A comparison of seasonal foliar chlorophyll change among ecotypes and cultivars of *Andropogon gerardii* (Poaceae) by using nondestructive and destructive methods

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Abstract

Leaf chlorophyll (Chl) concentration can be an indicator of plant health, including photosynthetic potential and nutrient status. In some cases, this measure can indicate the degree to which plants are water-stressed. Traditional methods of measuring Chl concentration have involved a destructive sampling technique: extraction and spectrophotometric analysis. A compatible nondestructive method to measure leaf Chl concentration exists and applies transmittance spectroscopy to plants with a *Minolta SPAD-502* meter. These techniques were evaluated by comparing leaf Chl concentration in big bluestem (*Andropogon gerardii*). Leaves were sampled from plants representing three ecotypes (originating from Central Kansas, Eastern Kansas, and Illinois, USA) and two cultivars of *A. gerardii* growing in Hays, Kansas, USA. Leaf Chl concentration was measured using nondestructive and destructive techniques. We documented a saturating relationship between destructively measured leaf Chl concentration and SPAD index resulting from a decelerating change in SPAD index as Chl concentration increased. The comparison of *A. gerardii* ecotypes and cultivars demonstrated highest Chl concentration in the ecotype and cultivar from areas with historically low precipitation, Central Kansas and *A. gerardii* var. *hallii*, respectively. A high ratio of Chl *a* to Chl *b* is an index of drought adaptation and was also manifested in *A. gerardii* from drier regions. Thus, drought-adapted ecotypes and cultivars might be able to maintain high photosynthetic productivity and protect photosystem II during dry periods. Conversely, the ecotypes and cultivar originating from areas with higher precipitation had lower leaf Chl and a lower Chl *a/b* ratio.

Additional key words: drought; grassland; populations; pigments; prairie; precipitation gradient; sand bluestem; tallgrass prairie.

Introduction

Leaf Chl concentration can provide valuable information on the physiological condition of plants, including leaf nitrogen status and photosynthetic capacity (Chang and Robison 2003, Gitelson *et al.* 2003, Lin *et al.* 2010). Traditional measurements of Chl concentration have involved spectrophotometric measures of Chl in acetone (Arnon 1949) or ethanol (Wintermans and De Mots 1965). These measures of leaf Chl are direct (Gitelson *et al.* 2003) and accurate (Monje and Bugbee 1992), but involve a time-consuming extraction of Chl from destructively harvested leaves. By contrast, the *SPAD-502* chlorophyll meter (*Konica Minolta*, Osaka, Japan) is an increasingly popular method for measuring Chl concentration in plants (Markwell *et al.* 1995, Ling *et al.* 2011). This nondestructive method illuminates and measures light passage (wavelengths of 650 and 940 nm) through the leaf (Markwell *et al.* 1995). The SPAD meter is portable, easy to operate (Chang and Robison 2003), and attractive to

research on endangered plants (Hawkins *et al.* 2009) and crops (Mulholland *et al.* 1997, Anand and Byju 2008, Kumagai *et al.* 2009). Nondestructive Chl measurement also facilitates repeated Chl sampling in response to environmental stresses (Mielke *et al.* 2010) or changing conditions (Mulholland *et al.* 1997). SPAD readings have also been used to assess nitrogen status of plants (Chang and Robison 2003, Lin *et al.* 2010). Thus, SPAD-based measures can provide an index of photosynthetic capacity of a leaf (Kumagai *et al.* 2009).

Despite the appeal of nondestructive Chl measures, some contradicting results have emerged about the accuracy of SPAD data (Monje and Bugbee 1992). Nonlinear relationships have been determined between extracted Chl and SPAD-based readings in a number of plant species (Monje and Bugbee 1992, Markwell *et al.* 1995, Ishida *et al.* 1996, Mulholland *et al.* 1997, Netto *et al.* 2002, Netto *et al.* 2005, Uddling *et al.* 2007, Hawkins *et al.* 2009, Mielke *et al.* 2010, Ling *et al.* 2011). In other cases, linear relationships were found between extracted Chl and SPAD-based readings (Papasavvas *et al.* 2008, Kumagai *et al.* 2009). Thus, the nature of this relationship needs to be determined for each focal species in botanical studies.

