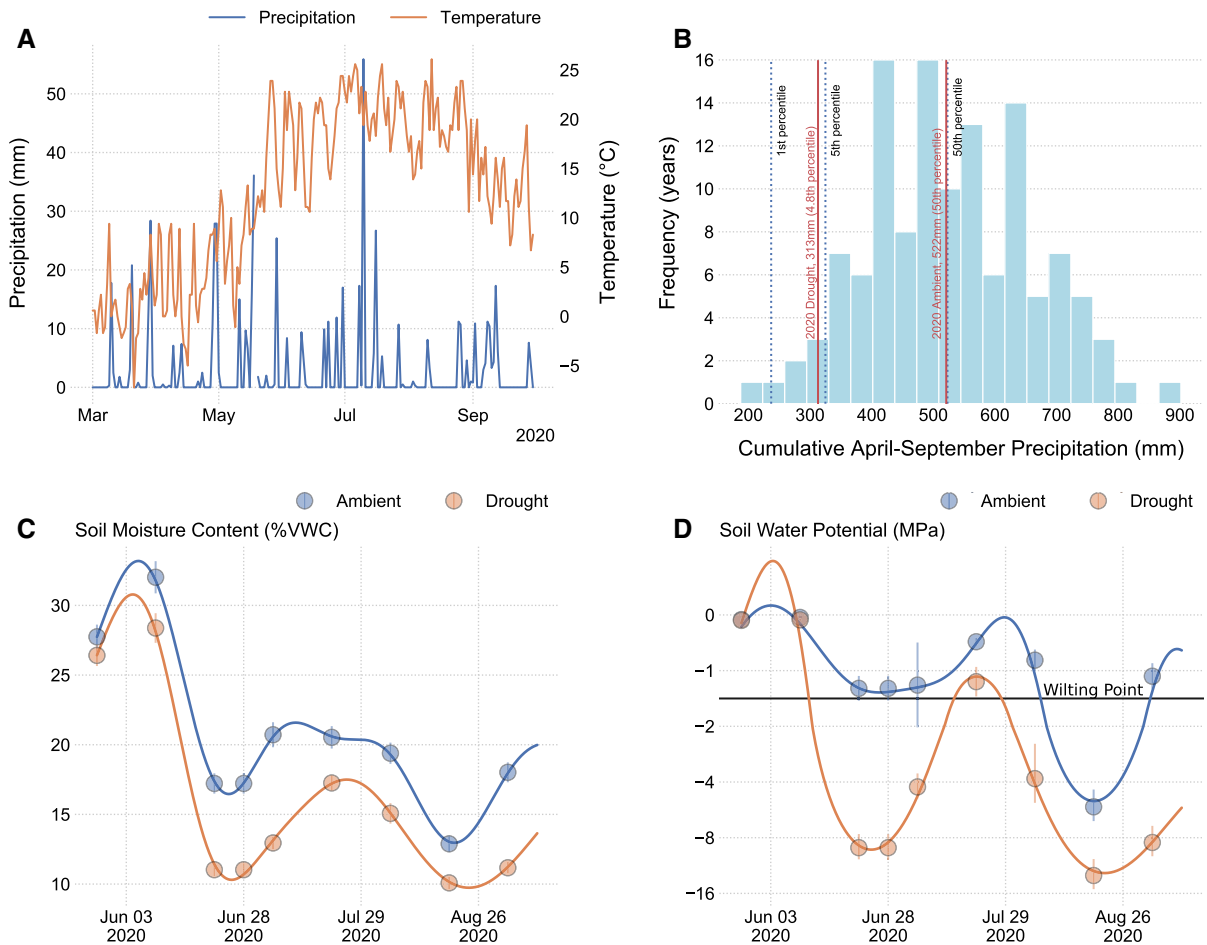


Fig. 1 Weather, climate, and soil moisture profiles during our experiment. **A** Daily total precipitation and average temperature for our study site at Oconomowoc, WI during the experimental year of 2020. Precipitation data come from a long-term weather station in Waukesha, WI (USC00478937), and temperature data were obtained from a nearby weather station in Brookfield, WI (USC00471062). **B** Histogram of total growing season (April–September) precipitation, based on 127 years of data from Waukesha, WI (USC00478937). Dotted blue lines show the 50th, 5th, and 1st percentiles of growing season pre-

cipitation. Red lines show growing season precipitation under ambient conditions and in our experimental manipulations (40% reduction from ambient). **C** Soil moisture profiles during the course of our experiment. Points and bars show means ± 1 SE, and trend lines were fitted via a Gaussian Process Model. **D** Soil water potential profiles during the course of our experiment, as estimated from a soil water release curve. Points and bars show means ± 1 SE, and trend lines were fitted via a Gaussian Process Model

drought in this time period (Sheffield and Wood 2008).

Rainfall reduction treatments

To reduce rainfall, we installed ten rainout shelters in the early spring of 2020 following the Drought-Net protocol. On 15 April 2020, we designated twenty 2×2 m experimental plots. Plots were randomly assigned to ‘Ambient’ or ‘Drought’ treatments ($n = 10$

per treatment). Ambient plots were left open to the environment and received 100% of growing season precipitation. In drought plots, growing season precipitation was reduced by 40% with a passive rainout shelter design (Yahdjian and Sala 2002). Based on 127 years of daily rainfall data from a nearby weather station (NOAA NCDC Station ID USC00478937, Waukesha WWTP, WI US), a 40% reduction in growing season precipitation during 2020 was below the 5th percentile and thus represents a severe drought

relative to the annual rainfall at our study site (Fig. 1B). Each passive rainout shelter consisted of a 2×2 m wooden frame enclosing a 1×1 m measurement plot topped with a roof. Roofs consisted of nine polycarbonate sheets (1.8 m long, 15 cm wide). Plastic strips were evenly spaced across the roof to cover 40% of shelter area, and angled to slope west with the high and low sides being 1.5 m and 1 m from the ground, respectively. Shelter roofs were left in place until 15 September 2020, after which they were removed and the experiment terminated. This chronic drought design (Carroll et al. 2021) replicates severe drought at our field site; drought years are typified by season-long chronic daily rainfall shortages (Fig. S1). To verify that our treatments imposed a drought, we measured soil volumetric water content (%VWC) every two weeks throughout the experiment using a Field Scout TDR 150 with 12 cm probes. On each sampling date, we recorded three %VWC measurements per plot and averaged the estimates to produce a single value for each plot. We converted %VWC to soil water potential using a soil water release curve (Fig. S2), measured by The METER Group. Prior to the experiment in May, we removed all aboveground biomass within the 2×2 m plot to accurately estimate aboveground net primary production (ANPP).

