Acta Oecologica 93 (2018) 14–20

Contents lists available at ScienceDirect

Acta Oecologica

journal homepage: www.elsevier.com/locate/actoec

Physiological and anatomical trait variability of dominant C_4 grasses

Seton Bachle*, Jesse B. Nippert

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

A R T I C L E I N F O

Keywords:
Climate variability
Gas exchange
Microanatomy
Grassland
Chlorophyll fluorescence

A B S T R A C T

Climate variability is a key driver of physiological responses in common grass species in grasslands of North America. Differences in microanatomical traits among coexisting species may influence physiological responses to climate variability over large geographic scales. The goal of this research was to determine leaf-level physiological and microanatomical trait variability among four dominant C_4 grass species across a natural precipitation gradient. Physiological traits were observed to vary significantly across the gradient with greater variability than microanatomical traits. Microanatomical traits were shown to predict physiological responses in *A. gerardii* and *P. virgatum*, but the nature of the relationships varied between species. These results illustrate that microanatomical and physiological traits vary across a precipitation gradient, there are clear linkages between microanatomy and physiology in grass species, and this evidence underscores the need for further investigation using phylogenetically diverse assemblages.

1. Introduction

North American grasslands of the Great Plains have been shaped by fire, grazing, and climatic variability, resulting in an environment with fluctuating resource availability and extremes in temperature and precipitation (Anderson, 2006; Axelrod, 1985; O'Keefe and Nippert, 2017; Weaver, 1968). The mid-continental climate of this region results in variable precipitation patterns and the warmest annual temperatures during the summer growing season (Borchert, 1950). Despite the natural occurrence of climate variability in this region, increased frequencies and magnitude of climate variability due to climate change may have negative consequences on grassland structure and function within the central Great Plains of North America (Houghton et al., 2001; Nippert et al., 2009).

Many grasses in the Great Plains have traits that confer tolerance to drought and fluctuations in water availability (Tucker et al., 2011). Traits such as increased bundle sheath cell area, enhanced water-use-efficiency, and decreased transpiration are generally associated with species that utilize the C_4 photosynthetic pathway which has come to dominate many grassland systems (Griffiths et al., 2013). C_4 photosynthetic traits also provide multiple adaptive benefits to plants in dry environments, including reduced photorespiration, increased drought tolerance, and greater growth efficiency (Brown, 1975; Lundgren et al., 2014). Species that utilize C_4 photosynthesis possess a modified leaf anatomical structure referred to as ‘Kranz’ anatomy (Brown, 1958, 1975), which is characterized by two spatially separate cell types that partition the biochemical reactions required for CO_2 carboxylation and assimilation (Edwards et al., 2001; Hatch, 1987). A ring of mesophyll cells is wrapped around a ring of bundle sheath cells that surround the vascular tissue (xylem and phloem). C_4 mesophyll cells are responsible for the carboxylation of atmospheric CO_2, and these cells contain large numbers of chloroplasts for initial carboxylation (Berry and Patel, 2008). Bundle sheath cells are responsible for the decarboxylation and assimilation of the C_4 acid assembled in the mesophyll cells (Berry and Patel, 2008; Sage, 2004). The separation of the biochemical reactions facilitates maximal carboxylation rates by the enzyme Rubisco via exposure to saturating concentrations of CO_2 (Christin et al., 2013; Kromdijk et al., 2014). During times of water-stress, plants strategically close stomata to reduce water loss, thus inhibiting the ability to take in atmospheric CO_2. Therefore, species that have an increased water-use-efficiency (WUE) are more likely to survive in water limiting conditions because less water is lost during carboxylation (Hatch, 1987; Taylor et al., 2014; Vico and Porporato, 2008). Due to the aforementioned carbon-concentrating mechanism of the C_4 photosynthetic pathway, these species typically have increased WUE as stomatal resistance can be increased, reducing water loss while maintaining carbon assimilation rates (Nelson et al., 2004). Monitoring C_4 grass gas exchange (H_2O vapor and CO_2) has been used to assess plant physiological response to drought and other altered climatic conditions (Fay et al., 2000; Knapp et al., 2002; Nippert et al., 2009). Gas exchange measurements allow instantaneous measurements of photosynthesis and water lost through transpiration, which aid in understanding water limitation during