Andropogon gerardii Vitman (big bluestem) is a warm-season grass with the C_4 photosynthetic pathway and a broad geographic distribution in North America. Ecotypes of this species were first described across a 2,000 km latitudinal gradient (McMillan 1959) and numerous cultivars have been developed for improving highly erodible land (USDA 1995). Variation in traits and productivity of *A. gerardii* ecotypes across a longitudinal gradient, corresponding to water availability, is the focus of recent and ongoing investigations (Goad 2011, Olsen 2012, Shelton 2012, Mendola 2013, Olsen *et al.* 2013, Gray *et al.* in press, Johnson *et al.* unpublished). When grown in a dry

climate, *A. gerardii* plants originating from the historically driest region were found to have higher photosynthetic rates compared to ecotypes from more mesic environments (Olsen 2012). We hypothesized this resulted (in part) from the presence of higher Chl concentrations in the dry-region ecotype. The *SPAD-502* Chl meter has rarely been used to measure variation in Chl concentration within a species (Hawkins *et al.* 2009), making data gained from this nondestructive measure of novel importance. One objective of this study was to compare nondestructive and destructive techniques of measuring leaf Chl in ecotypes of a prairie grass, *A. gerardii*, planted in a common garden. A second objective of this study was to examine Chl concentration among *A. gerardii* ecotypes collected from three climatically distinct regions and grown in a common garden located in the driest region of plant origin.

Conditions of low water availability often result in a decrease in Chl concentration of leaves (Huang *et al.* 1997, Nayyar and Gupta 2006), which helps reduce light absorption during times of drought (Estill *et al.* 1991, da Silva and Arrabaça 2004). It was hypothesized the most drought-adapted ecotype of *A. gerardii* would have the highest Chl concentration to resist drought-associated reductions in Chl. Conditions of low water availability also commonly result in decreases in the ratio of Chl *a/b* (Ashraf *et al.* 2001, Ghorbanli *et al.* 2012), indicating light-harvesting complexes of thylakoid membranes are changed during water stress (Parida *et al.* 2007). Consequently, it was hypothesized that the most drought-adapted ecotype of *A. gerardii* would have the highest ratio of Chl *a/b*. In few cases, ecotypic variations in Chl *a/b* have been measured in response to drought or between mesic and drought-adapted ecotypes (*e.g.*, Zhu *et al.* 2003, Shahbaz *et al.* 2011), but there has been little consideration of the mechanistic consideration of these differences.

Materials and methods

Seeds of three *A. gerardii* ecotypes were hand collected from 11 remnant prairies in 2008 from three regions (Table 1). Each region of origin or "ecotype" (collected from Central Kansas, Eastern Kansas, and Illinois) was represented by three or four remnant prairies, which we refer to as "populations" by each prairie name (Table 1). Seeds of two widely used cultivars, *A. gerardii* Kaw (big bluestem, accession 421276) and *A. gerardii* var. *hallii* Garden (sand bluestem, accession 421277), were obtained from the United States Department of Agriculture Plant Materials Center in Manhattan, KS, USA (Table 1).

In autumn 2008, seeds from each population and cultivars of *A. gerardii* were planted in *Metromix 510* potting mix (*Scotts Company*, Marysville, Ohio, USA) in 10 cm × 10 cm pots in a greenhouse at Kansas State University (39°11'N, 96°35'W). In August 2009, *A. gerardii* plants were transplanted from pots into a

common garden in Hays, KS, USA (38°51'N, 99°19'W) containing formerly cultivated silt loam soil. Plants were spaced 50 cm apart in landscape fabric to decrease competition from unwanted plants. The garden was arranged in 10 rows (blocks) with one plant from each population and cultivar source randomly assigned to each row. The garden was unshaded and grew under ambient conditions. In the year of study (2012), total precipitation in Hays, KS was 383 mm, of which 270 mm (70%) fell from April through September (data from Kansas State University Research and Extension).