Photosynthesis

We measured gas exchange on healthy, intact leaves using an LI-6800 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Every week, we measured one individual of both *B. curtispindula* and *S. scoparium* from three randomly selected plots for both Ambient and Drought treatments ($n=3$ per species per treatment, each week). Replicate numbers were limited by time and weather, as we measured curves on cloudless, rain-free days between 11:00 and 15:00. Prior to measurements, leaves acclimated to saturating light ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and atmospheric CO_2 ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 15 min. Leaf temperature and vapor pressure deficit were maintained at ambient conditions, and flow rate set to $400 \mu\text{mol s}^{-1}$. For *A-Ci* curves, we measured A under a CO_2 ramp of 400, 300, 200, 100, 50, 400, 400, 600, 800, 1000, 1200, $400 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$. Midway through the experiment, we added additional $400 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ during the middle and end to allow for more recovery time and improve curves. We allowed A and g_{sw} to

stabilize for 90–180 s prior to recording each measurement. Measurements occurred from early June through early September. In total, we collected 1859 measurements from 148 curves across both species.

ANPP

We estimated ANPP for *B. curtispindula* and *S. scoparium* by harvesting aboveground biomass at the end of the growing season on 14 September 2020. In each 1×1 m plot, we randomly placed two 20×50 cm (0.1 m^2) frames and harvested all aboveground biomass for *B. curtispindula* and *S. scoparium* from within each frame. Fresh biomass was dried at 60°C until mass was stable before being weighed. Biomass in each frame was standardized to g m^{-2} , and then the two frames averaged to produce a single estimate per plot.

Data analysis

To analyze soil moisture, we first aggregated plot measurements by week of the experiment. Because soil moisture data were a time series, we accounted for autocorrelation using a Gaussian Process Model (GPM) to interpolate soil moisture trends during unobserved weeks (*see below*).

We assessed the impact of drought on leaf biochemistry and photosynthesis by fitting empirical and biochemical models to each *A-Ci* curve. First, we calculated A and g_{sw} for each curve by averaging A and g_{sw} for all measurements made at 40 Pa (atmospheric CO_2). We next calculated intrinsic WUE as A/g_{sw} . Stomatal limitation was determined numerically following (Long and Bernacchi 2003). Briefly, for each *A-Ci* curve, we fit an asymptotic monomolecular function of the form:

$$A = a - (a - b)\exp(-cCi)$$

to quantify the degree of stomatal limitation as A_{obs}/A_{40} , where A_{obs} is net assimilation at the observed Ci when the leaf was held at 40 Pa CO_2 , and A_{40} is the what A would be in the absence of stomatal closure, when $Ci=40$ Pa. Thus, lower values of A_{obs}/A_{40} indicate stronger stomatal limitation. We also calculated CO_2 supply curves as $(1/g_{\text{sw}})(Ca - Ci)$, where Ca and Ci are ambient and intercellular CO_2 concentrations, respectively, in ppm. After fitting the empirical *A-Ci*

curve, we fit a biochemical C_4 model to each dataset (von Caemmerer 2000; Bellasio et al. 2016). See Supplemental Methods for details.

We analyzed the effects of phenology and drought on leaf biochemistry (A , g_{sw} , WUE, A_{obs}/A_{40} , V_{cmax} , J_{max} , R_d) using Gaussian Process Models (GPMs). We chose Gaussian Process Models because they automatically incorporate temporal autocorrelation and allow for nonlinear trends (Rasmussen and Williams 2005; Roberts et al. 2013). The advantage of GPMs is that the Bayesian treatment smooths outliers and thus avoids overfitting and mistaking noise for a signal (Lemoine et al. 2016; Lemoine 2019). Both the response variables and predictor (week of experiment) were standardized to $N(0,1)$ prior to analysis. GPM parameters were optimized via maximum likelihood. It is worth noting that GPMs emphasize accurately estimating effects, rather than statistical significance. See Supplemental Methods for more details.

We also examined correlations between A and each of g_{sw} , V_{cmax} , and J_{max} using Bayesian linear regressions. Regressions included drought treatment as an interactive covariate to determine whether drought altered the relationship between A and leaf biochemistry. We placed a hierarchical $N(0,1)$ prior on the standard deviation of regression coefficients to provide a degree of regularization (Lemoine et al. 2016; Lemoine 2019). We analyzed aboveground biomass with a Bayesian two-factor ANOVA that included both species identification, drought treatment, and their interaction as predictors. As with regressions, we place a hierarchical $N(0,1)$ prior on the standard deviation of regression coefficients. In all Bayesian models, both the predictor and response were standardized to $N(0,1)$ prior to analysis. Due to nonlinearities and heteroskedasticity, A was log-transformed then standardized before regressing against V_{cmax} .

To quantitatively analyze the pathways by which soil moisture influenced photosynthesis, we conducted a path analysis relating environmental variables to photosynthetic physiology for both *B. curtipendula* and *S. scoparium*. The model allowed both precipitation and air temperature to determine soil VWC, which in turn affected V_{cmax} and J_{max} , which both drove net CO_2 assimilation (A , see Fig. 6 for diagram of conceptual model). Because we were working with soil VWC measurements, our path analysis dataset was restricted to those alternating weeks during which we measured VWC. Also because soil

moisture was measured on a weekly basis, often on different days than A - C_i curves, we had to bin all weather, soil, and plant data by week. During each week, we quantified the abiotic environment as the average daily temperature and the sum of precipitation for that week. Soil VWC, V_{cmax} , J_{max} , and A were first averaged at the plot level, and then at the treatment level, such that each week contained two estimates of VWC and physiology, one for ambient and one for drought treatments. The path analysis was fit by minimizing the Wishart log-likelihood, and goodness of fit was evaluated using the χ^2 statistic. All variables were standardized prior to analysis to enable the direct comparison of path coefficients.

All analyses were performed in Python v3.7.9. Gaussian process models were fit using the *scikit-learn* module, and Bayesian models were fit using *cmdSTAN* 2.25.0 accessed via the *cmdstanpy* 0.9.67 module. Path analyses were fit using the *semopy* 2.0.18 module. All raw data, cleaned data, Python scripts, and figures (including raw A - C_i curves, cleaned A - C_i curves, A - C_i curves fitted with the empirical model, and A - C_i curves fitted with the biochemical model) are available on figshare at <https://doi.org/10.6084/m9.figshare.17209244.v1>.