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* Corresponding author.
E-mail address: sbachle@ksu.edu (S. Bachle).

https://doi.org/10.1016/j.actao.2018.10.007
Received 3 January 2018; Received in revised form 8 August 2018; Accepted 14 October 2018

drought. Chlorophyll fluorescence (measurement of electron's loss of excitement or energy) is also an indicator of the photosynthetic machinery involved in the light-dependent reactions of photosynthesis and has been observed to respond to water availability (Kakani et al., 2008; Maxwell and Johnson, 2000; Murchie and Lawson, 2013). A common metric for chlorophyll fluorescence is quantum yield (*QY*), which is the ability of photosystem II to convert light to usable energy (Krause and Weis, 1991). These physiological traits should be directly influenced by the microanatomical features of the species; more specifically, proportions and sizes of cell types in Kranz anatomy of C_4 plants should affect the rates of gas exchange and water use in leaves.

Plant physiological characteristics have been used to explain species-specific responses to varying conditions, but little work has described species-specific microanatomical traits. Key physiological traits may be influenced by the microanatomical structure that is characteristic of the species. Grass micro-anatomical features have been previously described for C_4 species (Christin and Osborne, 2014), but understanding variability in leaf microanatomy within broadly distributed species has seldom been described. Measuring physiological and microanatomical trait differences may give insight to their variability between and within species over the growing season, and across natural environmental gradients.

The objective of this research was to measure the variability of leaf-level physiological and anatomical traits among four common C_4 grasses across time and space. Four C_4 grasses were examined due to their dominance in Kansas' prairies systems: *Andropogon gerardii* Vitman, *Schizachyrium scoparium* (Michx.) Nash, *Sorghastrum nutans* (L.), and *Panicum virgatum* (L.) Nash. Leaf-level physiological traits measured included: Photosynthesis (A_n), stomatal conductance (g_s), and chlorophyll fluorescence; leaf microanatomical traits included: Bundle sheath area (BS_A) and Mesophyll area (MC_A). Here, we propose 3 hypotheses: (1) Leaf-level physiological traits will exhibit larger variation within/across species in Kansas due to the instantaneous nature of these physiological measurements (Epstein et al., 1996). (2) Microanatomical traits will vary greatly between species and across Kansas, but show less variability within a species and location (Carmo-Silva et al., 2009). (3) Changes in leaf-level microanatomy will constrain maximal physiological rates in all species measured (Kromdijk et al., 2014; Wright and Westoby, 2002).

2. Materials and methods

This research was conducted at three sampling locations in Kansas, USA. Albertson Prairie is located near Hays (38° N, 99° W), Konza Prairie Biological Station (40° N, 99.5° W) and Rockefeller Prairie site (39° N, 95° W). Three locations at the Konza Prairie Biological Station with varying fire frequency were used in this study (watersheds 4B, 1D, K20A). which were combined to more broadly represent within site variability of genotypes and phenotypes.

Measurements at each of the three locations were made during two separate sampling periods: June 1st, 3rd, 4th and August 9th, 10th, 11th in the summer of 2016. Precipitation and temperature data were collected from the PRISM climate database (PRISM Climate Group, 2014) and a long-term dataset from Konza LTER (AWE01) (Table 1).

Four C_4 grassland species were measured at each site: *Andropogon gerardii* (Big bluestem), *Sorghastrum nutans* (Indian grass), *Schizachyrium scoparium* (Little bluestem), and *Panicum virgatum* (Switchgrass). Ten individuals from each species were labelled with metal identification tags and marked via GPS at each sampling location. Species physiological and microanatomical samples were measured and collected on the same leaf; samples were collected from the majority of the research area in order to maximize heterogeneity. Soil moisture was measured at 0–5 cm depth adjacent to each individual sampled to account for differences in surface soil water availability. Soil moisture measurements were conducted with a HydraProbe II (Stevens, Inc., Portland, OR, USA).

Table 1
Mean annual precipitation and mean growing season (June, July, August) temperature data at each research location from 2000 to 2016. Data collected from the PRISM climate database, and Konza Prairie LTER database.