Leaves were harvested from individuals of *A. gerardii* three times during the 2012 growing season. One young, fully-expanded leaf was selected from each plant. The same 5 individual plants were sampled from each of the 13 populations in June, July, and August for a total of 195 leaves (13 populations × 5 replicates × 3 months) in

Table 1. Population source origins of three ecotypes and two cultivars of *Andropogon gerardii* planted in the common garden in Hays, KS, USA (38°51'N, 99°19'W). For statistical analysis, the two cultivars were treated as ecotypes.

Ecotype or cultivar	Collection site or population	County	Latitude (N)	Longitude (W)
Central Kansas	Relict Prairie	Ellis, KS	38°51'	99°22'
	Webster Reservoir	Rooks, KS	39°24'	99°32'
	Saline Expt. Range	Ellis, KS	39°02'	99°14'
	Cedar Bluffs Reservoir	Trego, KS	38°45'	99°46'
Eastern Kansas	Konza Prairie	Riley/Geary, KS	39°05'	96°36'
	Tallgrass Prairie Nat. Pk.	Chase, KS	38°25'	96°33'
	Top of the World Pk.	Riley, KS	39°13'	96°37'
Illinois	DeSoto Railroad Prairie	Jackson, IL	37°51'	89°14'
	Twelve Mile Prairie	Effingham, Fayette, and Marion, IL	38°46'	88°50'
	Fults Hill Prairie	Monroe, IL	37°58'	89°48'
	Walters Prairie	Jasper, IL	38°59'	88°09'
Kaw	Kaw	Riley, KS	USDA Plant Materials Center Accession 421276	
Sand bluestem	Sand bluestem	Garden, NE	USDA Plant Materials Center Accession 421277	

a randomized complete block design with repeated measures. Leaf samples were frozen in liquid nitrogen in the field and stored at –20°C for further analysis. In the lab, leaves were allowed to thaw, and Chl concentration was then measured nondestructively *via* a *SPAD-502* Chl meter. Each leaf was measured three times with the SPAD instrument in the lab for a per-leaf average (Chang and Robison 2003, Kumagai *et al.* 2009, Lin *et al.* 2010). Following this, Chl was extracted from the same leaves destructively and measured spectrophotometrically according to Maricle (2010). Extraction buffer was added to fresh leaf tissue (FM) at 10 mL g^{-1}(fresh mass, FM). The extraction buffer contained 50 mM Tris-HCl (pH 7.0), 5 mM MgCl$_2$, 2 mM cysteine hydrochloride, and 2% PVP-40 (w/v). Leaves were ground to complete maceration with a mortar and pestle. A 40 µL subsample of grindate was drawn up from the mortar and mixed with 960 µL of 100% ethanol. Chl concentrations [µg mL^{-1}] were determined spectrophotometrically according to Wintermans and de Mots (1965):

$$Chl\ a = (13.70 \times A_{665}) - (5.76 \times A_{649}) \quad (1)$$
$$Chl\ b = (25.80 \times A_{649}) - (7.60 \times A_{665}) \quad (2)$$
$$Total\ leaf\ Chl = (6.10 \times A_{665}) + (20.04 \times A_{649}) \quad (3)$$

Results and discussion

This study investigated the relationship between leaf Chl and water stress (a possible indicator of drought adaptation) originating from many populations of *A. gerardii*, a dominant species in tallgrass prairie. Two leaf Chl measurement methods were used to assess total leaf Chl, as light absorption by leaves is important during times of drought. Furthermore, these measures were accompanied

Chl concentrations were expressed on a tissue-level basis as:
Leaf Chl [mg(Chl) g^{-1}(FM)] = Chl concentration [µg(Chl) mL^{-1}] × (1 mg 1,000 µg^{-1}) ÷ (0.004 g) (4)

Differences among ecotypes (including the two cultivars) were analyzed according to a randomized complete block design by using an analysis of variance with repeated measures (*StatView 5.0*, 1998 *SAS Institute, Inc.*, Cary, NC, USA). Differences among populations were analyzed with a separate repeated measures analysis. Repeated measures were from five plants of the same population in each month. Post-hoc comparisons of means were performed with *Fisher*'s protected least significant difference. Significance was assigned at $P = 0.05$.