Results

Precipitation and soil moisture

During the 2020 growing season, our field site in Oconomowoc received 522 mm of rainfall (Fig. 1A). Daily rainfall during extreme drought years is typically 33% lower throughout the growing season (Fig. S1), and the 40% rainfall reduction imposed here simulated a drought falling just below the 5th percentile of growing season precipitation during the past 127 years (Fig. 1B). As a result, soil moisture was substantially lower in drought plots than in ambient plots. During late spring, soil moisture was ~27% in both treatments (Fig. 1C). By early summer, drought treatments had ~5% lower VWC than controls, though VWC was still above 25% in both treatments (Fig. 1C). By mid- and late-summer, soil moisture varied between ~15 and 20% in controls and between ~10–20% in drought treatments (Fig. 1C). In fact, drought plots reduced VWC to 40% of the ambient value throughout most of the growing season,

except late July/early August, closely corresponding to our 40% rainfall reduction (Fig. 1C). Using the soil water release curve (Fig. S2), we converted VWC to soil water potential as an estimate of water availability. In the ambient plots, soil water potential only once fell below the plant wilting point in early August (-1.5 MPa, Fig. 1D). In contrast, soil water potential was consistently below the plant wilting point, often at or below -4 MPa, except for a brief rainy period during mid-July (Fig. 1D). Thus, the reduction in soil moisture did impose conditions unfavorable to plant water uptake in the top 10 cm of soil.

Bouteloua curtipendula

Gas exchange and leaf biochemistry of *B. curtipendula* were both resilient to rainfall reductions. Drought affected neither A nor g_{sw} ; both stayed relatively constant at $15 \mu\text{mol CO}_2$ and $0.2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively, throughout the growing season (Fig. 2A, B). The temporal stability and lack of drought response in A and g_{sw} translated into a generally stable WUE that fluctuated between 80

and $100 \mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$ throughout the growing season (Fig. 2C). Stomatal limitation increased slightly throughout the growing season, as A_{obs}/A_{40} declined from $\sim 92\%$ to $\sim 85\%$ between late May and late August (Fig. 2D). A minimum of 85% is still fairly high, however, and indicates that CO_2 supply remained high enough to allow plants to operate near their theoretical maximum A . Indeed, averaged across the entire growing season, drought had no effect on any gas exchange parameter (Figs. S4–S6, Tables S1–S2). Leaf biochemistry was similarly resilient to drought averaged across the entire growing season (Figs. S4–S6, Tables S1–S2), but was more phenologically labile. Neither J_{max} (Fig. 2F) nor R_d (Fig. S3) changed directionally throughout the growing season, and neither were affected by the drought treatment. On the other hand, V_{cmax} did appear to be consistently lower in drought conditions throughout the growing season (Fig. 2E), but the $8 \pm 3\%$ reduction is not statistically significant (Table S2) and likely biologically irrelevant, especially compared to the 72% decline in V_{cmax} caused by phenology. From spring to late summer, V_{cmax} declined from a maximum of $35 \mu\text{mol}$

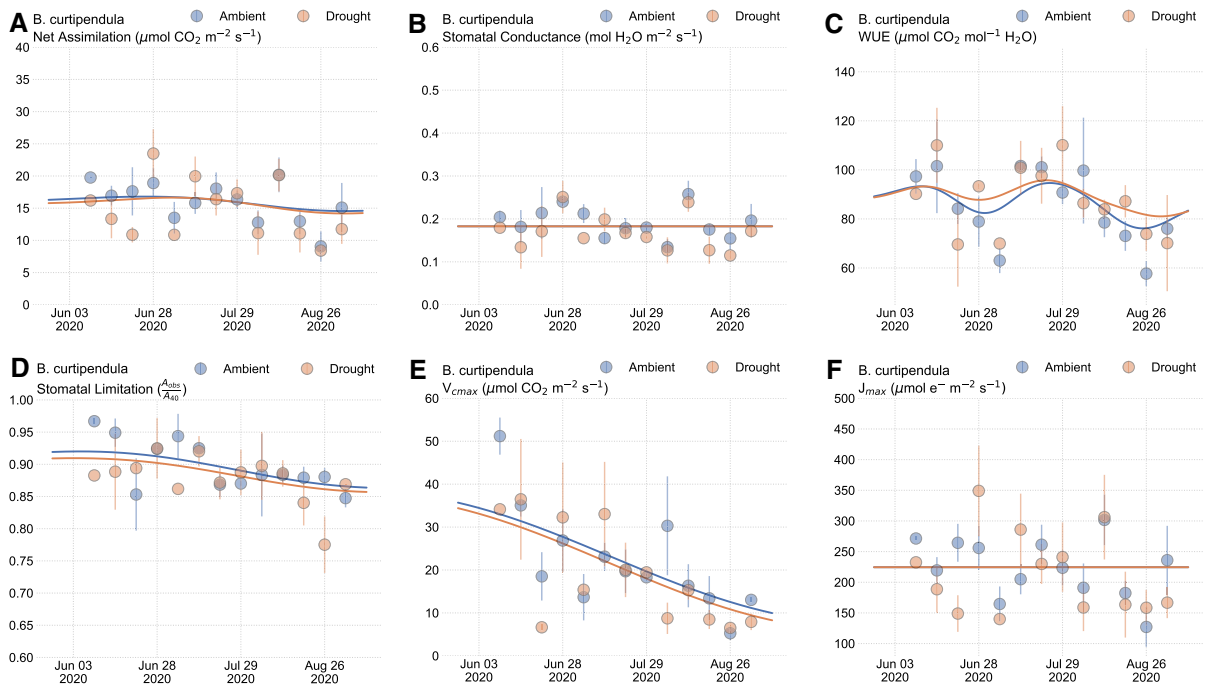


Fig. 2 Phenological patterns in gas exchange and leaf biochemistry, based on A - C_i curves, for *B. curtipendula*. **A** Net assimilation, **B** stomatal conductance, **C** water-use efficiency,

D stomatal limitation, **E** V_{cmax} , and **F** J_{max} . In all plots, points and bars represent the mean ± 1 SE, and the trend lines were fitted with Gaussian Process Models

$\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in spring to a minimum of $10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ by late summer (Fig. 2E). The steady decline in V_{cmax} does not appear to have been coupled to soil moisture, given that VWC dropped sharply at the beginning of the growing season and then remained stable (Fig. 1C). It also appears that the decline in V_{cmax} did not alter A , which remained stable despite the sharp reduction in Rubisco activity.