Location	Precipitation (mm)	Temperature (C°)
Albertson Prairie	596.66	24.835
Konza Prairie	828.65	24.575
Rockefeller Prairie	953.24	24.785

2.1. Trait measurements

All physiological trait data were collected between 10:00 and 18:00 CDT at each sampling site. For leaf-physiology measurements, the youngest, developmentally mature leaves were used in order to reflect the most recent environmental conditions. Gas exchange rates were measure using a LI-6400 system (LiCOR, Inc., Lincoln, NE, USA) equipped with an LED light source (light intensity was maintained at 2000 μmol m^{-2} s^{-1}, CO_2 concentration at 400 ppm, and relative humidity between 40 and 60%) and included rates of photosynthesis (A_n) and stomatal conductance (g_s). The environmental values used in the gas exchange cuvette were guided by previous ecophysiology research on these C_4 species, in these grasslands (Knapp, 1985; Knapp et al., 1994; Knapp and Smith, 1990). Measurements from the LI-6400 were logged when maximum photosynthetic rate remained stable for at least 1 min. Leaf chlorophyll fluorescence was measured using a miniaturized pulse-amplitude-modulated photosynthesis yield analyzer (Mini-PAM) (Walz, Hamburg, Germany). Quantum yield (*QY)* measurements were taken with actinic light intensity at values ranging from 0 to 1087 μmol m^{-2} s^{-1}; this range of light intensities was appropriate to minimize the risk of photoinhibition in the field.

After leaf-level physiological measurements were taken, leaf anatomical samples were collected by harvesting the same leaf previously measured for physiological characteristics. Leaf anatomy samples included leaf cross sectional tissue from the newest mature leaf tissue from each species (four samples of each species) and were collected at all sites and sampling periods. Leaf tissue samples were fixed in FAA (10% formalin/5% glacial acetic acid/50% ethanol (use 95% EtOH)/35% DI water) and sent to Kansas State's College of Veterinary Medicine Histopathology lab for paraffin mounting and cross-sectional slide staining with Safranin-O and Fast Green (Ruzin, 1999). Leaf cross sectional samples were scanned with a Panoramic MIDI (3DHistech Inc., Budapest, Hungary) and measured using IMAGEJ software (Rasband, 1997).

Due to the intricacies of leaf tissue, sub-samples were created to measure microanatomical traits (Fig. 1). A set distance between two major vascular bundles was selected on either side of the mid-rib; both major and minor vascular bundles were measured in this area. Bundle sheath and mesophyll tissues were the focus of this study (Fig. 1); as they are heavily influential on photosynthetic rates (Yin et al., 2011).

2.2. Statistical analysis

The primary goal was to elucidate differences in physiology and microanatomical among these C_4 grass species across research sites. Therefore, we used a repeated measures model analysis of variance (ANOVA). For each model, the physiological gas exchange, fluorescence, and microanatomical data measured were used as the response variables. Multiple comparison tests to identify differences among species and locations were conducted using post-hoc Tukey's HSD. BS_A data was also analyzed with a Kruskal Wallace Test in conjunction with a Wilcox test, due to its non-parametric distribution. Regression analysis was utilized to correlate physiological and microanatomical traits. All analyses and Fig. 4 were conducted and produced in R V3.4.3 (R

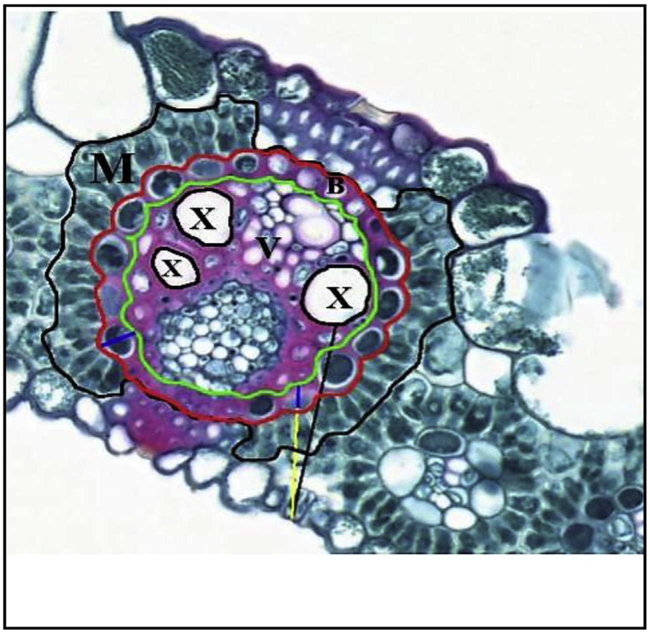

Fig. 1. Major vascular bundle from A. gerardii taken at 40X. Mesophyll (M); Bundle sheath (B); Xylem (X); Vein (V).