We modeled the relationship between the nondestructive (SPAD index) and destructive (spectrophotometric analysis of tissue extractions) methods to measure Chl concentration using *Table Curve 2D* software, *version 5.01* (*SYSTAT Software Inc*. 2002). All measurements from all populations over time were used to generate the model. We selected the simplest model to describe the relations based on the coefficient of determination (r^2) and significance (*P*-value).

by determination of Chl *a* and Chl *b* to investigate a finer scale of adaptation to drought in *A. gerardii*.

A single-term linear equation with an exponential function and intercept best described the relationship between the nondestructive and destructive methods of measuring Chl concentration in *A. gerardii* (Fig. 1). The model, significant at *P*<0.0001, demonstrated that as Chl
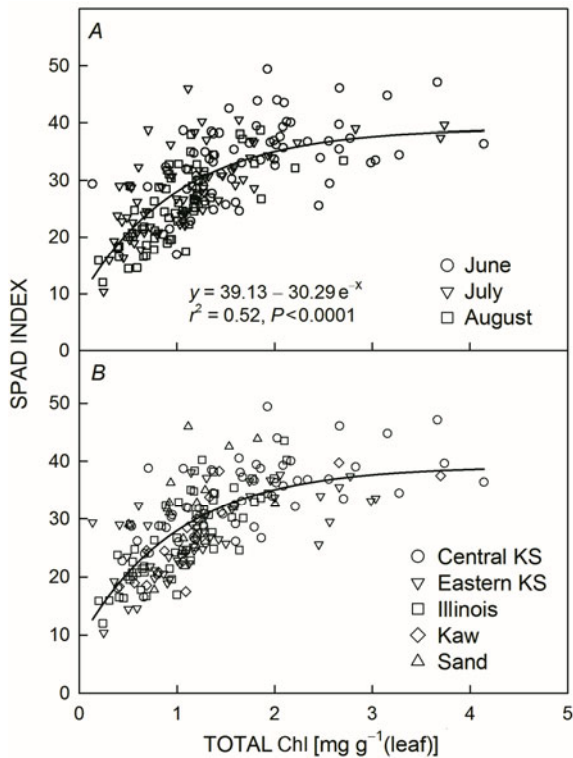
Fig. 1. Relationship between nondestructive measures of leaf chlorophyll (Chl) concentration in three ecotypes and two cultivars of *Andropogon gerardii* made with a *SPAD-502* Chl meter and destructive measures by Chl extraction. Points are separated by (*A*) month and (*B*) ecotype.

Fig. 2. Destructive (*A,C*) and nondestructive (*B*) measures of chlorophyll (Chl) concentration in leaves of three ecotypes and two cultivars of *Andropogon gerardii*. Shown are total leaf Chl per g of leaf tissue (*A*), leaf Chl concentration based on light absorption in a *SPAD-502* meter (*B*), and the ratio of Chl *a/b* (*C*). The three ecotypes each contained multiple populations of plants. Bars show the mean of 5–20 replicates ± SE.