The decoupling of A from V_{cmax} is apparent by examining relationships between A and stomatal/non-stomatal limiting factors. First, the relationship between A and g_{sw} was strongly positive for both control and drought conditions ($\text{Pr}(\beta_{g_{\text{sw}}} > 0) = 1.00$, Fig. 3A). However, drought appeared to slightly strengthen the dependence of A on g_{sw} ($\text{Pr}(\beta_{g_{\text{sw}}:\text{drought}} > 0) = 0.95$), but this effect was relatively weak compared to the overall relationship between A and g_{sw} (Fig. 3A). Surprisingly, V_{cmax} did not have a strong, linear relationship with A , but instead was best characterized by an asymptotic, log-linear response ($\text{Pr}(\beta_{V_{\text{cmax}}} > 0) = 1.00$, Fig. 3B). The log-linear relationship suggests that A might be strongly Rubisco limited when $V_{\text{cmax}} < 10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, but becomes relatively independent of Rubisco activity at higher V_{cmax} rates (Fig. 3B). Drought appeared to slightly strengthen the dependence of A on V_{cmax} by reducing the severity of the asymptote ($\text{Pr}(\beta_{V_{\text{cmax}}:\text{drought}} > 0) = 0.93$), but again the difference between ambient and drought conditions was relatively weak compared to the overall relationship (Fig. 3B). The power relationship explains why V_{cmax} could decline without affecting

A . While individual observations fell below the $10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ threshold and did likely reduce A on any given day, the mean trend never declined below the threshold value for V_{cmax} (Fig. 2E), such that phenological reductions in V_{cmax} never limited A . Instead, A appeared to be tightly coupled to J_{max} , with a strong positive relationship suggesting that electron transport is the primary factor limiting A in *B. curtispindula* ($\text{Pr}(\beta_{J_{\text{max}}} > 0) = 1.00$, Fig. 3C). Drought did not affect the dependency of A on J_{max} ($\text{Pr}(\beta_{J_{\text{max}}:\text{drought}} < 0) = 0.82$, Fig. 3C), which is unsurprising given that drought had no effect on J_{max} throughout the growing season (Fig. 2F). Thus, constant RuBP limitation throughout the growing season led to highly stable CO_2 assimilation for *B. curtispindula*.

The above analyses suggest that *B. curtispindula* maintains a stable rate of net CO_2 assimilation because A is limited entirely by electron transport rates (RuBP regeneration) and not by CO_2 supply (Rubisco carboxylation). Supply and demand curves for *B. curtispindula* support this argument. At no point during the summer did CO_2 supply fall below CO_2 demands (Figs. 6, S6), meaning that stomatal closure never reduced C_i to a point that would inhibit photosynthesis. The week in mid-July where the A - C_i curve had a higher asymptote under drought conditions (Fig. 6) matches the week in July where J_{max} in drought plots exceeded J_{max} in ambient plots (Fig. 2), further supporting the conclusion that A was limited by J_{max} .

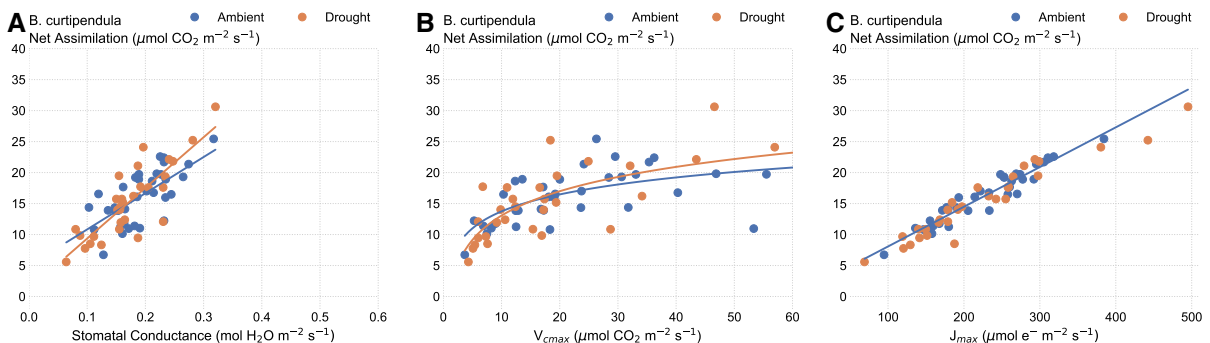


Fig. 3 Relationships between gas exchange and stomatal/non-stomatal processes for *B. curtispindula*. **A** Scatterplot showing the relationship between A and g_{sw} . **B** Scatterplot showing the relationship between A and V_{cmax} . Note that A and V_{cmax} were log-transformed prior to analysis to account for the asymptotic

relationship and heteroskedasticity. **C** Scatterplot showing the relationship between A and J_{max} . Points show individual plant measurements, lines show the best fit model from Bayesian regression. Two lines are shown only when there is a significant main effect or interaction effect of Drought

Schizachyrium scoparium

Like *B. curtipendula*, gas exchange of *S. scoparium* was resilient to experimentally reduced rainfall (Figs. S4–S5, Tables S1–S2), but did show a much tighter correspondence with seasonal changes in VWC. As with VWC, A was initially high in early spring but declined sharply as VWC declined and temperatures rose throughout late May into mid June (Fig. 4A). In late August, there was evidence of lower A in drought plots, but the effect size was a relatively small reduction of $\sim 2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, or roughly 13% of A in control plots (Fig. 4A). Unlike A , g_{sw} did not show either a response to drought or a seasonal pattern and remained constant at approximately $0.2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (Fig. 4B), roughly the same rate as for *B. curtipendula* (Fig. 2B). The constancy of g_{sw} throughout the growing season led WUE to generally reflect patterns in A , being highest in the early spring when soil moisture was high, declining in early summer, increasing in mid-summer with a brief rainfall pulse, and then declining towards the end of the growing season (Fig. 4C). It did appear that drought might

have affected WUE, but the effect was inconsistent and noisy throughout the experiment. In early summer, WUE was at most 10% lower in drought plots as VWC declined from early spring levels, before becoming $\sim 10\%$ higher in the mid-summer with a rainfall pulse, and eventually reaching parity with control plots in late summer (Fig. 4C). Shifting WUE and A were unrelated to A_{obs}/A_{40} , which was constant at $\sim 85\%$ across the entire growing season (Fig. 4D). The relatively constant A_{obs}/A_{40} throughout the growing season suggests that neither phenology nor drought affected the rate of CO_2 supply relative to demand.

Non-stomatal factors, however, were phenologically variable in *S. scoparium*, evidenced by strong changes in both V_{cmax} and J_{max} throughout the growing season. In early spring, both V_{cmax} and J_{max} were elevated, and then both declined into early summer (Fig. 4E, F). Following the mid-summer rainfall pulse, both V_{cmax} and J_{max} increased, but V_{cmax} increased by roughly 100% relative to early summer back to early spring levels, whereas the increase in J_{max} was a more modest 12.5% (Fig. 4E, F). In