Core Team, 2017); Figs. 2 and 3 were produced in SigmaPlot 12.5 (Sigma, St. Louis, MO, USA).

3. Results

3.1. Leaf level physiology

Gas exchange measurements varied significantly between species (Table 2; $P < 0.05$) for both A_n and g_s (Fig. 2A and B). A_n varied significantly by species (Table 2; $P < 0.05$), but not between research locations ($P < 0.053$). *S. nutans* had a higher A_n than *P. virgatum* ($P < 0.05$). However, there were no statistical differences of A_n within a single species across sites ($P > 0.05$). g_s displayed significant variation between location and species (Table 2; $P < 0.005$); this was influenced by the site differences between Konza and Rockefeller Prairies ($P < 0.005$). The source of variation was caused by *P. virgatum*, which had the lowest stomatal conductance rate (Fig. 2C; $P < 0.05$). Intraspecific comparisons by location revealed that *P. virgatum* on Konza Prairie had a significantly higher g_s than Rockefeller Prairie, but not the Albertson site ($P < 0.05$). *QY* assessed using fluorescence indicated differences among locations measured, but not differences among species. (Fig. 2E; Table 2). Within species observations of *QY* revealed *S. nutans* was less efficient at Rockefeller Prairie (Fig. 2E; $P < 0.05$). *QY* was significantly higher on Konza Prairie compared to Rockefeller, but not Albertson Prairie ($P < 0.05$). Overall coefficient of variation for all physiological traits was relatively small (Fig. 2B, D, F; CV < 0.70). *QY* was observed to increase in a stepwise fashion from western to eastern locations for all species excluding *P. virgatum* (Fig. 2F).

3.2. Leaf microanatomy

Bundle sheath cell area was observed to vary across species and location (Fig. 3A; Table 2). BS_A of *P. virgatum* was nearly twice that of any other species, ~40% of subsampled area (Fig. 3A; Kruskal-Wallis test: $P < 0.001$). This test also confirmed that grasses on Rockefeller contained a larger average BS_A compared to species in the western and central sites ($P < 0.05$). Within species comparisons of BS_A did not vary by location for any species (Fig. 3A). MC_A was also observed to vary by species and location (Fig. 3B; Table 2). Average mesophyll area was significantly lower in *P. virgatum* compared to other species, but it did not vary across locations (Fig. 3C). Rockefeller Prairie had the lowest MC_A in relation to Albertson and Konza Prairies ($P < 0.05$). MC_A across Kansas was static for most species measured except *S. nutans*, which was significantly lower at Rockefeller (Fig. 3C; $P < 0.05$). Although *S. scoparium* at Rockefeller Prairie was not represented due to a small sample size (Fig. 3B and D), coefficient of variation was small for leaf microanatomical traits at other locations. *A. gerardii* displayed the smallest coefficient of variation, while *P. virgatum* exhibited the largest due to variation in Rockefeller Prairie (Fig. 3B and D).

Regression analysis revealed that relationships exist between leaf-level physiological and microanatomical traits. Maximum photosynthetic rates for each location were found to exhibit a relationship with the amount of bundle sheath area (Fig. 4). The relationship between BS_A and A_n was observed to be species-specific. Fig. 4 illustrates the positive relationship found in *A. gerardii*, while *P. virgatum* was observed to have a negative relationship between BS_A and A_n (Fig. 4).