concentration in leaf tissue increased, the nondestructive SPAD measurements saturated. Thus, the SPAD index changed negligibly at high Chl concentration. This relationship is similar to previously published results on a wide variety of species, including several crop grasses (Monje and Bugbee 1992, Markwell *et al.* 1995, Mulholland *et al.* 1997, Uddling *et al.* 2007), forbs (Monje and Bugbee 1992, Markwell *et al.* 1995, Uddling *et al.* 2007, Ling *et al.* 2011), and woody trees and shrubs (Ishida *et al.* 1996, Netto *et al.* 2002, Netto *et al.* 2005, Uddling *et al.* 2007, Hawkins *et al.* 2009, Mielke *et al.* 2010). The *SPAD-502* Chl meter depends on transmission of light through a leaf, which saturates at higher Chl concentrations. In particular, sand bluestem had a SPAD index much higher than should be expected based on extractable Chl (Fig. 2*A,B*). This relationship is similar to what has also been observed for wheat (Monje and Bugbee 1992, Mulholland *et al.* 1997, Uddling *et al.* 2007), maize (Markwell *et al.* 1995), and rice (Monje and Bugbee 1992). Thus, for the most accurate determination of Chl concentration at higher concentrations in *A. gerardii*, Chl should be extracted from leaves and determined by spectrophotometric methods (Monje and Bugbee 1992, Markwell et al. 1995). The portability of the *SPAD-502* meter allows more convenient measures in the field,
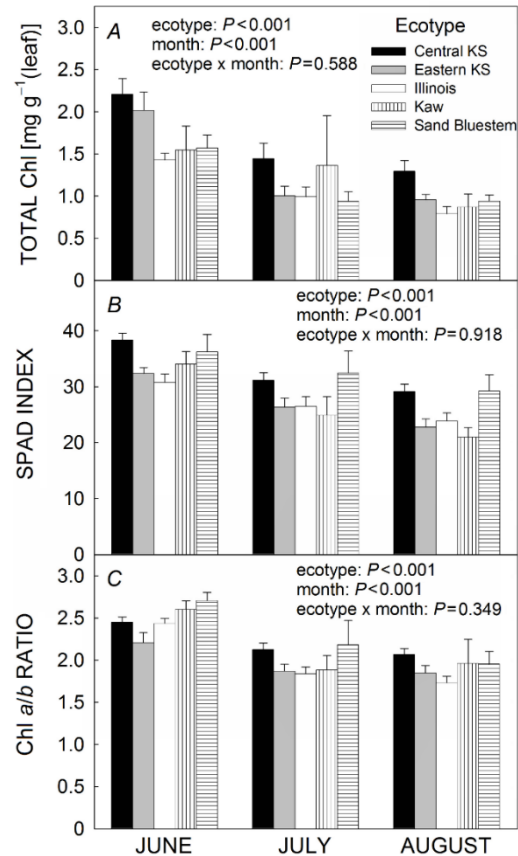
so *a priori* knowledge of when Chl concentrations might be highest in the plant (*e.g.*, early growing season, drought-adapted ecotypes) will assist decisions about which method to use and when the convenience of the SPAD meter will produce reliable results.

Chl concentration in *A. gerardii* varied among the ecotypes (including the cultivars), populations, and among measurement date (months) using both the destructive method (Fig. 2*A*) as well as the nondestructive method (Fig. 2*B*) of measurement. Leaf Chl concentration decreased in all ecotypes and cultivars as the growing season progressed ($F_{2, 120}$ = 28.18; $P<0.001$). According to destructive spectrophotometric measures, leaf Chl concentration in *A. gerardii* was significantly higher in June compared to August ($P<0.001$; Fig. 2*A*). According to nondestructive SPAD measures, leaf Chl concentration was significantly higher in June than July, in turn significantly higher than in August ($P\leq0.011$; Fig. 2*B*). A decrease in leaf Chl is typical late in the growing season in perennial grasses, as nitrogen is mobilized to

underground perennial structures (Baerlocher *et al.* 2004) or toward reproduction (Morash *et al.* 2007).

Total leaf Chl concentrations ranged from 0.79 to 2.21 mg g^{-1}(FM) in leaf tissue (Fig. 2*A*). This range was similar to concentrations measured in *Cynodon dactylon* (L.) Pers. by Riaz *et al.* (2010) and turfgrasses by Huang *et al.* (1997), but lower than measures of *A. gerardii* by Knapp and Gilliam (1985) or *Setaria sphacelata* (Stapf) Clayton by da Silva and Arrabaça (2004). Chl synthesis is reduced by drought (Sayed 2003), and drought commonly causes a decrease in leaf Chl concentration in grasses (García *et al.* 1987, Huang *et al.* 1997, da Silva and Arrabaça 2004, Nayyar and Gupta 2006, Shahbaz *et al.* 2011, Taylor *et al.* 2011). Indeed, leaf Chl concentrations for *A. gerardii* in the present study were similar to droughted turfgrass species reported by Huang *et al.* (1997) and water stressed *Zea mays* L. plants reported by Nayyar and Gupta (2006) and García *et al.* (1987). Reduction in photosynthetic pigment concentration has been suggested to be an adaptation to reduce light absorption during times of water stress (Estill *et al.* 1991, da Silva and Arrabaça 2004), and low Chl concentrations in *A. gerardii* in the present study were consistent with conditions of low water availability.