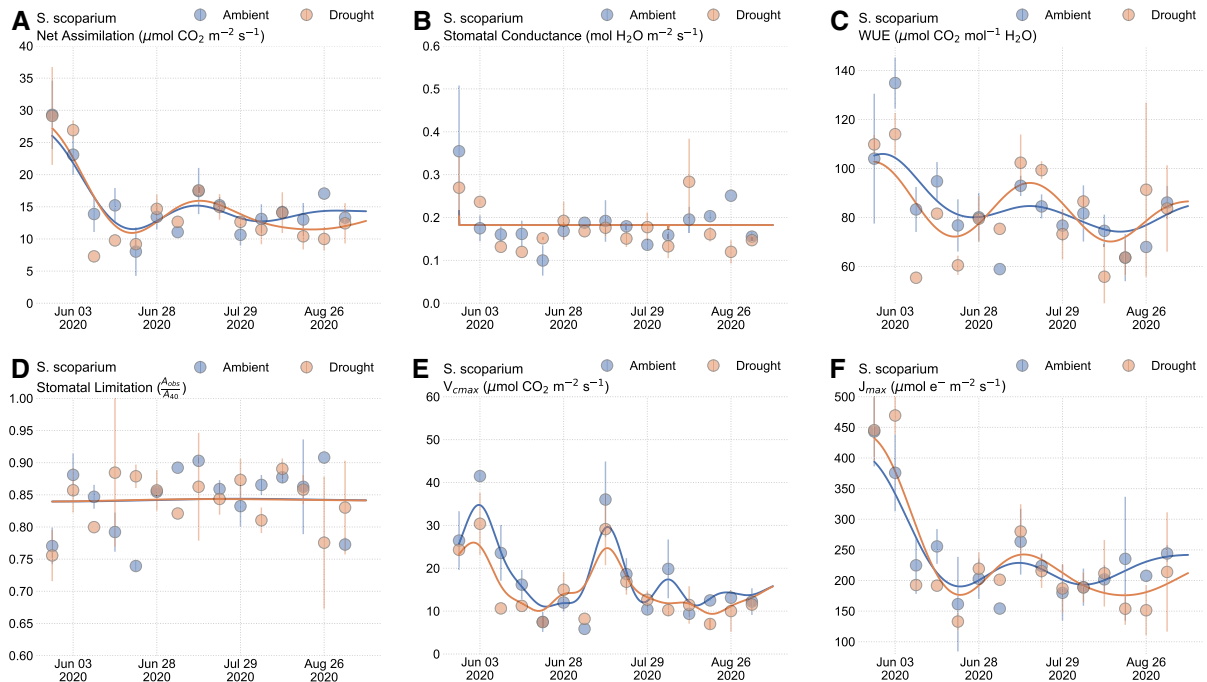


Fig. 4 Phenological patterns in gas exchange and leaf biochemistry, based on A - C_i curves, for *S. scoparium*. **A** Net assimilation, **B** stomatal conductance, **C** water-use efficiency,

D stomatal limitation, **E** V_{cmax} , and **F** J_{max} . In all plots, points and bars represent the mean ± 1 SE, and the trend lines were fitted with Gaussian Process Models

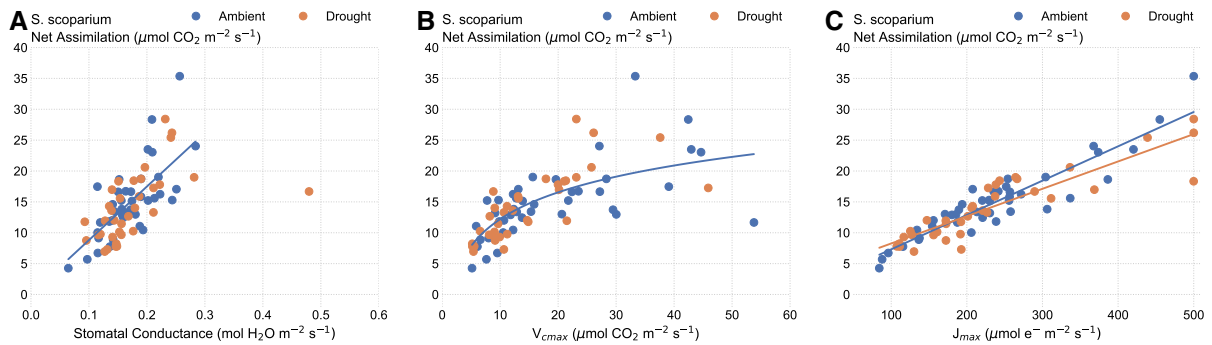


Fig. 5 Relationships between gas exchange and stomatal/non-stomatal processes for *S. scoparium*. **A** Scatterplot showing the relationship between A and g_{sw} . **B** Scatterplot showing the relationship between A and V_{cmax} . Note that A and V_{cmax} were log-transformed prior to analysis to account for the asymptotic

relationship and heteroskedasticity. **C** Scatterplot showing the relationship between A and J_{max} . Points show individual plant measurements, lines show the best fit model from Bayesian regression. Two lines are shown only when there is a significant main effect or interaction effect of Drought

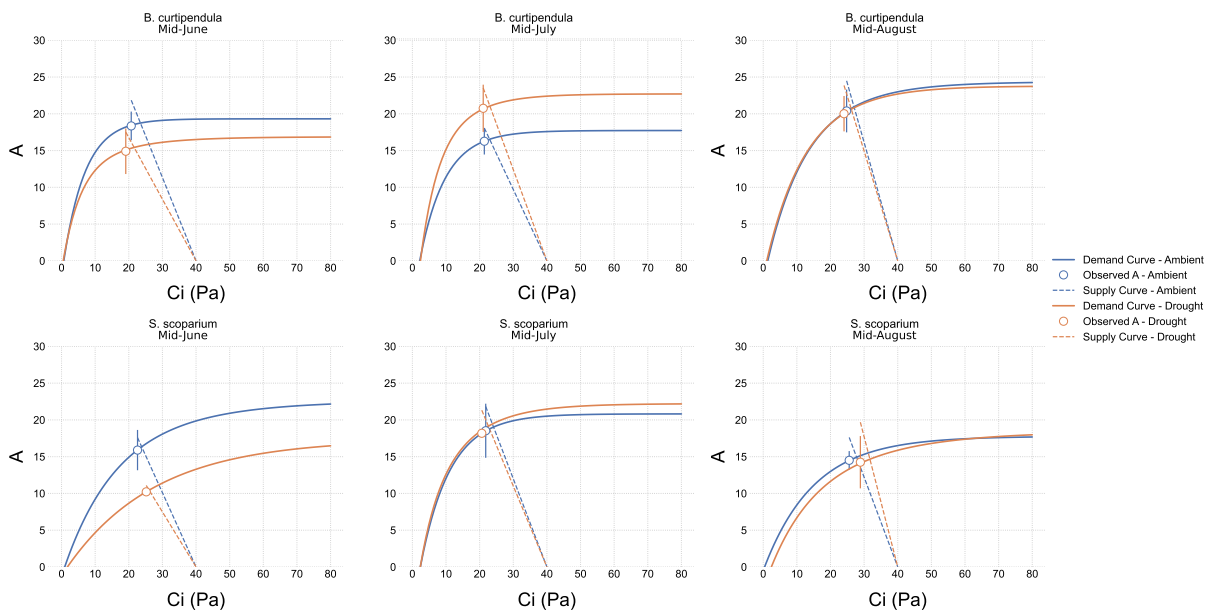


Fig. 6 Select A - C_i demand and CO_2 supply curves for *B. curtipendula* and *S. scoparium*. These curves represent the average curve for all measurements taken during the given week. Curves are the empirical fit, curves for the biogeochemical model are available at <https://doi.org/10.6084/m9.figshare.17209244.v1>. The CO_2 supply curve was estimated as