4. Discussion

The impacts of rainfall variation on leaf physiology and microanatomy of grassland species has been observed in previous research (Edwards et al., 2001; Fay et al., 2002; Ocheltree et al., 2012; Olsen et al., 2013; Schroeder-Georgi et al., 2016; Tucker et al., 2011). However, there are few studies that document intra- and interspecific relationships between leaf-level physiology and anatomy across environmental gradients. This study examined the inherent variability of leaf – level physiology and microanatomy among four dominant C$_4$ grass species across a natural rainfall gradient, diverse landscapes, and land-management practices; which allowed this project to addressed three hypotheses, resulting in novel findings. Species' physiology was determined to vary significantly within the Kansas rainfall gradient with greater variability than leaf-level anatomy, which may be explained by the instantaneous nature of leaf-level gas exchange measurements that are reflecting an individual's immediate physiology. While leaf microanatomical traits were observed to vary between species, within species variation was relatively low across Kansas. Leaf microanatomical traits are highly influenced by regional genotypes (i.e. ecotypes), which have been shaped through generations of both biotic and abiotic factors (Gibson, 2009; Gray et al., 2014). Leaf anatomical traits were predicted to significantly predict leaf physiology traits. The data illuminated significant relationships between micro-anatomy and physiology within species, but trends were not consistent among all species selected. Results from this study support previous research findings indicating a tight coupling between C$_4$ grasses physiological and microanatomical relationships in response to short-term changes in climate (Christin et al., 2013; Ocheltree et al., 2012; Smith and Knapp, 1999; Volder et al., 2010).

Physiological trait measurements varied significantly by location and between species (Fig. 2; Table 2). Gas exchange data indicates large variability between research locations (Fig. 2B and D), this may reflect site differences in topography, management practices, or inherent climate conditions (Baer et al., 2003; Nippert et al., 2011; Towne et al., 2005). Measurements made on Konza Prairie included three watersheds that include a variety of topographical positions, water availability, and burning regimes. In contrast, Albertson and Rockefeller Prairie have characteristically reduced topographic variability and similar management practices. Fire management may be responsible for the observed physiological differences between the sites; both Albertson and Rockefeller Prairies had not experienced fire or haying in over two years. In contrast, two watersheds on Konza Prairie were burned in early spring, which could have allowed for an increased nutrient availability (Baer et al., 2003), decreased competition for light, and overall increase in site variability of physiological traits (Wilson, 1988). However, A_n on Konza Prairie was likely driven by fire's removal of the top litter layer, allowing a faster growth rate than other sites (Gilliam et al., 1987).