Chl concentration and content, like other physiological variables, might differ between ecotypes of certain species (Hawkins *et al.* 2009). There were significant differences in Chl concentration between *A. gerardii* ecotypes and cultivars, based on both destructive ($F_{4, 120} = 3.90$; $P=0.007$) and nondestructive ($F_{4, 120} = 4.78$; $P=0.002$) measures. *Andropogon gerardii* plants adapted to areas of low precipitation, such as the Central KS ecotype and sand bluestem, had the highest total leaf Chl concentration (Fig. 2*A*). According to nondestructive SPAD measures, leaf Chl concentration was not different between the Central KS ecotype and sand bluestem ($P=0.902$), but leaf Chl was significantly lower in ecotypes from the more mesic region of tallgrass prairie (Eastern KS and Illinois), as well as the Kaw cultivar ($P\leq0.009$) that originated from the Flint Hills region of Eastern KS. According to destructive spectrophotometric measures, leaf Chl
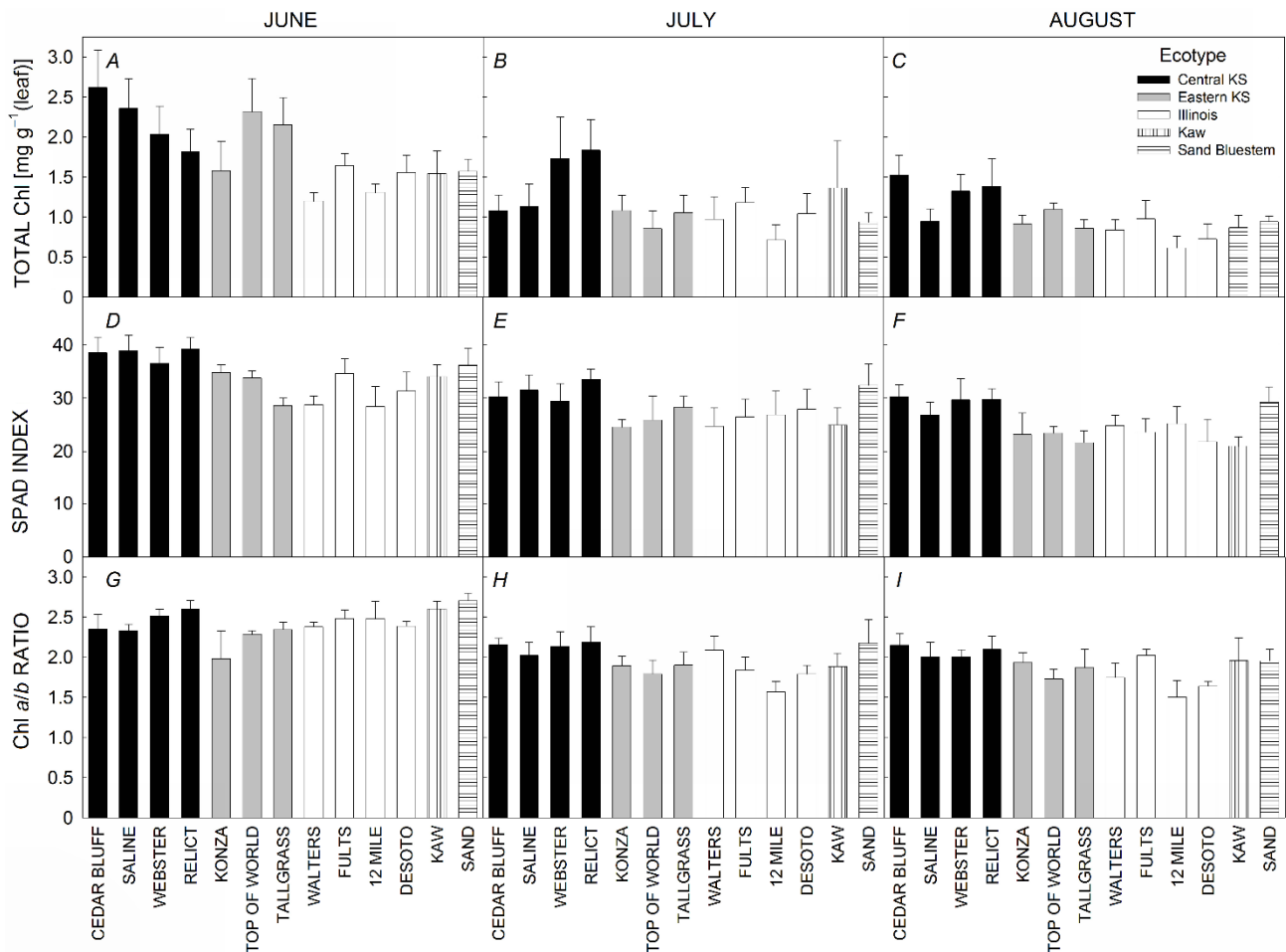