$A = (1/g_{sw}) (C_a - C_i)$ where C_i is internal CO_2 concentrations as measured by the LI6800 (ppm) and C_a was set to 400 ppm CO_2 , representing ambient concentrations. The CO_2 demand is the mean $\pm$ one standard error A when chamber conditions were set at 400 ppm CO_2 . Curves for all weeks are presented in Figs. S6–S7

fact, the seasonal pattern in J_{max} closely mirrored seasonal patterns in both VWC and A , indicative of a strong dependence of J_{max} on seasonal soil moisture, which in turn drove seasonal variation in A (Figs. 2, 4A, F). As with *B. curtipendula*, drought appeared to impose a consistent, but biologically

unimportant reduction in V_{cmax} of *S. scoparium* throughout the experiment. The effect of drought on J_{max} tracked the drought effect on A , being generally unimportant throughout the growing season except for an $\sim 20\%$ reduction towards late summer (Fig. 4A, F).

As with *B. curtipendula*, a decoupling of A from V_{cmax} suggests that electron transport rate was the strongest limiting factor for *S. scoparium* photosynthesis. First, A was linearly related to g_{sw} ($\Pr(\beta_{g_{\text{sw}}} > 0) = 1.00$), and drought did not affect this relationship ($\Pr(\beta_{g_{\text{sw}}:\text{drought}} > 0) = 0.65$) (Fig. 5A). As with *B. curtipendula*, the relationship between A and V_{cmax} for *S. scoparium* was log-linear, suggesting strong Rubisco control of A at low V_{cmax} but relatively little effect of Rubisco activity on A beyond a threshold of 10–20 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ($\Pr(\beta_{V_{\text{cmax}}} > 0) = 1.00$, Fig. 5B). Drought did not impact the shape of this relationship ($\Pr(\beta_{V_{\text{cmax}}:\text{drought}} > 0) = 0.84$). The asymptotic relationship explains why the large mid-summer increase in V_{cmax} to 20–30 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ did not result in a concomitant large increase in A ; V_{cmax} exceeded the threshold, and the relatively small increase in J_{max} during this time period limited the responsiveness of A (Figs. 4E, F, 5B). Indeed, A was strongly and linearly related to J_{max} ($\Pr(\beta_{J_{\text{max}}} > 0) = 1.00$), confirming that seasonal changes in J_{max} drove changes in A (Fig. 5C). Drought did slightly weaken the A – J_{max} relationship ($\Pr(\beta_{J_{\text{max}}:\text{drought}} < 0) = 0.98$), but this effect was minimal compared to the overall relationship (Fig. 5C). Based on these relationships, it appears that seasonal changes in VWC led to near identical changes in J_{max} , which in turn drove seasonal changes in A .

That J_{max} , rather than CO_2 limitation of V_{cmax} , drove changes in A for *S. scoparium* is further supported by supply–demand analysis. As for *B. curtipendula*, CO_2 supply for *S. scoparium* never fell below CO_2 demand throughout the growing season (Figs. 6, S7). This matches our observations of relatively low stomatal limitation; stomatal closure during the summer did not inhibit uptake of CO_2 enough to inhibit carboxylation, which is to be expected for C_4 grasses (Figs. 5, 6).

Path analysis

Our path analysis allowed us to determine whether phenological changes were driven by changes in soil moisture, air temperature, or both, since the seasonal decline in soil moisture also corresponded with a seasonal increase in ambient air temperature (Fig. 1). Our conceptual model allowed ambient air temperature to indirectly influence photosynthesis via changes in

soil moisture and to directly impact photosynthesis by increasing V_{cmax} , J_{max} , and A , all of which are known to be temperature-dependent (Fig. 7). For *B. curtipendula*, the conceptual model fit the data well ($\chi^2 = 4.94$, $p = 0.551$). A negative relationship between air temperature and soil moisture suggests that increasing air temperatures during the growing season drive declines in soil VWC, while the relative consistency of weekly precipitation decoupled changes in soil VWC from rainfall (Fig. 7). The resultant decline in soil VWC then caused declines in both V_{cmax} and J_{max} , but only J_{max} appeared to influence A in *B. curtipendula* (Fig. 7). A similar pathway occurred for *S. scoparium* photosynthesis, with two main differences: both V_{cmax} and J_{max} influenced A , and air temperature also had a direct effect on A in addition to an indirect pathway via soil VWC (Fig. 7). Indirectly, air temperature had a negative effect on A by reducing soil VWC, which in turn limited V_{cmax} which then caused a decline in A ($\beta_{\text{airtemp} \rightarrow \text{VWC} \rightarrow V_{\text{cmax}} \rightarrow A} = -0.167$). A pathway of similar strength passed through J_{max} ($\beta_{\text{airtemp} \rightarrow \text{VWC} \rightarrow J_{\text{max}} \rightarrow A} = -0.149$). The direct effect of air temperature on A was of similar strength to the two indirect effects ($\beta_{\text{airtemp} \rightarrow A} = 0.209$), which means that the combined indirect negative effects of air temperature on A were 50% stronger than the direct positive effect ($\beta_{\text{airtemp indirect}} = -0.316$, $\beta_{\text{airtemp direct}} = 0.209$). This suggests that the overall effect of rising air temperatures in our analysis was to suppress photosynthesis of *S. scoparium* by reducing soil VWC and limiting both V_{cmax} and J_{max} .

Aboveground biomass

Drought did not affect ANPP of either *B. curtipendula* or *S. scoparium* ($\Pr(\beta_{\text{drought}}) > 0 = 0.76$, Fig. 8). *Bouteloua curtipendula* was more productive than *S. scoparium*, with the two species averaging ~100 and 40 g m^{-2} , respectively ($\Pr(\beta_{\text{species}} < 0) = 0.982$, Fig. 8).