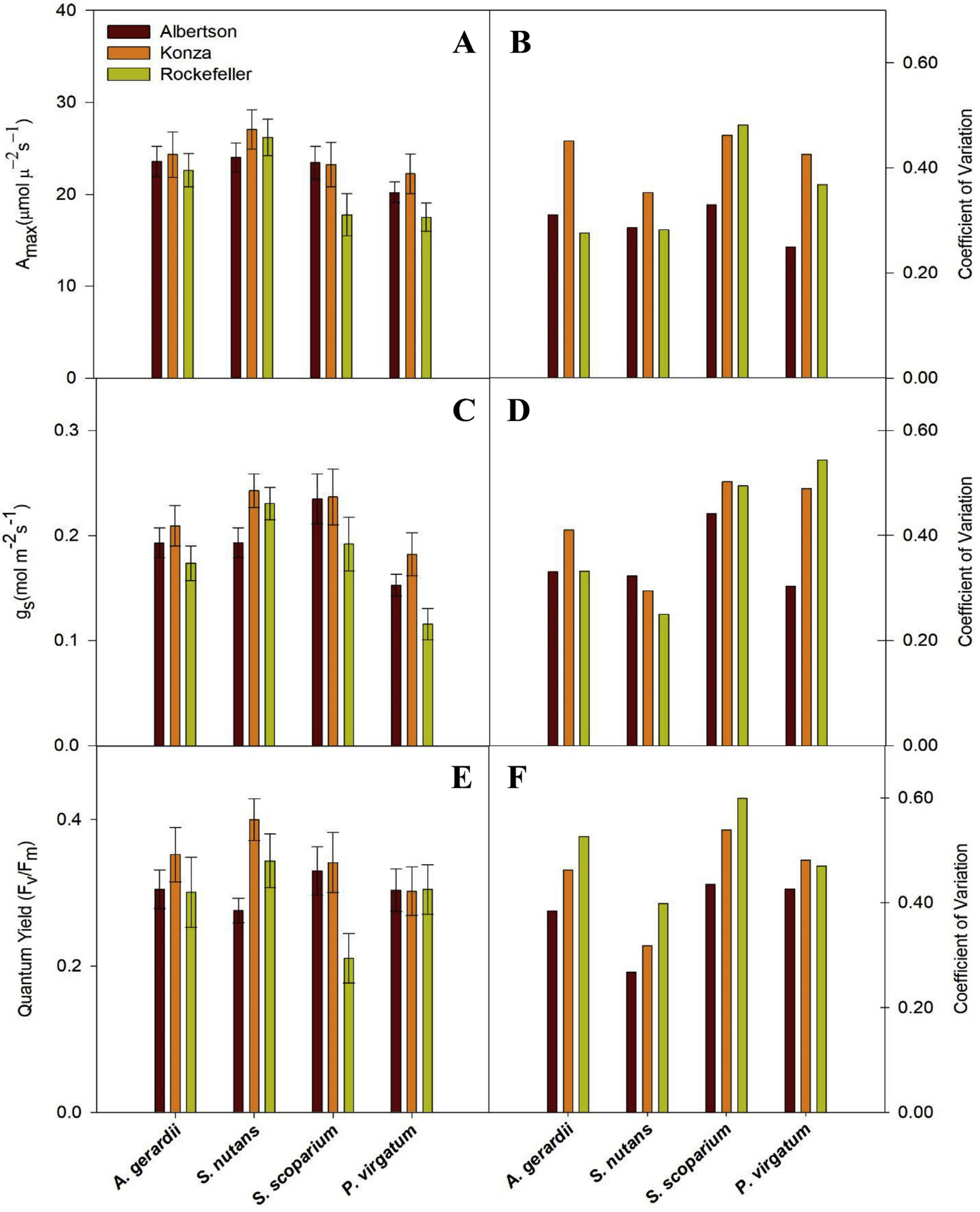

Fig. 2. Physiological traits measured across all research locations and species (means ± SE). (**A**) represents the photosynthetic rate; (**C**) represents the stomatal conductance rate; (**E**) represents the quantum yield extrapolated from chlorophyll fluorescence. Panels B, D, and F represent the coefficient of variation of selected trait.

Leaf-level microanatomical traits were observed to vary significantly between species and location (Fig. 3; Table 2). These traits indicate small variability for all grass species sampled. *A. gerardii* exhibited an extremely small amount of variation in photosynthetic tissue across Kansas (Fig. 2B and D), demonstrating that some traits may be conserved in response to environmental factors, whereas other traits are variable (Liancourt et al., 2015). Relationships between leaf microanatomical traits and physiological traits were not consistent among C_4 species in this study, with both positive and negative relationships between the same traits (Fig. 4). Known relationships between cell types have varying effects on species physiology. For instance, increasing size of BS_A may correlate with increased photosynthetic rate (Dengler et al., 1994). In the results shown here, the relationship between BS_A (measured as a percentage) and maximum