Fig. 3. Mean (± SE; *n* = 5) leaf chlorophyll (Chl) concentration from destructive sampling methods (*A–C*), leaf Chl concentration based on light absorption using the *SPAD-502* meter (*D–F*), and ratio of Chl *a/b* (*G–I*) in all populations of three ecotypes and two cultivars of *Andropogon gerardii*.

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concentration was higher in the Central KS ecotype compared to all other ecotypes and cultivars ($P \leq 0.027$). SPAD measures consistently over-estimated Chl concentration in sand bluestem leaves. Sand bluestem has five times more epicuticular wax compared to big bluestem (Shelton 2012), which could potentially impede passage of light through the leaf. How cuticle architecture and thickness influence absorption of light, and how this relates to drought adaptation in grasses like *A. gerardii* deserves further investigation.

There was variation in leaf Chl concentration among the 13 population sources of *A. gerardii*. Both destructive ($F_{12, 104} = 2.92$; $P=0.001$) and nondestructive ($F_{12, 104} = 3.28$; $P<0.001$) measures (Fig. 3*A–F*) demonstrated minor differences within the Central KS, Eastern KS, and Illinois ecotypes. However, in most cases there was less variation among populations within an ecotype relative to variation among ecotypes (Fig. 3*A–F*). Furthermore, Chl *a/b* ratios also showed less variation within an ecotype *vs.* among ecotypes (Fig. 3*G–I*). This is evidence that similar conditions within each collection region resulted in similar adaptations among populations within a region but different from other collecting regions.

This suggests conditions are more similar among prairies within each collection region relative to the other collection regions and there are similar adaptations among populations within each region, conforming to the definition of an ecotype (Lowry 2012).