Discussion

Increased drought frequency poses a severe threat to the stability and function of grasslands throughout the world. Understanding how dominant grass species physiologically respond to drought provides a mechanistic understanding of ecosystem-level drought responses, and enables us to accurately model and

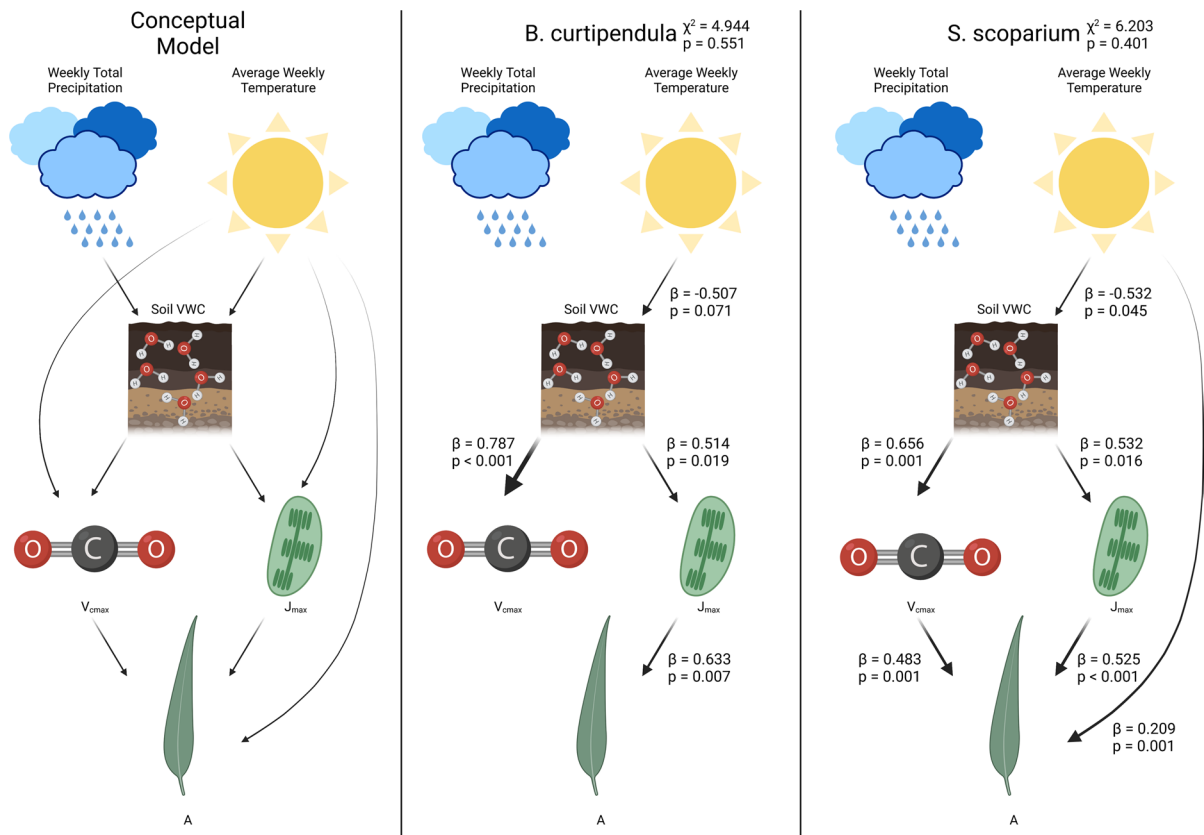


Fig. 7 Path analysis relating abiotic drivers, soil moisture, and leaf biochemistry as drivers of photosynthesis in *B. curtipendula* and *S. scoparium*. Arrows are scaled according to the

Z-score of the effect, with larger arrows representing stronger effects. Only significant arrows are shown in the species panels

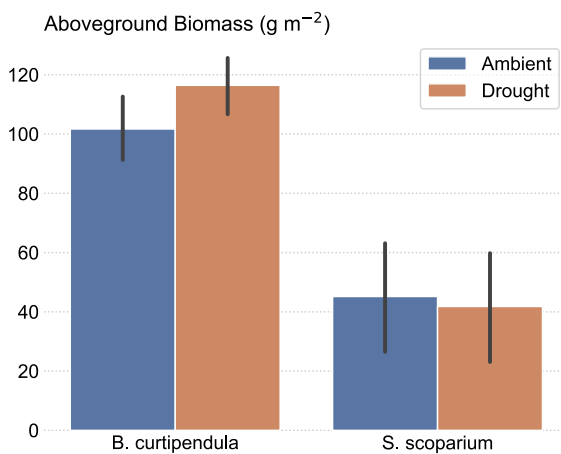


Fig. 8 Effect of drought on aboveground biomass production for *B. curtipendula* and *S. scoparium*. Bars and points represent means ± 1 SE

predict how grasslands will be affected by water shortages in the future. However, few data exist on the metabolic and stomatal responses of dominant C_4 grasses to rainfall shortages. Based on these limited data, we made three predictions: (H1) Drought will reduce A and g_{sw} for C_4 grasses during the mid- and late-summer months, (H2) the reduction in A will be driven in large part by metabolic limitations, such as reduced $V_{\max}$ and $J_{\max}$, and H3) drought-induced metabolic limitations of photosynthesis will reduce end-of-season biomass for both *B. curtipendula* and *S. scoparium*. Our results illustrate that co-occurring, dominant C_4 grasses experience strong metabolic limitation of photosynthesis, particularly by RuBP regeneration via electron transport. Interestingly, $J_{\max}$ was not reduced by drought for either *B. curtipendula* or *S. scoparium*, enabling both species to maintain constant A under experimentally reduced precipitation. However, seasonal changes in soil moisture did affect

leaf biochemistry, but only for *S. scoparium*; low soil moisture during summer months decreased $J_{\max}$, which in turn reduced A . Photosynthesis of *B. curtipendula*, on the other hand, remained stable throughout the growing season, reflecting relatively constant leaf biochemistry that was decoupled from seasonal declines in soil moisture. That two common C_4 species possess such different biochemical and photosynthetic responses to soil moisture highlights the physiological variability inherent within plant functional groups, and underscores the need for more field studies of C_4 biochemistry.

The metabolic limitation of *B. curtipendula* and *S. scoparium* described here supports previous observations that C_4 photosynthesis is rarely inhibited by gas exchange. Low stomatal limitation rates of 10–20% (i.e. $A_{\text{obs}}/A_{40}=80\text{--}90\%$) for *B. curtipendula* and *S. scoparium* are within the range reported for other tallgrass C_4 species, including *Themeda triandra* and *Hemarthria altissima* (Ripley et al. 2010; Zhong et al. 2019). Thus, C_4 photosynthesis appears to be primarily limited by leaf biochemistry, but only one study we know of estimated both V_{cmax} and $J_{\max}$ for a C_4 species. Maximum Rubisco carboxylation rates of C_4 species are generally low; our V_{cmax} range of 10–40 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ appears typical for C_4 species (Simioni et al. 2004; Ripley et al. 2010; Pinto et al. 2014). Indeed, a meta-analysis of V_{cmax} for both wild and agricultural C_4 plants found a maximum range of 20–60 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ across all species (Pignon and Long 2020), substantially narrower than the 10–200 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ range of C_3 species (Wullschlegel 1993). Less is known about $J_{\max}$ for C_4 species, in part due to the difficulties of fitting the C_4 biochemical model. Studies can rarely estimate all six unknowns of the C_4 model (V_{pr} , V_{pmax} , V_{cmax} , $J_{\max}$, R_{d} , R_{m}) because estimating V_{pr} and V_{pmax} requires numerous measurements at low mesophyll CO_2 pressures (<5 Pa), which standard A - C_i measures do not include (Yin et al. 2011). Instead, most studies use a C_4 biochemical model (Collatz et al. 1992) that estimates the initial slope (k) of the A - C_i curve (Ripley et al. 2010), or estimate V_{pmax} in lieu of $J_{\max}$ (Zhong et al. 2019; Pignon and Long 2020). Studies using a more recent C_4 biochemical model that includes $J_{\max}$ are rare (Yin et al. 2011), and the only other estimate of $J_{\max}$ for a non-agricultural grass species we could find might be incorrect because it was derived using the C_3 model (Ulrich et al. 2019). Yet our study