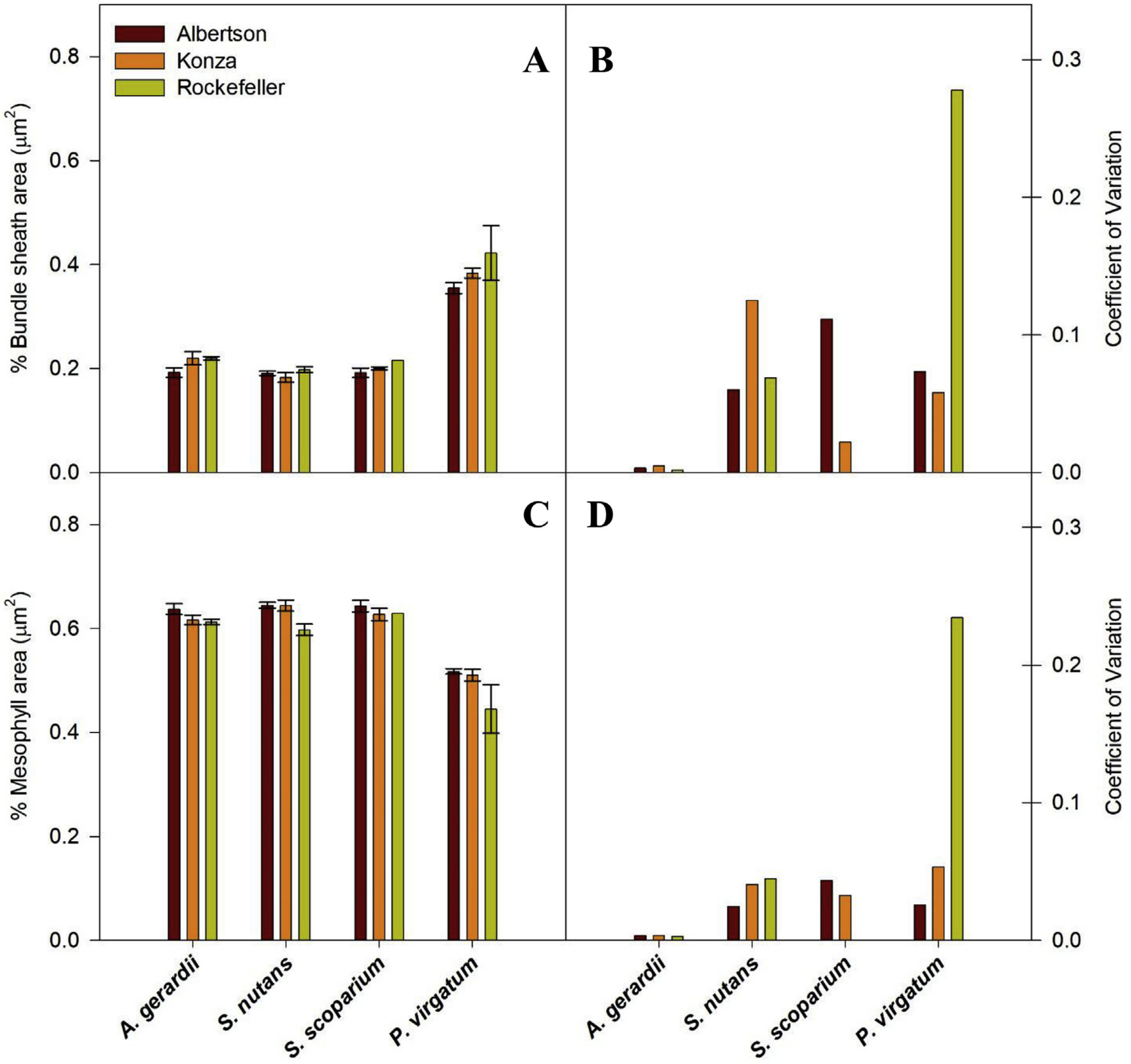

Fig. 3. Microanatomical traits calculated from subsampled (means ± SE). (**A**) represents the bundle sheath area as a percent of total leaf area; (**C**) mesophyll cell area as a percent of total leaf area. (**B**) and (**D**) represent the coefficient of variation of selected trait.

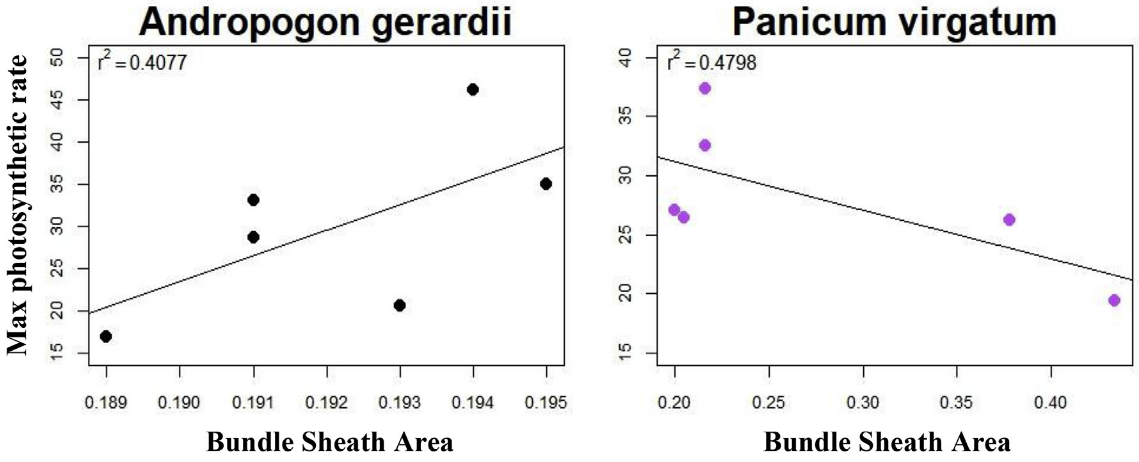

Fig. 4. Comparison of the relaionship between bundle sheath area (expressed as a percent of subsampled area) and the maximum photosynthetic rate measured from each species at both sampling times.