Another facet of drought adaptation in *A. gerardii* is a high ratio of Chl *a/b*, consistent with characteristics of sun leaves (Givnish 1988). Drought leads to a reduction in Chl binding proteins (Zhu *et al.* 2003), which results in a preferential reduction of light-harvesting pigment-proteins in PSII (Sayed 2003). Reductions in PSII might lead to increases in the Chl *a/b* ratio, which has been documented in droughted *Cicer arietinum* L. (Rahbarian *et al.* 2011). However, it is more common to see decreased (Estill *et al.* 1991, Ashraf *et al.* 2001, Parida *et al.* 2007, Ghorbanli *et al.* 2012) or unchanged (da Silva and Arrabaça 2004, Kholová *et al.* 2011) ratios of Chl *a/b* in droughted plants, which suggests protecting PSII is involved in drought tolerance (Ghorbanli *et al.* 2012). Having a high ratio of Chl *a/b* might suggest a plant has a smaller LHCII, making PSII less susceptible during drought (Zhu *et al.* 2003). Therefore, a high ratio of Chl *a/b* might be an adaptation for drought. In the present study, there were significant differences in the Chl *a/b* ratio among ecotypes and cultivars ($F_{4, 120} = 4.07$; $P=0.006$). The mean Chl *a/b* ratio was higher in sand bluestem and the Central KS ecotype from the driest collection region relative to the Eastern KS and IL ecotypes ($P \leq 0.001$). The Chl *a/b* ratio in the Kaw cultivar was intermediate compared with the dry and mesic region sourced ecotypes (Fig. 2*C*). These results compare to Zhu *et al.* (2003), where a dune-adapted ecotype of *Phragmites communis* L. had a higher ratio of Chl *a/b* compared to a swamp ecotype. Futhermore, the Chl *a/b*

ratio in *A. gerardii* became significantly lower as the growing season progressed (Fig. 2*C*).

There were temporal differences in the ratio of Chl *a/b*. In June, the ratios were 2.2 to 2.7 and were significantly higher ($P<0.001$) than ratios of 1.7 to 2.2 in July and August ($P=0.279$; Fig. 2*C*). These Chl *a/b* ratios were lower than those reported for *Paspalum dilatatum* Poir. by Soares-Cordeiro *et al.* (2010), *P. communis* by Zhu *et al.* (2003), or *A. gerardii* by Knapp and Gilliam (1985), but similar to values for *Pennisetum glaucum* (L.) R.Br. by Ashraf *et al.* (2001), *Panicum antidotale* Retz. by Shahbaz *et al.* (2011), and *Cynodon dactylon* by Riaz *et al.* (2010). Growing conditions in Hays, KS were consistent with those that commonly lead to low Chl *a/b* ratios.

Lower Chl *a/b* ratios have been documented in response to decreases in both sunlight and soil moisture. A lower Chl *a/b* ratio is consistent with shade leaves (Givnish 1988). Although our common garden was in full sunlight, it is possible plants shaded each other to a small extent. Shading between plants would become more evident later in the growing season when plants became larger, which would be consistent with decreasing Chl *a/b* ratios in *A. gerardii* plants as the growing season progressed. Chl *a/b* ratios have also been shown to decrease in a number of plants under water stress (Estill *et al.* 1991, Ashraf *et al.* 2001, Parida *et al.* 2007, Shahbaz *et al.* 2011, Ghorbanli *et al.* 2012). Protein complexes in thylakoid membranes are thought to be sensitive to drought conditions (Sayed 2003, Parida *et al.* 2007). Specifically, protection of PSII is suggested to be important for tolerating drought conditions (Zhu *et al.* 2003, Ghorbanli *et al.* 2012). The high ratio of Chl *a/b* in the drought-adapted ecotype of *A. gerardii* could indicate it is able to protect PSII during drought with a smaller light-harvesting complex. Similarly, a drought-adapted ecotype of *P. communis* had a higher ratio of Chl *a/b* compared to a swamp ecotype (Zhu *et al.* 2003). Maintaining a smaller LHCII might be an adaptation for drought in grasses such as *A. gerardii*, and measuring the Chl *a/b* ratio could serve as an indication of water stress or drought adaptation in grasses.

These data provide valuable information about the accuracy of available techniques for measuring Chl concentration in plants. This study suggested more accurate data through the destructive method of leaf Chl analysis. This is most likely due to limitations of light passage through leaves with high Chl concentration. Additionally, this study found leaf Chl concentration to differ among ecotypes and cultivars of *A. gerardii*, contributing a broader idea of the physiological differences accounting for drought tolerance among ecotypes within a species. The ecotype and cultivar from the driest regions, and presumably more drought-adapted, had higher Chl concentration and Chl *a/b* ratio compared to more mesic ecotypes, suggesting light absorption and maintenance of PSII is related to drought tolerance in *A. gerardii*.

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