suggests that $J_{\max}$ plays a crucial role in limiting C_4 photosynthesis and might be responsible for interspecific differences in seasonal assimilation rates. *Bouteloua curtipendula* and *S. scoparium* exhibited different phenological patterns in $J_{\max}$, which paralleled their phenological patterns in A . Moreover, the higher sensitivity of $J_{\max}$ to soil moisture for *S. scoparium* might explain why quantum efficiency ($\mu\text{mol CO}_2 \mu\text{mol e}^{-1}$) is more sensitive to soil moisture for *S. scoparium* than for *B. curtipendula* (Maricle et al. 2015). Interspecific differences in $J_{\max}$ should be examined more thoroughly, because such differences could underpin how C_4 photosynthesis, and therefore the productivity of warm-season grasslands, depends on soil moisture.

Given the strong dependence of $J_{\max}$ and A on seasonal soil moisture for *S. scoparium*, we were surprised at the high degree of physiological drought resistance demonstrated by both species. This was especially surprising in light of the widespread findings that drought adversely impacts both photosynthesis and biomass production for C_4 grasses. A 50% decline in V_{cmax} under drought can impose significant metabolic limitations on photosynthesis, decoupling the relationship between A and g_{sw} (Ripley et al. 2010). Indeed, reduced A is a common consequence of drought for both C_3 and C_4 species (Taylor et al. 2011). Yet we found no evidence that drought affected A , g_{sw} , V_{cmax} , $J_{\max}$, or biomass production, nor did drought appear to strengthen metabolic limitations by weakening the A - g_{sw} relationship. Given the lack of studies on other C_4 species, it is difficult to ascertain whether our findings reflect an underlying variability in C_4 drought physiology. Indeed, *Bouteloua gracilis*, another dominant C_4 species, showed only negligible declines in A , g_{sw} , and V_{cmax} when subjected to an extreme, long-term drought, similar to our findings (Ulrich et al. 2019). It is also difficult to compare our results to previous studies because ours is the first in situ measurement of C_4 leaf biochemistry under drought conditions; other studies used either growth chambers or pots kept outdoors. Pot experiments are notoriously difficult to compare to field experiments (Nowak and Caldwell 1984; Rode et al. 2017). This might be especially true for drought studies, given the way pots alter growth habits. Roots tend to be underdeveloped in pots due to constricted space (Poorter et al. 2012), and perennial field plants have had years to develop robust root and mycorrhizal

networks. Soil moisture in pots also drains more rapidly than in natural soils (Ray and Sinclair 1998). Thus, pots might artificially impose stronger water limitation than field drought experiments, evidenced by plants typically having lower A in pots than in field conditions (Poorter et al. 2016). Indeed, comparative studies generally find little to no correlation between results of field and pot studies (Mead and Pritchett 1971; Gunes et al. 2006), so it is likely that drought responses in natural settings might be tempered compared to pot experiments. Still, other field studies in tallgrass prairies found strong declines in both A and biomass under drought (Dietrich and Smith 2016; Carroll et al. 2021). Plants at our study might also have deeper rooting depths than at other sites. Our Waukesha site is characterized by deep agricultural soils, while the best lowland soils at Konza prairie, for example, have soils only 2 m deep and upland sites where most drought experiments are conducted are much shallower, often with bedrock less than 1 m deep (Dietrich and Smith 2016). Given that the root systems of both *B. curtipendula* and *S. scoparium* can extend beyond 1 m, it is possible that the deeper soils of Waukesha allowed *B. curtipendula* and *S. scoparium* to access deeper water layers, which could be a critical component of ecosystem drought resistance. The discrepancy from our study might also reflect experimental differences; experiments at Konza prairie, for example, imposed a much more severe (i.e. 66%) reduction in rainfall than the 40% reduction used here (Carroll et al. 2021). The discrepancy might also reflect underlying biological differences; cool-season grasslands appear particularly resilient to drought (Knapp et al. 2020; Carroll et al. 2021). Though C_4 grasses dominate our site, it is climatically similar to cool-season grasslands; in Oconomowoc, the average daily high during July and August is 5 °C cooler than in Manhattan, KS and similar to Cheyenne, WY. As a result, potential evapotranspiration is markedly lower throughout Wisconsin than Kansas (Sanford and Selnick 2013). Unfortunately, drought studies from the Great Lakes and northeastern Great Plains region (i.e. Wisconsin, Minnesota, Illinois) are rare, and more such studies are needed to confirm that cooler temperatures can mitigate drought stress.

In summary, climate change is likely to fundamentally alter water dynamics in grasslands around the world. Grasslands appear to differ in their sensitivity to drought, yet no study has yet provided

a physiological basis underlying this differential drought sensitivity. Our results have two important implications. First, cold climate grasslands might be physiologically resilient to drought because their underlying biochemistry appears insensitive to reduced rainfall. In our experiment, we reduced rainfall to below the 5th percentile of growing season rainfall in Wisconsin, which then reduced soil VWC by 5–10% throughout the growing season (Fig. 1B, C). This reduction in soil VWC was enough to impart severe water limitation within the top 10 cm of soil, as soil water potential fell below – 2 MPa in drought plots for most of the growing season (Fig. 1D). Yet despite the reduction in water availability in the drought treatment, photosynthesis and biomass production of both *B. curtipendula* and *S. scoparium* were not affected. Second, two co-occurring species within the same plant functional type are limited by the same metabolic process ($J_{\max}$), but that metabolic process exhibits markedly different sensitivities to soil moisture among species. Therefore, to provide a mechanistic understanding of ecosystem drought responses, we must continue to understand the stomatal and non-stomatal limitations of C_4 photosynthesis.

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Author contributions NPL planned the research. NPL and MLB designed the experiment. MLB conducted all gas exchange measurements, NPL and MLB conducted all soil moisture measurements. NPL and MLB both measured biomass. NPL analyzed the data and wrote the manuscript.

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Data availability All data, code, and manuscript files available on Figshare.

Code availability All code available on Figshare.

Declarations

Conflict of interest We have no conflicts of interest to report.

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