photosynthetic rate varied between *A. gerardii* and *P. virgatum* (Fig. 4). This may indicate that *A. gerardii* is more sensitive to photosynthetic responses when small changes in bundle sheath area are present. In addition, these differences among species illustrate the importance of phylogenetic differences in plant physiological studies (Edwards et al., 2007; Edwards and Still, 2008).

Differences in anatomical and physiological relationships between species may reflect phylogenetic differences, which result in contrasting

Table 2
ANOVA results for the chosen physiological and anatomical traits by species, location, and species x location interaction. ˆ P < 0 0.10, *P < 0.05, **P < 0.01, ***P < 0.001.

	A_n	G_s	QY	MC_A	BS_A
Species (S)	*F = 4.41***	*F = 9.64****	*F = 0.87*	*F = 102.65****	*F = 12.75****
Location (L)	*F = 2.97ˆ*	*F = 5.37***	*F = 3.92**	*F = 20.46****	*F = 215.89****
S x L	*F = 0.69*	*F = 1.01*	*F = 1.89ˆ*	*F = 0.79*	*F = 0.53*

arrangements of decarboxylating tissues of C_4 grasses (Rao and Dixon, 2016). In this study, we included grasses that have NAD-dependent malic enzyme (which use malate as a transported metabolite) and NADP-dependent malic enzyme (which use aspartate as the transported metabolite) decarboxylating enzymes. *P. virgatum* is a NAD-ME grass that holds similar tissue arrangement as classical Kranz anatomy but has two distinct layers of bundle sheath cells. *A. gerardii*, *S. nutans*, and *S. schizachyrium* are NADP-ME grasses which contain one layer of both mesophyll and bundle sheath cells. While contributions from aspartate and malate are considered equal in their ability to transfer CO_2 for decarboxylation (Meister et al., 1996; Rao and Dixon, 2016), there may be differences in nitrogen-use efficiency due to the reduction of cellular aspartate in NAD-ME grasses (Bräutigam and Gowik, 2016; Rao and Dixon, 2016). These enzymatic alterations may be a primary driver for a majority of the anatomical and physiological differences shown here.

Grassland ecosystems in Kansas typically encounter periods of summer drought. During the 2016 growing season sampled here, rainfall for all locations measured was above-average. Each site experienced abnormally high levels of rainfall in the latter half of the growing season. The impacts of higher late-season rainfall were atypical for Albertson Prairie (Hays, KS), which characteristically experiences drier conditions. Thus, the conditions at this site in 2016 may have increased photosynthetic, stomatal conductance, and transpiration rates than in previously measured years (Maricle and Adler, 2011).

5. Conclusion

The two main results gained from this research are (1) Although the selected C_4 grasses displayed differences in plant traits across location and between species, there was little variation exhibited within species at individual sites. This supports the concept of conserved adaptive traits which are influenced by phylogenetic relationships. (2) Because the selected C_4 grasses have varying responses (physiological, anatomical, and anatomy predicting physiology), models estimating a “general C_4 grass response” are likely underestimating actual interspecific variability and the influence of phylogenetic relatedness. The significance of missing the unique interspecific variability within the functional type (C_4 grass) has yet to be determined, and will require further research.

Acknowledgements

We thank Fort Hays University, Kansas State University, and University of Kansas for allowing access to the research locations. In particular, we'd like to thank Brian Maricle and Vaughn Salisbury for providing management history and/or general aid; and Mark Ungerer and Carolyn Ferguson for comments that improved this manuscript. This project was supported by the NSF award #002893. The LTER long-term dataset incorporated was AWEO1 (DOI: 10.6073/pasta/0e0c648068f3f2755e0b500062a4f7e9).

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